Development of Biomimetic Extracellular Matrix Scaffolds for Tissue Engineering

Hongxu Lu

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Development of Biomimetic Extracellular Matrix Scaffolds for Tissue Engineering

Hongxu Lu

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List of abbreviations

ABC	Avidin-biotinylated peroxidase complex
AESca	Autologous extracellular matrix scaffold
AESca-C	Autologous extracellular matrix scaffold derived from chondrocytes
AESca-F	Autologous extracellular matrix scaffold derived from fibroblasts
AESca-M	Autologous extracellular matrix scaffold derived from MSCs
AESca-mF	Autologous extracellular matrix scaffold derived from mouse fibroblasts
ANOVA	Analysis of variance
ATR-FTIR	Atenuated total reflection fourier transform infrared spectroscopy.
BCSp	Bovine collagen sponge
BSA	Bovine serum albumin
C-DMEM	Medium for chondrocyte expansion
cDNA	Complementary DNA
DAB	Diaminobenzidine
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EOG	Ethylene oxide gas
F-actin	Fillament actin
FBS	Fetal bovine serum
GAG	Glycosaminoglycan
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HE staining	Hematoxylin and eosin staining
HESca	Homologous (allogeneic) extracellular matrix scaffold
HESca-mF	Homologous extracellular matrix scaffold derived from mouse fibroblasts
IgG	Immunoglobulin type G
IL-10	Interleukin-10
ITS+1	(Insulin, transferrin, sodium selenite + linoleic acid) liquid media supplement
LSCM	Laser scanning confocal microscope
mAb	Monoclonal antibody
MHC II	Major histocompatibility complex class II
mRNA	Messenger RNA
MSC	Human mesenchymal stem cell
MSCBM	Mesenchymal stem cell basal medium
MSCGM	Mesenchymal stem cell growth medium

NHAC	Normal human articular chondrocyte
NHDF	Normal human dermal fibroblast
PBS	Phosphate buffered saline
PI	Propidium Iodide
PLGA	Poly(DL-lactic-co-glycolic acid)
RNA	Ribonucleic acid
RT	Room temperature
S-DMEM	Medium for ECM scaffold fabrication
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
TBS	Tris buffered saline
TGF-β3	Transforming growth factor-β3
TNF-α	Tumor necrosis factor-α

Chapter 1

General introduction

The loss or failure of an organ or tissue is a frequent, devastating, and costly problem in health care, occurring in millions of patients every year. In the United States, approximately 9 million surgical procedures are performed annually to treat these disorders, and 40 to 90 million hospital days are required. The total national health-care costs for these patients exceed $500 billion per year [1-3]. Organ or tissue loss is currently treated by transplanting organs from one individual to another or performing surgical reconstruction by transferring tissue from one location in the human body to the diseased site. Furthermore, artificial devices made of plastic, metal, or fabrics are utilized. Mechanical devices such as dialysis machines or total joint replacement prostheses are used, and metabolic products of the lost tissue, such as insulin, are supplemented. Although these therapies have saved and improved millions of lives, they have their respective limitations. Organ/tissue transplantation is heavily limited by shortage of donor. And artificial prostheses can't produce "true and living" organs or tissues for the patients, which means only partial functions can be provided. Therefore new therapies are desired to solve the problems. Tissue engineering has been proposed and rapidly developed in recent years as a promising approach without the above limitations.

1.1 Tissue engineering

1.1.1 Basic concept of tissue engineering

The term "Tissue Engineering" was initially defined by the attendees of the first National Science Foundation (NSF) of U.S.A. sponsored meeting in 1988 as the "application of the principles and methods of engineering and life sciences toward fundamental understanding of structure-function relationship in normal and pathological mammalian tissues and the development of biological substitutes for the repair or regeneration of tissue or organ function". Tissue engineering differs from standard therapies in that engineered tissues become integrated within the patient, affording a potentially permanent and specific treatment of the disease state. "Tissue engineering is an emerging multidisciplinary field that applies the principles of biology

and engineering to the development of viable substitutes that restore, maintain or improve the function of human tissues" [1].

During the 90s, Tissue Engineering progressed rapidly and biological substitutes were developed for several tissues in the body. Tissue Engineering-products have reached the market and in little over a decade, the Tissue Engineering-industry has grown to become a $3.5 billion worldwide R&D effort by over forty biotech start-ups and business units [4,5]. Tissue Engineering has been developed as a potential alternative to tissue or organ transplantation and tissue loss or organ failure may be treated either by implantation of an engineered biological substitute or by ex vivo perfusion systems. Tissue Engineering products may be fully functional at the time of treatment (e.g. liver assist devices, encapsulated pancreatic islets), or have potential to integrate and form the expected functional tissue upon implantation.

In the most frequent paradigm of tissue engineering (Figure 1.1), isolated living cells are used to develop biological substitutes for the restoration or replacement of tissue or organ function. Generally, cells are seeded on biodegradable scaffolds, a tissue is matured in vitro, and the construct is implanted in the appropriate anatomic location as a prosthesis. Cells used in tissue engineering may come from a variety of sources including application-specific differentiated cells from the patients themselves (autologous), human donors (allogeneic) or animal sources (xenogeneic), or undifferentiated cells comprising progenitor or stem cells. The use of isolated cells or cell aggregates enables manipulation prior to implantation, e.g., transfection of genetic material or modulation of the cell surface in order to prevent immunorecognition.

Figure 1.1 The concept of tissue engineering. In general, tissue engineering involves the expansion of cells from a small biopsy, followed by culturing of the cells in temporary three-dimensional scaffolds to form the new organ or tissue. By using the patient's own cells, this approach has the advantages of autografts, but without the problems associated with limitation of donor supply.

1.1.2 Cell sources used in tissue engineering

To create a functional, durable tissue engineered construct, the establishment of cultured cells is a priority. To avoid rejection, cells should be autologous [6]. The use of autologous cells has many advantages, including ethical considerations. Candidate cell types for tissue engineering include somatic and stem cells.

Stem cells are undifferentiated cells that are capable of self-renewing and differentiating into specialized cells types. Thus, stem cells are ideal cells for the repair and regeneration of disordered or lost tissues/organs. Stem cells can be classified into three types, based on the stage of development: embryonic, fetal, and adult stem cells. Embryonic stem cells are cells derived primarily from an early embryonic structure, known as the inner cell mass of the blastocyst, and from embryonic germ cells. These cells are pluripotent cells that can develop into all specified cell types for peripheral tissues and organs [7,8].

Fetal stem cells are cells found in various tissues of the fetus and are committed to differentiation into specified cell types within a given tissue. Note that in humans the fetal stage is defined from the formation of the tissue and organ systems (at about the end of the second month) to the birth (at about the end of the ninth month) and the period before the fetal stage is known as the embryonic stage. Because of ethical concerns, fetal stem cells have not been extensively used for regenerative medicine and engineering.

Adult stem cells are committed stem cells found in a mature tissue or organ. These cells are capable of renewing and differentiating into specialized cells. Adult stem cells have been found in a number of tissues, including the bone marrow, blood, brain, liver, skin, intestine, stomach, and pancreas. While certain adult stem cells from a given tissue may be multipotent, that is, capable of differentiating into cells for a different type of tissue, most adult stem cells are committed to give rise to specialized cells in the same type of tissue. Compared to embryonic stem cells, adult stem cells are scarce and difficult to identify. Nevertheless, because of ethical concerns about using embryonic and fetal stem cells, adult stem cells are still valuable candidates for cell regenerative engineering. Mesenchymal stem cells (MSCs) are a prospective cell source for the tissue engineering. They are relatively easy to obtain from a small aspirate of bone marrow and have multiple potentials to differentiate into different cell lineages such as osteoblasts, chondrocytes, adipose cells, ligament cells, and neural cells [9-12].

Somatic cells are defined as cells other than germ cells, including the egg and sperm, in a mature animal. Most somatic cells are specified cells that constitute tissues and organs. These cells have limited capability of proliferation, differentiation, and regeneration. Certain types of somatic cell, such as hepatocytes, smooth muscle cells, and epithelial cells, are capable of differentiating and/or proliferating within specified tissues. These cells may be used for cell regenerative engineering. For instance, hepatocytes can be used for liver repair and regeneration. Smooth muscle cells may be used for the construction of artificial blood vessels. Other types of somatic cell, such as the neurons and cardiomyocytes, exhibit minimal capacity for differentiation and proliferation. These cells are not suitable for tissue engineering. Chondrocytes are other

important somatic cell applied in tissue engineering. The major problem is the dedifferentiation in the two dimensional culture. Overall, somatic cells are not considered preferred candidates for cell regenerative engineering.

Figure 1.2 Pluripotent stem cells in tissue engineering.

Recently a new kind of cell source defined as induced pluripotent stem (iPS) cells have attracted many attentions. The iPS cells are human somatic cells that have been reprogrammed to a pluripotent state [13]. This occurs by the introduction of a defined and limited set of transcription factors and by culturing these cells under embryonic stem (ES)-cell conditions. The method was first described by Shinya Yamanaka and colleagues [14] using mouse fibroblasts, in which it was demonstrated that the retroviral-mediated introduction of four transcription factors—octamer-binding transcription factor-3/4 (OCT3/4), SRY-related high-mobility-group (HMG)-box protein-2(SOX2), MYC and Kruppel-like factor-4 (KLF4)—could induce pluripotency. There are several hurdles to be overcome before iPS cells can be considered as a potential patient-specific cell therapy, and it will be crucial to characterize the developmental potential of human iPS cell lines. As a research tool, iPS-cell technology provides opportunities to study normal development and to understand reprogramming [15]. iPS cells can have an immediate impact as models for human diseases, including cancer and should be a promising cell source for tissue engineering.

1.2 Scaffolds development in tissue engineering

The scaffold or three-dimensional (3-D) construct provides the necessary support for cells to proliferate and maintain their differentiated function, and its architecture defines the ultimate shape of the new bone and cartilage. Transplanted cells adhere to the temporary scaffold in three dimensions, proliferate, and secrete their own ECM, replacing the biodegrading scaffold. And finally a new tissue or organ is regenerated while the scaffold is degraded.

1.2.1 Requirements of scaffolds

With the approach of tissue engineering, porous three-dimensional temporary scaffolds play a critical role in manipulating cell function and guidance of new organ formation. Isolated and expanded cells adhere to the temporary scaffold in all three dimensions, proliferate, and secrete their own extracellular matrices (ECM), replacing the biodegrading scaffold. Significant challenges to this approach include the design and fabrication of the scaffolds. Ideally, scaffolds for tissue engineering should meet several design criteria [16]: (1) the surface should permit cell adhesion, promote cell growth, and allow the retention of differentiated cell functions; (2) the scaffolds should be biocompatible, neither the polymer nor its degradation by-products should provoke inflammation or toxicity in vivo; (3) the scaffold should be biodegradable and eventually eliminated; (4) the porosity should be high enough to provide sufficient space for cell adhesion, extracellular matrix regeneration, and minimal diffusional constraints during culture, the pore structure should allow even spatial cell distribution throughout the scaffold to facilitate homogeneous tissue formation. A number of three-dimensional porous scaffolds fabricated from various kinds of biodegradable materials have been developed and used for tissue engineering of liver, bladder [17,18], nerve [19], skin [20,21], bone [22], cartilage [23-26], ligament [27,28], and etc.

1.2.2 Biodegradable biomaterials used for scaffolds

Biomaterials play central roles in modern strategies in regenerative medicine and tissue engineering as designable biophysical and biochemical milieus that direct cellular behavior and function. The guidance provide by biomaterials may facilitate restoration of structure and function of damaged or dysfunctional tissue. Such materials should provide a provisonal 3D support to interact biomoleculary with cells to control their function, guiding the spatially and temporally complex multicellular processes of tissue formation and regeneration. Both biologically derived and synthetic materials have extensively explored to fabricate scaffolds in regenerative medicine and tissue engineering (Figure 1.3).

1.2.2.1 Synthetic polymers

Biodegradable synthetic polymers such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and their copolymer of poly(lactic-co-glycolic acid) (PLGA) are most commonly used for tissue engineering. They have gained the approval of the US Food and Drug Administration for certain human clinical use, such as surgical sutures and some implantable devices. PLA undergoes hydrolytic scission to its monomeric form, lactic acid, which is eliminated from the body by incorporation into the tricarboxylic acid cycle. The principal elimination path for lactic acid is respiration, and it is primarily excreted by lungs as CO_2. PGA can be broken down by hydrolysis, nonspecific esterases and carboxypeptidases. The glycolic acid monomer is either excreted in the urine or enters the tricarboxylic acid cycle.

Although the applications of biodegradable synthetic polymer have got great successes in both research and clinical applications, there are some drawbacks. One of the major drawbacks of the non-informational synthetic polymers (e.g., PLGA, PLA, PGA) is that they can't provide an extracellular matrix (ECM) and informational signals (such as the Arg-Gly-Asp sequence), which facilitate cell attachment and proliferation [29]. Another major problem is the *in vivo* degradation. *In vivo*, massive release of acidic degradation and resorption by-products results in inflammatory reactions, as reported in the bioresorbable device literature [30-32]. If the capacity of the surrounding tissue to eliminate the by-products is low, due to the poor vascularization or low metabolic activity, the chemical composition of the by-products may lead to local temporary disturbances. Potential problems of biocompatibility in tissue engineering bone and cartilage, by applying degradable, erodable, and resorbable polymer scaffolds, may also be related to biodegradability and bioresorbability. Therefore, it is important that the 3-D scaffold/cell construct is exposed at all times to sufficient quantities of neutral culture media, especially during the period where the mass loss of the polymer matrix occurs [33].

1.2.2.2 Natural polymers

The natural polymers include proteins of natural extracellular matrices such as collagen and glycosaminoglycan, alginic acid, chitosan, polypeptides, and etc. Yannas et al. conducted pioneering studies on collagen-glycosaminoglycan scaffolds to induce the regeneration of dermis of skin, sciatic nerve and knee meniscus [34]. Chemical crosslinking by glutaraldehyde has been proposed to control the stability and degradation rate of these matrices, whereas porosity has been changed by both chemical and physical techniques. Alginic acid [35], a polysaccharide from seaweed, is a family of natural copolymers of β-D-mannuronic acid (M) and α-L-guluronic acid (G). They have been processed in gel beads encapsulating living cells as a means of immunoprotection [36,37]. Alginates crosslinked with calcium sulfate have been recently used as cell delivery vehicles for tissue engineering, however gelation kinetics by the use of calcium sulfate is difficult to control, and the resulting structure is not uniform. Chitosan is a natural polysaccharide, whose structural characteristics are similar to glycosaminoglycans. It has been used in a variety of biomedical applications, such as hemodialysis membranes, drug delivery systems, orthopaedic and dental coating materials and artificial skin [38-40]. Polypeptides with some amino acid sequences can be favorable to cell adhesion and function and thus they may have potential for cell attachment and transplantation. The poor control of enzymatic degradation and poor mechanical performance are however two major objections to the use of naturally derived polymers.

Figure 1.3 Micrographs of porous synthetic scaffolds made by (a)freeze-drying (collagen-GAG); (b)fibre bonding (PGA); (c)foaming(PLLA); (d)salt-leaching (tyrosine-derived polycarbonate) [41].

1.3 Extracellular matrix as biomaterials for tissue engineering

Cells *in vivo* are surrounded with extracellular matrix (ECM). The ECM is a complex chemically and physically crosslinked network of proteins and glycosaminoglycans (GAG) [42]. The ECM serves to organize cells in space as a physical support, and to provide cells with environmental signals to adjust cellular functions and maintain metabolic activities. ECM is lost when a tissue or organ is defected. Therefore, a porous three-dimensional temporary scaffold is necessary to function as an artificial ECM to assemble transplanted cells, control cell function and guide new organ formation.

The extracellular matrix is a secreted product of cells that populate a given tissue or organ. The composition and ultrastructure of the ECM are determined by factors that influence the phenotype of these cells including mechanical forces, biochemical milieu, oxygen requirements, pH and the inherent gene expression patterns. In turn, the ECM influences the behavior and phenotype of the resident cells. Cell attachment, migration, proliferation and three-dimensional spatial arrangement are strongly affected by matrix composition and structure. Stated differently, the ECM is in a state of 'dynamic equilibrium' with the cells, tissues and organs of which it is a part and it serves as an information highway or medium between cells [43-45]. For these reasons, the ECM has been developed as a biologic scaffold for tissue engineering applications in virtually every body system. Figure 1.4 is an illustration a ECM-Cell interaction. Individual components of the ECM such as collagen I or fibronectin have been used to modulate cell behavior and augment

the in vivo performance of alternative scaffold materials. Methods of chemically cross-linking the ECM for purposes of increased material strength, slower in vivo degradation or passification of antigenic epitopes have uniformly resulted in a scaffold material that is less biocompatible than ECM that is preserved in its native state.

The mammalian response to tissue injury involves a complex sequence of events that includes vascular, cellular and humoral components; the outcome of which is either tissue necrosis with scarring or reconstruction of the tissue with return of function. ECM scaffolds can modulate the wound healing response toward tissue reconstruction rather than scar tissue formation [46].

Figure 1.4 Illustration of the individual cell behavior and the dynamic state of multicellular tissues, that are regulated by intricate reciprocal molecular interactions between cells and their surroundings. This extracellular microenvironment is hydrated protein and proteoglycan-based gel network composing soluble and physically bound signals as well

as signals arising from cell-cell interactions. Specific binding of these signaling cues with cell-surface receptors induces complex intracellular signaling cascades that converge to regulate gene expression, establish cell phenotype and direct tissue formation, homeostasis and regeneration. Ellipsis(...) indicates that the list of signals is not intended to be complete. PLC, phospholipase C; GAGs, glycosaminoglycans; PGs, proteoglycans; CAMs, cell adhesion molecules. Image from Lutolf M. P. [47] et al.

1.3.1 ECM components

The composition of the ECM is a complex mixture of structural and functional proteins, glycosaminoglycans, glycoproteins and small molecules arranged in a unique, tissue specific three-dimensional architecture [48-50]. The logical division of the ECM into structural and functional components is not possible because many of these molecules have both structural and functional roles in health and disease.

1.3.1.1 Collagen

Collagen is the most abundant protein within the mammalian ECM. Greater than 90% of the dry weight of the ECM from most tissues and organs is represented by collagen. More than 20 distinct types of collagen have been identified, each with a unique biologic function. Type I collagen is the major structural protein present in tissues and is ubiquitous within both the animal and plant kingdoms. Type I collagen is abundant in tendinous and ligamentous structures and provides the necessary strength to accommodate the uniaxial and multiaxial mechanical loading to which these tissues are commonly subjected. These same tissues provide a convenient source of collagen for many medical device applications. Bovine type I collagen is harvested from Achilles tendon and is perhaps the most commonly used xenogeneic ECM component intended for therapeutic applications.

Type I collagen is a compound of three chains two of which are identical, termed α1(I), and one α2(I) chain with different amino acid composition or it can rarely represent a trimer built of three α1(I) chains. Type II collagen is essentially unique to hyaline cartilage and the α1(II) subunit is believed to be similar to α1(I). Type III is found in limited quantities (approximately 10%) in association with type I. Thus, type III can be a minor contaminant of type I collagen prepared from skin . In addition blood vessels predominantly contain type III. Collagen types I, II, and III have large sections of homologous sequences, independent of the species [51] . Type IV is a highly specialized form found only as a loose fibrillar network in the basement membrane. For the other interstitial collagen types which occur in small quantities and are associated with specific biological structures the author refers to pertinent connective tissue research literature [52] . Each type of collagen is of course the result of specific gene expression patterns as cells differentiate and tissues and organs develop and spatially organize. In nature, collagen is intimately associated with glycosylated proteins, growth factors and other structural proteins such as elastin and laminin to provide unique tissue properties. The diversity of collagen types, the association with non-collagenous molecules and the tissue specific ultrastructure of ECM are predictive of the

difficulty in recreating such a composite scaffold in vitro (i.e. an artificial ECM).

1.3.1.2 Fibronectin

Fibronectin is second only to collagen in quantity within the ECM. Fibronectin is a dimeric molecule of 250 000 MW subunits and exists both in soluble and tissue isoforms and possesses ligands for adhesion of many cell types [53-56]. The ECM of submucosal structures, basement membranes and interstitial tissues all contain abundant fibronectin [55,56]. The cell friendly characteristics of this protein have made it an attracttive substrate for in vitro cell culture and for use as a coating for synthetic scaffold materials to promote host biocompatibility. Fibronectin is rich in the Arg-Gly-Asp (RGD) subunit; a tripeptide that is important in cell adhesion via the αβ integrin. Fibronectin is found [57] at an early stage within the ECM of developing embryos and is critical for normal biologic development, especially the development of vascular structures. The importance of this molecule and its interactions with other matrix components cannot be overstated with regard to cell–matrix communication.

1.3.1.3 Laminin

Laminin is a complex adhesion protein found in the ECM, especially within basement membrane ECMs. This protein plays an important role in early embryonic development and is perhaps the best studied of the ECM proteins found within embryonic bodies[58]. The prominent role of laminin in the formation and maintenance of vascular structures is particularly noteworthy when considering the ECM as a scaffold for tissue reconstruction [59,60]. The crucial role of the beta-1 integrin chain in mediating hematopoietic stem cell interactions with fibronectin and laminin has been firmly established. Loss of the beta-1 integrin receptors in mice results in intrapartum mortality. This protein appears to be among the first and most critical ECM factors in the process of cell and tissue differentiation. The specific role of laminin in tissue reconstruction when ECM is used as a scaffold for tissue and organ reconstruction in adults is unclear but its importance in developmental biology suggests that this molecule is critical for organized tissue development as opposed to scar tissue formation.

1.3.1.4 Glycosaminoglycans

The ECM contains various mixtures of glycosaminoglycans (GAGs) depending upon the tissue location of the ECM in the host, the age of the host and the microenvironment. The GAGs bind growth factors and cytokines, promote water retention and contribute to the gel properties of the ECM. The heparin binding properties of numerous cell surface receptors and of many growth factors (e.g. fibroblast growth factor family, vascular endothelial cell growth factor) make the heparin-rich GAGs useful components of naturally occurring substrates for cell growth. The glycosaminoglycans present in ECM include chondroitin sulfates A and B, heparin, heparan sulfate and hyaluronic acid. Hyaluronic acid has been most extensively investigated as a scaffold for tissue reconstruction and as a carrier for selected cell populations in therapeutic tissue

engineering applications. The concentration of hyaluronic acid within ECM is highest in fetal and newborn tissues and tends therefore to be associated with desirable healing properties. The specific role, if any, of this GAG upon progenitor cell proliferation and differentiation during adult wound healing is unknown.

1.3.1.5 Growth factors

An important characteristic of the intact ECM that distinguishes it from other scaffolds for tissue reconstruction is its diversity of structural and functional proteins. The bioactive molecules that reside within the ECM and their unique spatial distribution provide a reservoir of biologic signals. Although cytokines and growth factors are present within ECM in very small quantities, they act as potent modulators of cell behavior. The list of growth factors found within ECM is extensive and includes vascular endothelial cell growth factor (VEGF), the fibroblast growth factor (FGF) family, epithelial cell growth factor (EGF), transforming growth factor beta (TGF-beta), keratinocyte growth factor (KGF), hepatocyte growth factor (HGF), platelet derived growth factor (PDGF) and bone morphogenetic protein (BMP) among others [61-63]. These factors tend to exist in multiple isoforms, each with its specific biologic activity. Purified forms of growth factors and biologic peptides have been investigated in recent years as therapeutic methods of encouraging blood vessel formation (e.g. VEGF), inhibiting blood vessel formation (angiostatin), stimulating deposition of granulation tissue (PDGF) and bone (BMP), and encouraging epithelialization of wounds (KGF). However, this therapeutic approach has struggled with determination and delivery of optimal dose, the ability to sustain and localize the growth factor release at the desired site, and the inability to turn the factor 'on' and 'off' as needed during the course of tissue repair.

An advantage of utilizing the ECM in its native state as a substrate or scaffold for cell growth and differentiation is the presence of all the attendant growth factors (and their inhibitors) in the same relative amounts that exist in nature and perhaps more importantly, in their native three-dimensional ultrastructure. The ECM presents these factors efficiently to resident cell surface receptors, protects the growth factors from degradation, and modulates their synthesis. If one considers the ECM to be a substrate for in vitro and in vivo cell growth, it is reasonable to think of the ECM as a temporary (i.e. degradable) controlled release vehicle for naturally derived growth factors. As our understanding of the differences in ECM as a function of tissue type and age increases, our ability to modulate the differentiation (or lack thereof) of host reparative cells, including progenitor cells, within ECM scaffolds at sites of tissue reconstruction will improve.

1.3.2 Acellular matrix scaffolds for tissue engineering

The use of these natural biomaterials has typically required chemical or physical pretreatment aimed at (1) preserving the tissue by enhancing the resistance of the material to enzymatic or chemical degradation, (2) reducing the immunogenicity of the material, and (3) sterilizing the tissue [64]. Multiple crosslinking techniques have been explored in an attempt to find the ideal procedure to stabilize the collagen-based structure of the tissue while maintaining its

mechanical integrity and natural compliance. In addition to crosslinking techniques, decellularization approaches may reduce host immune response to bioprosthetics and generate natural biomaterials for use in cell seeding and tissue engineering applications. Naturally derived materials offer many mechanical, chemical and biological advantages over synthetic materials, and thus hold tremendous potential for use in tissue engineering therapies.

Chemical treatments designed to stabilize and reduce collagen immunogenicity, for example, glutaraldehyde fixation, are often cytotoxic and prevent cell migration into the processed tissue [65-68]. Thus, tissue function through remodeling may never be achieved. As these limitations have become apparent, more recent treatment methodologies have aimed to decrease residual toxicity (inherent with many aldehyde-based crosslinking mechanisms), minimize thrombogenicity, maintain tissue-specific mechanics, and ideally, promote acceptance of the biomaterial by the host. A number of alternatives to aldehyde-based treatments have been reported that may improve graft patency [68-71]. For example, clinical evaluation of processed blood vessels has included cryopreserved vessels such as the saphenous and umbilical veins. Although cryopreserved vessels are a promising option, they are limited by supply.

As an alternative to a single crosslinking step, the addition of decellularization steps aims to remove immunogenic foreign epitopes. A variety of methodologies have been described to specifically remove lipids, host cells, and cellular components to generate immune tolerant materials (Figure 1.5 showed decellularized esophagus for tissue engineering). Decellularization methodologies include: osmotic shock [72,73], solvent extraction [74-76], ionic and nonionic detergents [77,78], acid/alkaline treatments [79], and enzymatic digestion with DNase, RNase, lipase, and proteases [80]. These are promising studies with cells migrating into and populating the matrix material, showing that crosslinking is not necessarily a vital step. However, despite decellularization, immunogenic proteins associated with the distinct extracellular arterial matrix can remain localized within the vascular graft media [81]. Although these materials have shown promise, problems of thrombosis, neointimal hyperplasia, and graft degradation continue to limit the use of these approaches.

Figure 1.5 Gross appearances of native rat esophagus (A), esophagus decellularized by DEOX (B), and Triton X-100 (C) [82].

Decellularized collagen scaffolds are prepared by decellularizing tissues and organs while retaining the extracellular matrices. Removal of cells from a tissue or organ leaves a complex mixture of structural and functional proteins that constitute the extracellular matrix [83,84]. The collagen is in a complex network with other ECM molecules. The tissues from which the ECM is harvested, the species of origin, the decellularization methods, and the methods of terminal sterilization for the acellularized scaffolds vary widely. The efficiency of cell removal from a tissue is dependent on the origin of the tissue and the specific physical, chemical, and enzymatic methods that are used [85,86]. Each of these treatments affects the biochemical composition, tissue ultrastructure, and mechanical behavior of the remaining extracellular matrix (ECM) scaffold which, in turn, affect the host response to the material. Acellularized scaffolds derived from decellularized tissues and organs have been used successfully in both pre-clinical animal studies and in human clinical applications. ECM from a variety of tissues, including heart valves [87,88], blood vessels [89,90], skin [91], nerves [92,93], tendon [27], ligament [94], small intestinal submucosa [95-97], urinary bladder (Figure 1.6) [18,98], vocal fold [99], have been studied for tissue engineering and regenerative medicine applications.

Figure 1.6 Gross appearance of decellularized porcine urinary bladder [100]. This thin (60 μM) sheet of ECM is entirely free of any cellular component, has a multidirectional tensile strength of approximately 40N, and has not been chemically cross linked or modified from its native structure.

1.4 Cell-derived autologous ECM scaffolds for tissue engineering

The ECM plays a central role in mammalian development and physiology and the amino acid sequence and quaternary structure of many components of ECM such as collagen are highly conserved across species lines [101]. This high degree of sequence homology is one reason that xenogeneic ECM can function as a constructive scaffold in mammalian recipients rather than

inciting a destructive inflammatory reaction. However, despite the fact that complete removal of all the cellular components seems to be impossible even with the most rigorous processing methods [102], the differences of ECM molecules between the species and individuals are potential to provoke adverse immune reactions [103-105].Furthermore, the allogeneic and xenogeneic scaffolds have the possibility of disease transmitting [106]. However, the development of autologous ECM-based scaffolds, which are considered as the good standard, is still a great challenge due to the limited supply of autologous materials.

Researchers have developed various methods to omit the immune reaction of the scaffolds. An alternative and novel approach is the production of autologous tissue constructs without the use of a supporting scaffold [107]. The scaffold-free engineering for skin [20,108], bone [109] and cartilage [110] have been reported. Furthermore, cell sheet engineering have been studied allover the world [111,112]. But it's difficult to generate 3D structures. And the advantages of using a scaffold are still attractive for the field of tissue engineering. It has been demonstrated that human aortic myofibroblasts are capable of forming sheets in culture, which can be layered and cultured on frames forming solid flexible and elastic tissues [113]. Ascorbic acid was added to promote ECM production and the constructs resembled native tissue possessing bundles of mature collagen fibers. Using the same technique, it was reported that folding and framing the sheets of cells enhanced collagen production through the creation of tension and that cells of aortic origin produced more collagen than those of venous origin [114].

Other attempts have been tried by many researchers. Matsusaki et al. have reported a hydrogel template leaching technique for tissue engineering [115]. And clotted autologous serum and plasma have been used as autologous scaffolds for tissue engineering [116,117]. However, the clotted serum or plasma couldn't mimic the in vivo microenvironment for the cells. Jin et al [118] have reported to use cell-derived ECM as scaffolds, but they didn't induce the autologous cells to produce the autologous ECM. The cell-derived autologous ECM scaffolds are supposed to be a breakthrough in tissue engineering.

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Chapter 2

Fabrication of biomimetic extracellular matrix scaffolds

2.1 Summary

In this chapter, ECM biomimetic scaffolds were fabricated by using three-dimensional cell culture and decellularization techniques. Cells were cultured in a biodegradable synthetic polymer porous skeleton to generate ECM structure. The introcellular components and the biodegradable polymer skeleton were selectively removed to obtain the ECM scaffolds (Figure 2.1).

Several kinds of ECM biomimetic scaffolds were prepared from MSCs, dermal fibroblasts and articular chondrocytes. At first, the cells were cultured in a PLGA knitted mesh for a specific period. The cells proliferated and excreted ECM in the PLGA knitted mesh. Subsequently, the intracellular components such nucleus and actin were selectively removed by decellularization; while the deposited ECM was maintained in the mesh. Finally, the PLGA knitted mesh was selectively removed by treatment with 0.5 M trisodium phosphate aqueous solution. The MSC-, fibroblast- and chondrocyte-derived ECM scaffolds were prepared after freeze-drying.

To decide the optimal decellularization method, several protocols were compared. The decellularization effects were confirmed by observation with phase contrast microscope, HE staining, and fluorescent staining for cell nuclei, F-actin and cell membrane. The freeze-thaw cycling with ammonium hydroxide could get rid of all the cellular components without any toxic chemical remained. Treatment of 0.1%Triton X-100 with 1.5M KCl aqueous solution completely removed the cellular components. However, the Triton X-100-treated ECM might cause adverse effects to the host cells and tissues because of the toxicity of Triton X-100 residue. 0.1% Triton X-100 alone and freeze-thaw cycling couldn't completely remove the cell nuclei even if the Triton X-100 concentration and freeze-thaw cycle number were increased. Therefore, treatment by freeze-thaw cycling with ammonium hydroxide was used for decellularization for the preparation of ECM biomimetic scaffolds.

The structures of MSC-, fibroblast- and chondrocyte-derived ECM biomimetic scaffolds were confirmed by a scanning electron microscope (SEM). The ECM scaffolds showed porous structures that were similar to that of the PLGA knitted mesh. Microscale and nanoscale ECM

fibers were observed in the scaffolds. The complete removal of PLGA knitted mesh from the scaffolds was confirmed by ATR-FTIR. There was no significant difference among the morphologies of the three ECM scaffolds.

ECM components such as type I collagen, type II collagen, type III collagen, fibronectin, vitronectin, laminin, decorin, versican and aggrecan were stained with their respective antibodies and observed under fluorescent microscope. The ECM scaffolds showed highly organized networks of the ECM components. Most of theses ECM molecules were detected in the MSC-, fibroblast- and chondrocyte-derived ECM scaffolds. Quantitative analysis of glycosaminoglycans (GAG) showed that there was still some GAG remained in the ECM scaffolds. The GAG content decreased after decellularization.

Figure 2.1 Preparation scheme of the ECM scaffolds.

2.2 Introduction

Scaffolds used for tissue engineering should mimic the natural microenvironments where cells reside. *In vivo*, cells are surrounded with specific microenvironments consisting of soluble molecules such as cytokines and growth factors as well as non-soluble extracellular matrices [1]. Therefore, the extracellular matrices have been considered as an ideal materials or scaffolds for tissue engineering because of their native-like structure and chemical components, excellent biocompatibility and harmless degradation products [2-4].

At present, the extracellular matrices are mainly derived from decellularized allogeneic or xenogeneic tissues and organs. The high conservation of ECM molecules gives the possibility of exogenous acellular matrices to be used in research and clinical applications. Although the allogeneic and xenogeneic acellular matrices have been frequently used and shown some interesting effects, the differences of ECM molecules between the species and individuals are potential to provoke adverse immune reactions [5-7]. Furthermore, the allogeneic and xenogeneic scaffolds have the possibility of disease transmitting [8]. Therefore, development of autologous ECM-based scaffolds is considered as the good standard and has been a great challenge due to the limited supply of autologous materials.

In this study, a new approach was proposed to fabricate autologous ECM scaffolds. Culture cells were used to produce the ECM scaffolds instead of usage of autologous tissues or organs. The autologous biomimetic ECM scaffolds were developed by using three-dimensional cell culture in a polymeric template and decellularization. Three kinds of human cells were used. To obtain the ECM scaffolds, the cellular components were removed by decellularization and the biodegradable polymer template was selectively removed by treatment with an alkaline aqueous solution. In the tissue engineering application, ECM scaffolds will be developed from autologous cells and they are so called as autologous ECM scaffolds (AESca).

2.3 Materials and methods

2.3.1 Cell expansion

In this study, human mesenchymal stem cells (MSCs), chondrocytes and fibroblasts were used to fabricate the ECM scaffolds. Human bone marrow-derived mesenchymal stem cells (MSCs) and normal human articular chondrocytes (NHACs) were obtained from Lonza (Walkersville, MD, USA) at passage 2. Normal human dermal fibroblasts (NHDFs, origin from neonatal foreskin) were purchased from Cascade Biologics (Invitrogen, Portland, OR, USA). The cells were seeded in 75 cm^2 tissue-culture flasks and cultured using the respective proliferation media under an atmosphere of 5% CO_2 at 37°C. The cells were further subcultured twice after confluence. Trypsin/EDTA was used to detach the cells during subculture. The cell sources are listed in Table 2.1 and the medium compositions for cells expansion are listed in Table 2.2.

Table 2.1 Cell source and expansion culture media

Cell Type	Origin	Medium
MSC	Lonza, p2	MSCGM
NHAC	Lonza, p2	C-DMEM
NHDF	Cascade Biologics, p2	Medium 106

Table 2.2 Composition of the expansion culture media

Name	Composition
MSCGM	440 ml bottle of mesenchymal stem cell basal medium and the following growth supplements; mesenchymal stem cell growth supplement, 50 ml; L-Glutamine, 10 ml; penicillin-streptomycin, 0.5 ml. All products were purchased from Lonza.
C-DMEM	DMEM (high glucose, Sigma), 10% fetal bovine serum (FBS), 4mM L-glutamine, 0.4mM L-proline, 50μg/mL ascorbic acid, 0.1mM MEM non-essential amino acid, 1mM sodium pyruvate, 100U/mL penicillin and 100μg/mL streptomycin
Medium 106	Medium 106 supplemented with low serum growth supplement (FBS, 2% v/v; hydrocortisone, 1μg/ml; human epidermal growth factor, 10ng/ml; basic fibroblast growth factor, 3ng/ml; heparin, 10 μg/ml), 10μg/ml gentamicin and 0.25μg/ml amphotericin B. All products were purchased from Cascade Biologics.

FBS, fetal bovine serum

MSCGM, mesenchymal stem cells growth medium

C-DMEM, medium for chondrocytes

2.3.2 Cell culture in polymer templates

Passage 5 cells were collected by treatment with trypsin/EDTA solution and resuspend in the medium for scaffold fabrication (S-DMEM) to a certain cell density (Table 2.3). The medium for scaffolds fabrication (S-DMEM) was composed of C-DMEM supplemented with 150μg/mL L-ascorbic acid phosphate magnesium salt n-hydrate (Wako Pure Chemicals, Japan). The L-ascorbic acid phosphate was used to promote the production of ECM.

A Vicryl knitted mesh made of polyglactin 910 (a 90:10 copolymer of glycolic acid and lactic acid, PLGA) (Johnson & Johnson, USA) was cut into small discs with a diameter of 10.4mm. After sterilized by ethylene oxide gas (EOG), the meshes were immersed in S-DMEM in 12-well tissue culture plate (Falcon) and covered with glass rings (inner Φ=10mm, outer Φ=12mm). The glass rings were used to avoid the leakage of cells from the mesh. After 6 hours, the cells were seeded on another side of the mesh by the same method. The meshes were moved into a new plate on the second day and the media were exchanged every 3 days during the culture.

Pre-experiments were carried out to evaluate the favorite culture conditions. Three kinds

of cells were seeded on the PLGA template at various densities (3.0×10^5cell/mL, 5.0×10^5cell/mL, 7.5×10^5cell/mL and 1.0×10^6 cell/mL, 200μl/side) and cultured for certain days (2d, 3d, 4d, 5d, 6d and 7d). The porous structure and the mechanical strength were compared to choose the best conditions. According to determined conditions, MSCs, NHACs and NHDFs were cultured in the meshes for 5, 6 and 6 days, respectively, to obtain their respective Cell-ECM-Template complexes. The cell seeding number and culture time are listed in Table 2.3.

Table 2.3 Cell seeding and culture time for Cell-ECM-Template complex fabrication

Cell Type	Passage	Cell Seeding	Fabrication Medium	Culture Time
MSC	5	3.0×10^5cells/ml, 200μl/side, double sides	S-DMEM	5d
NHAC	5	5.0×10^5 cells/ml, 200μl/side, double sides	S-DMEM	6d
NHDF	5	5.0×10^5cells/ml, 200μl/side, double sides	S-DMEM	6d

2.3.3 Decellularization

After cultured for a specific time as shown in Table 2.3, the Cell-ECM-Template complexes were washed by phosphate buffered saline (PBS) thrice and MilliQ water thrice in 100 mm tissue culture dish (Falcon). And the cellular components of each ECM-Cell-Template complexes were removed by decellularization. Twelve complexes were put in one dish. To achieve a complete decellularization, several protocols were applied.

2.3.3.1 Freeze-thaw cycling plus ammonium hydroxide treatment

The treatment of freeze-thaw cycling with ammonium hydroxide was used as the first method for the decellularization. After washing, 40ml MilliQ water was added into the tissue culture dish. And then dish was frozen at -80°C for 4h. The complex was thawed at room temperature and washed by MilliQ water for 3 times, 10 min each time on a slowly moving shaker). The freeze-thaw cycles were repeated for 6 times. Finally, the complexes were immersed in 25mM NH_4OH for 30 min on a slowly moving shaker and washed by MilliQ water for 6 times (10 min each time).

2.3.3.2 Triton X-100 plus osmotic shock treatment

The second decellularization method used in this study is a method combining detergent

and osmotic shock according to the reported protocol [7] with some modifications. Briefly, the complexes were immersed in 0.1% Triton X-100 with 1.5M KCl in 50mM Tris buffer (pH 8.0) for 6h on ice on a slowly moving shaker after washing. The complexes were then washed with 10mM Tris buffer (pH 8.0) for 3 hours followed washing with MilliQ water for 3h.

2.3.3.3 Other decellularization methods

Furthermore, we have tried decellularization by freeze-thaw cycling without NH_4OH. Triton (in 50mM or 10mM Tris buffer, pH 8.0) alone and osmotic shock (1.5M KCl or 3M NaCl: pure water) were also carried out for the decellularization. 0.1% sodium dodecyl sulfate (SDS) alone was tried too.

2.3.4 PLGA Template removal and ECM scaffold harvest

After decellularization, the complexes were put into in new 100 mm tissue culture dishes containing 50mL 0.5M trisodium phosphate (Na_3PO_4) aqueous solution and incubated at 37°C for 48h to remove the PLGA template. However, the ECM could be reserved after the alkaline treatment.

The ECM mesh was washed for 6 times with MilliQ water (5 min each time) and frozen at -80°C for 12h. In this step, the ECM meshes were handled with a stainless spoon. The scaffolds were moved onto water droplets spreading on a stainless plate wrapped by Saran wrap and frozen at -80°C in a deep freezer. The ice blocks containing the ECM scaffolds were transferred into 35mm tissue culture dishes (-80°C pre-cold) by tweezers. Then the ECM-Scaffolds were freeze-dried at 7.7Pa for 24h on a EYELA FDU-2200 freeze-drier (Tokyo Rikakikai, Tokyo, Japan).

Ethylene oxide gas (EOG) was used to sterilize the scaffolds for the *in vivo* implantation and cell culture. The ECM scaffolds were put in cell culture dishes and packaged in ELK bags. They were sterilized by EOG at 40°C for 22h 44min under Eogelk SA-1000 sterilizer (ELK corp., Osaka, Japan).

After sterilization, the ECM scaffolds were stored at 4°C until usage.

2.3.5 Confirmation of decellularization

The decellularized ECM scaffolds were observed by phase contrast optical microscope. Hematoxylin and eosin (HE) staining and fluorescent staining of cell nucleus, filament actin (F-actin) and cell membrane were performed to confirm the removal effects of cellular components by decellularization.

2.3.5.1 HE staining

To perform HE staining, the ECM scaffolds were fixed by 10% formalin followed by a

washing with MilliQ water. The fixed scaffolds were spread on slide glasses and air-dried for 24h at 37°C. The scaffolds attached tightly on the slide glasses. The attached scaffolds were stained with hematoxylin and eosin.

2.3.5.2 Fluorescent staining of cell nuclei, F-actin and cell membrane

Double staining by Alexa Fluor-488 labelled Phallodin (staining for F-actin) and Hoechst 33258 (staining for cell nuclei) was used to confirm the removal of cell nuclei and actin filaments. The ECM scaffolds were fixed by 0.1% glutaraldehyde followed by a washing with MilliQ water. The fixed scaffolds were spread on slide glasses and air-dried for 24h at 37°C. Before staining, the samples were incubated with 0.5mg/ml $NaBH_4$/PBS to quench the auto-fluorescence induced by aldehyde group. And then the samples were penetrated by 0.1% Triton X-100 in PBS for 5min and blocked with 1% bovine serum albumin (BSA) for 30min. After the pre-treatments, the samples were firstly stained with 5U/mL Alexa Fluor-488 labelled Phallodin for 20min. After being washed thrice with PBS, the slides were then incubated with 10μg/mL Hoechst 33258 for 10min. After being washed for 3 times, the slides were observed under Olympus BX51 fluorescent microscope with a DP-70 CCD camera (Olympus). The samples without decellularization were used as controls. Cell-ECM-PLGA complexes were fixed by 0.1% glutaraldehyde and incubated in Na_3PO_4 solution to remove the PLGA by the same processes as those used for preparation of ECM scaffolds

Cell membrane is any important indicator of decellularization. The DiI (Molecular Probe, USA) was used to specifically stain the cell membrane. The ECM scaffolds were firstly fixed with 0.1% glutaraldehyde followed by a washing with MilliQ water. The fixed scaffolds were spread on slide glass and air dried for 24h at 37°C. Before staining, the samples were incubated with 0.5mg/ml $NaBH_4$/PBS to quench the auto-fluorescence induced by aldehyde group. The samples were then stained with 5U/mL DiI for 20min. After being washed for 6 times, the slides were observed under Olympus BX51 fluorescent microscope with a DP-70 CCD camera (Olympus, Japan). The samples without decellularization as described above were used as controls.

2.3.5.3 DNA quantification

The DNA contents in the ECM scaffolds were measured with Hoechst 33258-based quantitation kit (Sigma, USA). ECM Scaffolds were digested in 100μl papain solution at 60°C for 6h. The Cell-ECM-Template complexes were used as controls and dissolved in 500μl papain solution. Papain (Sigma) was dissolved at 400μg/mL in sterile 0.1M phosphate buffer, pH 6.0, with 5 mM cystein・HCl and 5mM EDTA. Eight pieces were combined to ensure the DNA amount is enough for test. Five micro liter lysate was added to 2mL Hoechst 33258-containing working solution and fluorescent intensity was recorded by FP-6500 spectrofluorometer (JASSO, Japan). The excitation wavelength was set at 360 nm and the emission wavelength was set at 460 nm. The DNA amount was calculated by using a standard curve obtained with the standard DNA supplied by the kit (n=3).

2.3.6 Confirmation of PLGA template removal

The removal of PLGA was examined by ATR-FTIR on IR prestige-1 FTIR spectrophotometer (Shimadzu, Japan). The freeze-dried ECM scaffolds were put on the ATR-FTIR stage and scanned at wavenumber from 0 to 4000 cm^{-1}. The air was used for background. PLGA and Cell-ECM-Template complexes were compared with the ECM scaffolds.

2.3.7 Structure observation and Components analysis

2.3.7.1 Structure observation

The gross appearance of the AESca was recorded by a digital camera (Canon, Japan) held on camera photo-taking stand (Hakuba, Japan). The microstructure of the AESca was observed by a scanning electron microscope (SEM). The freeze-dried ECM scaffolds were coated with Pt using a sputter coater and observed with SEM at 10kv.

2.3.7.2 GAG amount measurement

The sulfated-glycosaminoglycan (sGAG) amount was measured with Blyscan™ sulfated sGAG assay kit (Biocolor, United Kingdom) according to the manufacturer's manual. The specimen lysate (digested by Papain as described above) was mixed with Blyscan™ dye to bind the sGAG. Then the sGAG-dye complex was collected by centrifugation. After removing the supernatant and draining the tube, the dissociation reagent was added. Two hundred microliter solution was transferred into 96-well plate. The absorbance against the background control was obtained at a wavelength of 656 nm on a Benchmark Plus™ microplate spectrophotometer (Bio-Rad, Japan) and the GAG amount (n=3) was calculated by using a standard curve obtained with the standard sGAG supplied by the kit.

2.3.7.3 Immunostaining of ECM molecules

The immunofluorescent staining was performed to detect the ECM components in the scaffolds. Type I collagen, type II collagen, type III collagen, fibronectin, vitronectin, laminin, versican, decorin, aggrecan were examined. The antibodies used for fluorescent staining were listed in

Table 2.4. The ECM scaffolds were fixed by 10% formalin followed by a washing with MilliQ water. The fixed scaffolds were spread on slide glasses and air-dried for 24h at 37°C. Scaffolds on the slides were incubated with proteinase K for 10min for antigen retrievals, and blocked with 2% BSA/PBS at room temperature for 30min. The slides were sequentially incubated with 1st antibodies for 1h and 2nd antibodies for 40 min at 37°C in dark. The slides were mounted

with Vectashield ® mounting media (Vector, USA) and observed under Olympus BX51 fluorescent microscope with a DP-70 CCD camera (Olympus, Japan). Type I collagen stained slides were also observed by a laser scanning confocal microscope (LSCM) (LSM 510 Meta, Zeiss, Germany). The images obtained by LSCM were manipulated by the LSM image browser.

Table 2.4 Antibodies used for ECM components analysis

Name	Inoculated animal	Species Reactivity	Company	Dilution	2nd antibody
Anti-collagen I (monoclone)	Mouse	Human, bovine, rat, rabbit, dog, pig, rhesus monkey	Abcam	1:500	FITC conjugated rabbit anti-mouse IgG (whole molecule) (Sigma, 1:50)
Anti-collagen II (monoclone 6B3)	Mouse	Human, mouse, chicken, salamander	Neomarker	1:200	FITC conjugated rabbit anti-mouse IgG (whole molecule) (Sigma, 1:50)
Anti-collagen III (affinity purified polyclone)	Rabbit	Human, mouse, rat	Abcam	1:500	TRITC conjugated goat anti-rabbit IgG(H+L) (Invitrogen, 1:100)
Anti-Fibronectin (affinity purified)	Rabbit	Human, bovine, mouse, goat, sheep, pig, rat, chicken	Sigma	1:200	TRITC conjugated goat anti-rabbit IgG(H+L) (Invitrogen, 1:100)
Anti-Vitronectin (whole serum)	Rabbit	Human, bovine, mouse, porcine, sheep, goat, canine, rabbit, rat	LSL	1:500	TRITC conjugated goat anti-rabbit IgG(H+L) (Invitrogen, 1:100)
Anti-Laminin (affinity purified)	Rabbit	Human, mouse, mammal, avian, reptilian and amphibian	Sigma	1:100	TRITC conjugated goat anti-rabbit IgG(H+L) (Invitrogen, 1:100)
Anti-Aggrecan (monoclonal)	Mouse	Human, bovine, rabbit, canine, chicken	Chemicon	1:250	FITC conjugated rabbit anti-mouse IgG (whole molecule) (Sigma, 1:50)
Anti-Vesican (affinity purified polyclone)	Rabbit	Human, mouse, rat	Santa Cruz	1:50	TRITC conjugated goat anti-rabbit IgG(H+L) (Invitrogen, 1:100)
Anti-Decorin (affinity purified polyclone)	Rabbit	Human, mouse, rat	Santa Cruz	1:50	FITC conjugated sheep anti-rabbit IgG(whole molecule) (Sigma, 1:100)

2. 4 Results

2.4.1 Cell-ECM-Template fabrication

Three kinds of cells, MSCs, NHACs and NHDFswere cultured in PLGA meshes for 5 or 6 days to form a three-dimensional structure of cells and ECM. SEM observation demonstrated that the three kinds of cells adhered in the PLGA meshes and produced flake like ECM (Figure 2.2). The cells and ECM surrounded the fibers of the PLGA meshes.

Figure 2.2 SEM images of Cell-ECM-Template complexes. (a) PLGA template; (b) Cell-ECM-Template complex formed by MSC; (c) Cell-ECM-Template complex formed by NHAC; (d) Cell-ECM-Template complex formed by NHDF. Scale bar = 100μm.

2.4.2 Decellularization

2.4.2.1 Decellularization by freeze-thaw cycling and NH_4OH

In the Cell-ECM-Template complexes, cells proliferated and deposited ECM surrounding the template fibers (Figure 2.3a). Cell membrane was disrupted and cell nuclei were exposed

during the freeze-thaw cycling (Figure 2.3b). After the treatment with NH_4OH, the cell nuclei couldn't be observed by phase contrast microscope (Figure 2.3c). Cell nuclei were removed from the complexes with the NH_4OH treatment. The ECM scaffold became transparent when the PLGA mesh template was removed (Figure 2.3d).

Figure 2.3 Phase-contrast micrographs of the ECM scaffolds during decellularization by the method of freeze-thaw cycling plus NH_4OH. (a) Cell-ECM-Template complex before decellularization (b) Complex after freeze-thaw cycling. Cell membrane was broken and cell nuclei were exposed. (c) After being treated with NH4OH, no cell nuclei could be observed. (d) After removal of PLGA template, ECM scaffold was clear without obvious cell debris. Scale bar = 200 μm.

The decellularization effect was further confirmed by fluorescent staining of cell nuclei, F-actin and cell membrane. Figure 2.4 shows the fluorescent staining of MSC-derived ECM scaffolds. Before decellularization, cell nuclei (blue dot), F-actin (green fiber) and cell membrane (red) were observed. After decellularization, no cell nucleus, F-actin and cell membrane was observed. The same results were observed with NHAC- and NHDF- derived ECM scaffolds. These results indicated that the cellular components of nuclei, intracellular proteins and cell membrane were removed by the freeze-thaw cycling plus NH_4OH.

Figure 2.4 Fluorescent staining of cell nucleus, F-actin and cell membrane to confirm the decellularization of MSC-derived ECM scaffolds by freeze-thaw cycling with NH_4OH. Scale bar=100μm.

DNA amount remained in the ECM scaffolds was further quantitatively analyzed (Table 2.5).The Cell-ECM-Template complexes before decellularization contained high amount of DNA. However, the DNA amount dropped to very low levels after decellularization. It indicated a successful decellularization was achieved. The DNA quantification and fluorescent staining results indicated that the treatment of freeze-thaw cycling plus ammonia could remove the cellular components.

Table 2.5 DNA amount (ng/mesh) in the ECM scaffolds (mean ± SD, n=3)

	Cell-ECM complexes	ECM scaffolds
AESca-M	670.11±51.54	4.38±1.67
AESca-C	2677.89±511.35	2.79±0.63
AESca-F	2689.33±567.10	4.35±0.81

From all the decellularization results, it could be concluded that the freeze-thaw cycling plus NH_4OH could achieve a successful decellularization. And the ECM scaffolds in this work was prepared by this method except of special description.

2.4.2.2 Decellularization by Triton X-100 plus 1.5M KCl

The decellularization effect of Triton X-100 with 1.5M KCl is shown in Figure 2.5. The cells were broken and cell nuclei are exposed after MSC-derived ECM-Cell-Template complexes were treated with Triton X-100 (Figure 2.5b). Then, with cooperation of the osmosis shock of 1.5M KCl, the nuclei were destroyed (Figure 2.5c). After removal of PLGA, the ECM scaffolds were prepared and no cell nuclei were observed (Figure 2.5d).

Figure 2.5 Phase-contrast micrographs of the MSC derived ECM scaffolds during decellularization by the treatment of Triton X-100 plus 1.5M KCl aqueous solution. (a) Cell-ECM-Template complex before decellularization (b) Complex treated by Triton X-100 alone. (c) Complex treated with Triton X-100 and 1.5M KCl (d) ECM scaffold after removal of PLGA template. Scale bar = 200 μm.

No cell nucleus could be found by HE staining (Figure 2.6) and the F-actin was absent by the fluorescent staining with phalloidin (Figure 2.7). Although we didn't measure the DNA content in Triton X-100 decellularized scaffolds, it's reasonable that DNA will remain in a very low level.

Figure 2.6 Micrographs of HE staining for the confirmation of decellularization. (a) Control; (b) Cell nuclei existed only freeze-thaw cycling. No cell debris could be observed after decellularization by freeze-thaw cycling plus NH_4OH (c) and Triton X-100 plus 1.5M KCl (d). Scale bar = 100 μm.

Figure 2.7 F-actin staining by AlexaFluor-488 labelled Phallodin for the confirmation of decellularization with Triton X-100 plus 1.5M KCl. F-actin existed before decellularization (a); while no F-actin could be observed after decellularization by Triton X-100 plus 1.5M KCl (b). Scale bar = 100 μm.

2.4.2.3 Decellularization by other methods

Freeze-thaw cycling could not achieve a complete decellularization (Figure 2.8a) even if the cycling number was increased to 10 time (Figure 2.8b). Triton X-100 alone couldn't get an ideal decellularization. For examples, after removal the PLGA, clear nuclei could be observed if only Triton X-100 was used (Fig. 6c). Cell debris couldn't be removed only by osmosis shock of

1.5M KCl or 3M NaCl (Fig. 6d). And if we combine the freeze-thaw with osmosis shock, cell nucleus couldn't be removed from the 3D matrix yet (Fig. 6e). We have used sodium dodecyl sulfate (SDS) for the decellularization. No nucleus could be observed if the complexes were treated with SDS alone (Fig 6f).

Figure 2.8 The decellularization results by other methods. (a) freeze-thaw cycling (6 times) without NH_4OH. (b) freeze-thaw cycling (10 times) without NH_4OH (Stained by HE staining). Arrows show the cell nuclei. (c) 0.1% Triton X-100 (in 10mM Tris, pH 8.0) only without 1.5M KCl. (d)Osmotic shock by 1.5M KCl (opposite to pure water). (e) Freeze-thaw cycling with 3M NaCl. (f) 0.1% SDS used ECM scaffold. Scale bar = 200 μm.

But the decellularization by SDS alone and Triton X-100 with 1.5M KCl was different

(Figure 2.9). SDS is dramatic than Triton X-100, so SDS could destroy DNA. But Triton X-100 with 1.5M KCl just broke the cell nuclei, while large amount of DNA was remained in the ECM.

Figure 2.9 Hoechst 33258 staining showed the differences of between SDS and Triton X-100 plus 1.5M KCl. (a), (b) SDS decellularization; No cell nuclei was observed (a), and DNA was destroyed by SDS (b). (c), (d) Triton X-100 with 1.5M KCl. No cell nuclei was observed (c), but large amount of DNA was remained in the places of cell nuclei (d). Scale bar = 100μm.

2.4.3 Removal of PLGA

By the SEM images (Figure 2.13), we couldn't find the PLGA fibers. And it was confirmed that PLGA was completely removed from AESca-M by ATR-FTIR (Figure 2.10). The most important indicator of PLGA, ester bond, which was shown by the peak at 1740 cm^{-1}, disappeared in the ECM scaffolds. While in the PLGA mesh and Cell-ECM-Template complexes, that band was detected. The ATR-FTIR results of AESca-C and AESca-F (Figure 2.11) also reveal that PLGA templates were completely removed.

Figure 2.10 AIR-FTIR spectra of PLGA, Cell-ECM-PLGA complex and ECM scaffold derived from MSC (ASEca-M). Disappearance of ester band (1740cm^{-1}) in AESca-M reveals that the biodegradable polymer, PLGA, was removed from the ECM scaffold.

Figure 2.11 AIR-FTIR spectra of PLGA, Cell-ECM-PLGA complex and ECM scaffolds derived from NHAC (ASEca-C, upper one) and NHDF (AESca-F, lower one). Disappearance of ester band (1740cm-1) reveals that PLGA template was removed from the ECM scaffold.

2.4.4 ECM scaffold fabrication

After decellularization and removal of the PLGA, the ECM scaffolds exhibited a mesh-like porous structure. The ECM scaffolds were in white color (Figure 2.12). The autologous ECM scaffolds derived from MSC, NHAC and NHDF were named as AESca-M, AESca-C and AESca-F respectively.

Figure 2.12 Gross appearance of ECM scaffolds in (a) (c) (e) water and (b) (d) (f) dry condition. (a),

(b) ECM scaffolds derived from MSC; (c), (d) ECM scaffolds derived from NHAC; (e), (f) ECM scaffolds derived from NHDF. (a), (c) and (e) were in water while (b), (d) and (f) were in dry condition. Scale bar= 4mm.

SEM observation showed that all the ECM scaffolds have mesh-like porous structures. ECM flakes and micro-scale and nano-scale fibers were observed in the scaffolds (Figure 2.13 and Figure 2.14). The ECM scaffolds derived from MSC, NHAC and NHDF were similar in structure. The cell type did not obviously affect the structure of the ECM scaffolds.

Figure 2.13 SEM images of the ECM scaffolds. (a), (b), (c) ECM scaffolds drived from MSC at different magnifications; (d), (e), (f) ECM scaffolds derived from NHAC at different magnifications; (g), (h), (i) ECM scaffolds derived from NHDF at different magnifications.

Figure 2.14 SEM image of the ECM scaffold derived from MSC that was observed at a 45° angle. Scale bar=200μm.

We also used a laser confocal microscope to observe the structure of the scaffolds. The type I collagen in the ECM scaffolds was stained by anti-type I collagen antibody. The type I collagen showed a highly organized fibrous structure (Figure 2.15), which was similar to its natural structure in human body.

Figure 2.15 Scanning laser confocal micrograph of the ECM scaffold derived from MSC. Scale bar=20μm.

2.4.4 GAG amount and ECM components

The GAG amount remained in the ECM scaffolds was listed in Table 2.6. Although a lot of GAG has been removed during the process for scaffold fabrication, there was still some GAG remained in the ECM scaffolds. These GAG were potentially for the cell seeding and tissue reconstruction. Thus, the ECM scaffolds would benefit from the remained GAG.

Table 2.6 GAG amount (μg/mesh) in the ECM scaffolds (mean ± SD, n=3)

	Cell-ECM-Template	ECM scaffolds
AESca-M	1.83±0.08	0.21±0.12
AESca-C	3.87±0.21	0.24±0.10
AESca-F	4.87±0.24	0.21±0.04

Nine ECM molecules were examined in this study. They are type I collagen, type II collagen, type III collage, fibronectin, vitronectin, laminin, versican, decorin and aggrecan. From the results, the ECM scaffolds were mainly composed of collagen I and fibronectin. The immunostaining results of ECM scaffolds from different cell types were shown in Figure 2.16, Figure 2.17 and Figure 2.18.

No type II collagen could be detected in the ECM scaffolds, even in the chondrocytes derived ECM scaffolds. Laminin was strongly detected in the fibroblast-derived ECM scaffold but only slightly detected in the MSC- and chondrocyte-derived ECM scaffolds. On the other hand, vitronectin was strongly detected in the MSC- and chondrocyte-derived ECM scaffolds but only slightly detected in the fibroblast-derived ECM scaffolds. No versican existed in the MSC- and fibroblast-derived ECM scaffolds. The ECM molecules composing the ECM scaffolds were summarized in the Table 2.7.

Figure 2.16 ECM molecules of MSC-derived scaffolds investigated by fluorescent staining. Scale bar = 100μm.

Figure 2.17 ECM molecules of NHAC-derived scaffolds investigated by fluorescent staining. Scale bar = 100μm.

Figure 2.18 ECM molecules of NHDF-derived scaffolds investigated by fluorescent staining. Scale bar = 100μm.

Table 2.7 Summary of ECM components in the ECM scaffolds

	AESca-M	AESca-C	AESca-F
Type I collagen	+++++	+++++	+++++
Type II collagen	×	×	×
Type III collagen	+	+	++
Fibronectin	++++	++++	++++
Vitronectin	+++	+++	+
Laminin	+	+	++
Aggrecan	+	++	+
Versican	×	+	×
Decorin	+	+	+

+ Positive × Negative

2.5 Discussion

2.5.1 ECM scaffolds derived from cultured cells

The ECM is custom-designed and manufactured by the resident cells of each tissue and organ and is in a state of dynamic equilibrium with its surrounding microenvironment[9]. The structural and functional molecules of the ECM provide the means by which adjacent cells communicate with each other and with the external environment [10,11]. The ECM is obviously biocompatible since host cells produce their own matrix. The ECM also provides a supportive medium or conduit for blood vessels, nerves and lymphatics and for the diffusion of nutrients from the blood to the surrounding cells. In other words, the ECM possesses all of the characteristics of the ideal tissue engineered scaffold or biomaterial.

The autologous ECM scaffolds derived from MSC, NHAC and NHDF were successfully fabricated. MSC, NHAC and NHDF are three important cell sources for tissue engineering. Autologous ECM would like to avoid the immune rejections and give the reseeded cells similar *in vivo* environments. The ECM scaffolds derived from these cells are prospective in tissue engineering.

PLGA was approved by Food and Drug Administration (FDA) for certain human clinical application, such as surgical sutures and some implantable devices. While PLGA showed good

biocompatibility and achieve many successes in both research and clinical applications, the degradation of PLGA can evoke severe inflammation that could lead to the final failure of implantation [12,13]. In this study, we used a new method to dissolve the PLGA: Na_3PO_4 aqueous solutions were used to take place of the commonly used organic solvent. By this method, the PLGA could be removed while the ECM was preserved.

Individual components of the ECM such as collagen, laminin, fibronectin and hyaluronic acid can be isolated and used both in vitro and in vivo to facilitate cell growth and differentiation. The complex three-dimensional organization of the structural and functional molecules of which the ECM is composed has not been fully characterized; therefore, synthesis of this biomaterial in the laboratory is not possible[3].

Collagen is the most abundant protein within the mammalian ECM. Collagen can be used alone or in combination with other extracellular matrix components such as glycosaminoglycan and growth factors to improve cell attachment and proliferation. Glycoproteins, such as fibronectin, vitronectin, laminin, are important for cell adhesion, proliferation and differentiation. Proteoglycans, such as versican, decorin and aggrecan, are critical for the maintenance and regulation of cell functions.

2.5.2 Decellularization

Decellularization was applied to remove cellular components in order to reduce the immunogenicity of allogeneic and xenogeneic biologic scaffolds. Besides, decellularization could facilitate the tissue remodeling because cellular debris could clog cell invasion and inhibit the cell repopulation [14]. Furthermore, cell debris has been speculated to constitute the early nuclei of calcification especially in the crosslinked acellular matrices [15,16].

Many methodologies have been described to selectively remove cellular component including freezing-thaw cycling, osmotic shock, acid or alkali treatment, detergent extraction, enzymatic digestion and the combination of these methods. They are reviewed in detail by Gilbert *et al* [17]. The principle of decellularization is to remove the cellular component as completely as possible while minimize the influences on the structure and components of ECM. The side-effect induced by decellularization should remain in a very low level as well.

It has been reported that cells could be removed by freeze-thaw cycling and ammonium hydroxide while ECM was kept in 2D culture systems [18]. To our knowledge, it's the first time to induce this method in the complicit 3D matrices. The treatment of freeze-thaw cycling plus ammonium could remove the cellular components while no toxic chemicals remains like the most commonly used detergent method.

Compared with other intracellular proteins (ex. F-actin), DNA is relative difficult to be removed due to its "stick" nature and tendency to adhere to ECM proteins (DNA is negatively charged) [17]. The remnant DNA was implicated as the cause of inflammatory reactions following the implantation [19]. However, despite the universal presence of DNA remnants in ECM scaffolds, the clinical efficacy of these devices for their intended application has been largely positive [20,21]. It is considered that there is a threshold amount of DNA to provoke the immune reaction [21]. And the

non-crosslinked scaffolds will degrade rapidly after implantation *in vivo* and the remnant DNA is logically subject to the same degradation fate via enzymatic breakdown [20]. DNase and RNase were widely used to degrade the nucleic acid. However, Na_3PO_4 was used to remove the PLGA in this study; the high pH environment of 0.5M Na_3PO_4 solution will cause the degradation of DNA as well. But it should be confirmed at first that the cell nucleus is broken so that DNA is not in a condensation status. Freeze-thaw cycling with Na_3PO_4 and Na_3PO_4 alone could not remove all the cell nuclei if the nuclei were not broken.

Although the freeze-thaw cycling could break the cells and release intracelluar proteins, the cellular components with tight connections to the ECM couldn't be removed, such as the cell nucleus. Some researchers used snap freeze to devitalize the cell without remove the cell debris [22-24]. However, freeze-thaw cycling combined other methods should be used to achieve a desired decellularization. It has been reported that freeze-thaw cycling was combined with detergent and nucleases to achieve a decellularization of menisci [25]. And Freeze-thaw cycling was combined with nuclease to achieve a decellularization of embryoid body [26].

The freeze-thaw cycling cause cryoinjuries to cells through solution effects, extracellular ice formation, dehydration and intracellular ice formation [27]. The formation of external ice and the efflux of water from the cells cause the solute toxicity and excessive cell shrinkage, which are very damaging to the cells. Although extracellular ice crystals don't work as punchers to cell membrane, too much extracellular ice crystals can cause mechanical damage to the cell membrane due to crushing. While some organisms and tissues can tolerate some extracellular ice, any appreciable intracellular ice is almost always fatal to cells. Many cells might contain ice crystals by –15°C, but for intracellular ice formation to be certain, the temperature needs to drop to below –40°C [28]. So we freeze the scaffolds in -80°C. Liquid nitrogen decellularization[29] has also been tried but it could destroy quite amount of ECM and mechanical strength. Although it has been reported the innocuous intracellular ice formation has recently been shown to exert a cryoprotective effect[30], the freeze-thaw cycling at -20°C and -30°C was not enough to achieve complete decellularization in this work (data not shown). While the mechanisms of cryoinjuries still need to be resolved, the freeze-thaw cycling does work in the decellularization.

Ammonium hydroxide has been used to remove the cells while keep the extracellular matrix [31-33]. We have tried to use NH_4OH to decellularize the scaffolds, but the results weren't ideal. It might due to the complicity of 3D matrix is able to inhibit the perfusion of NH_4OH into the scaffolds. And the extended exposure to NH_4OH would like remove more ECM molecules such as glycosaminoglycans. Therefore, we hypothesized that the effects of ammonium hydroxide highly depended on the types of tissues, which have different structures and chemical composition. The alkalescence of NH_4OH (pK_b= 4.75) is stronger than that of Na_3PO_4 (pK_b= 2.23), so the NH_4OH is able to disrupt the cell nucleus.

Since Meezan et al [34] have used detergent (sodium deoxycholate) to remove the cellular components; a lot of methods based on detergent have been developed for the decellularization. And Triton X-100-based decellularization was widely used for the preparation of biological scaffolds.

The non-ionic detergent, Triton X-100, could disrupt lipid–lipid and lipid–protein

interactions, but leave protein–protein interactions intact so that proteins within a tissue or organ following non-ionic detergent treatment should be left [35]. Therefore, Triton X-100 alone couldn't get an ideal decellularization. In our experiments, after removal the PLGA, clear nuclei could be observed if only Triton X-100 was used even if we increased the concentration of Triton X-100 to 1%. Many researchers [36,37] have combined DNase and RNase that could degrade nucleic acid with Triton X-100. But we didn't want to use the DNase and RNase because of their xenogenesis and difficulty to be removed [17]. Triton X-100 with NH_4OH could also remove the cell debris [38,39]. But we want to reduce the use of chemicals, so we didn't apply this method in our decellularization.

Other detergents have also been used in the decellularization. Sodium dodecyl sulfate (SDS) alone [40] and the methods combining nuclease [41] have been reported to reach a complete decellularization SDS alone could get a good decellularization for our ECM scaffolds. SDS, as an ionic detergent, is effective for solubilizing both cytoplasmic and nuclear cellular membranes, but tends to denature proteins by disrupting protein–protein interactions [35]. Thus it's more drastic than Triton X-100. In this study, the SDS decellularized ECM scaffolds were weaker than those from other methods. So we didn't choice the SDS treatment for the further studies.

The osmotic shock for the decellularization has been reported in other studies. But the results weren't always ideal, so did this work. And if we combine the freeze-thaw with osmosis shock, cell nucleus couldn't be removed from the 3D matrix yet. It might indicated that a decellularization methodology just broke the cells could achieve a successful result because of connections of cellular molecules with ECM.

ECM scaffolds were successfully decellularized by the treatment of freeze-thaw cycling plus ammonium hydroxide. The method didn't introduce exogenous or harmful chemicals into the scaffolds. Although Triton X-100 with 1.5M KCl also could get a good decellularization results, but Triton X-100 is toxic to the cells and very difficult to be completely removed. But it should be kept in mind that the decellularization effects differ quite a lot for different application. To choose a proper decellularization method should consider many criteria.

2.6 Conclusions

In this chapter, ECM scaffolds were prepared by using cultured cells and decellularization method. Decellularization with freeze-thaw cycling＋NH_4OH was the most optimal method. And the PLGA template was completely removed. All the MSCs, chondrocytes and fibroblasts could be used to prepare the ECM scaffolds. The ECM scaffolds showed mesh-like porous structures. The ECM scaffolds were mainly composed of type I collagen, fibronectin and other ECM molecules. A certain amount of GAG could be reserved in the ECM scaffolds. The composition of the ECM scaffolds changed a little depending on the cell types.

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Chapter 3

Biocompatibility of ECM scaffolds

3.1 Summary

In this chapter, the biocompatibility of ECM biomimetic scaffolds was investigated by implanting the autologous ECM scaffolds in mice.

ECM biomimetic scaffolds were prepared from fibroblasts isolated from mice by the method described in Chapter 2. The mouse fibroblast-derived autologous ECM scaffolds were implanted subcutaneously in the dorsa of the respective mice. Allogeneic ECM scaffolds, bovine collagen sponge and PLGA knitted mesh were used as controls. Histological examination for morphology, immunohistochemical staining for cell classification and gene expression analysis for the cytokines were carried out to evaluate the host tissue response.

The autologous ECM scaffolds showed the most moderate inflammation than did the allogeneic ECM, bovine collagen and PLGA. And there was no chronic inflammation sign for the autologous ECM scaffolds. These results suggest that the autologous ECM scaffolds were well accommodated by the host and the most biocompatible scaffolds.

Figure 3.1 Illustration of the experiment for the compatibility test of the autologous ECM scaffolds.

3.2 Introduction

The biocompatibility of a scaffold or matrix for a tissue engineering product refers to the ability to perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without eliciting any undesirable local or systemic response in the eventual host [1]. Biologic ECM scaffolds composed of extracellular matrix are commonly used in numerous tissue engineering and regenerative medicine applications, and in many reconstructive surgical procedures. These biologic materials composed of extracellular matrix are typically processed by methods that include decellularization and/or chemical crosslinking to remove or mask antigenic epitopes, DNA, and damage-associated molecular pattern (DAMP) molecules [2,3]. The effect of such scaffolds upon the host immune response is very important for the evaluation of ECM scaffolds. An improved understanding of the biocompatibility of biologic scaffold materials can only lead to greater safety and efficiency of devices and applications that utilize such materials [4,5].

In this chapter, ECM biomimetic scaffolds were prepared from fibroblasts isolated from mice by the method described in Chapter 2. The mouse fibroblast-derived autologous ECM scaffolds were implanted subcutaneously in the dorsa of the respective mouse. Homologous (thesaurus of allogeneic) ECM scaffolds, bovine collagen sponge and PLGA knitted mesh were used for the comparison. Histological examination for morphology, immunohistochemical staining for cell classification and gene expression analysis for the cytokines were carried out to evaluate the host tissue response.

3.3 Materials and methods

3.3.1 Experiment design

Twelve-weeks old (at implantation date) Clrj:CD1 (ICR) mice were randomly divided into 6 groups (6 mice per group). AESca-mF groups were implanted with AESca derived from autologous mouse fibroblast with freeze-thaw cycling plus NH_4OH decellularization. A Triton X-100 with 1.5M KCl aqueous solution treatment was also used for decellularization. This group was named as AESca-mF (Triton) group. HSca-mF groups were the mice implanted with ECM scaffolds derived from fibroblasts isolated form other mice (Homologous or allogeneic).

Bovine collagen sponge (BCSp, Koken, Japan) and PLGA knitted mesh (Johnson & Johnson, USA) were also implanted to the mice separately. They were cut into 8mm discs sterilely. The mice whose dorsa were opened and sutured but without implantation (sham operation) were used as controls.

3.3.2 Isolation of mouse fibroblasts

The animal experiment was conducted according to the committee guidelines of the National Institute for Materials Science for animal experiments. Surgical plane anesthesia in each

animal was induced and maintained with 2% isoflurane in air. The biopsies were taken by aseptic operation from mouse ears. The biopsies were sterilized again with 70% ethanol. The epidermal cells were removed by trypsin/EDTA and hair was shaved off. The biopsies were further digested with 1000 U/ml type I collagenase at 37°C for 5h in DMEM to dissociate the cells from the ECM. The digest solution was filtered with 70-μm cell strainer to remove non-digested tissues. The cell suspension was centrifuged at 200×*g* for 5min. The cells were resuspended and plated in 75 cm^2 tissue culture flasks with complete growth medium. The complete growth medium was composed of DMEM (high glucose, Sigma), 10%fetal bovine serum (FBS), 4mM L-glutamine, 0.1mM MEM non-essential amino acid, 1mM sodium pyruvate, 100U/mL penicillin and 100μg/mL streptomycin.

The viable fibroblasts attached on the flask within 24 h and showed the spindle-shape morphology in 2 to 3 days. The cells were subcultured twice and P2 cells were used for the scaffold fabrication.

3.3.3 Fabrication of ECM scaffolds derived from mouse fibroblasts

The twice subcultured mouse fibroblasts were harvested by trypsin/EDTA treatment. The cells were seeded in the PLGA mesh template at a cell density of 5×10^5 cells/ml (200μl/mesh, double sides). The cells were cultured in S-DMEM for 10 days. After 10 days culture, the Cell-ECM-Template complexes were decellularized and PLGA templates were removed by the methods described in Chapter 2.

3.3.4 Evaluation of ECM scaffolds derived form mouse fibroblasts

The decellularization was confirmed by HE staining by the method described in Chapter 2. The removal of PLGA was examined by ATR-FTIR. And the ECM molecules composing the scaffolds were detected by fluorescent immunostaining. Antibodies against collagen I, collagen II, collagen III, fibronectin, vitronectin, laminin, decorin, versican, aggrecan were used for the component analysis. All the antibodies applied in this chapter were as same as those in Chapter 2 except the anti-collagen I antibody (affinity purified polyclone, Santa Cruz, 1:50).

3.3.5 In vivo implantation

After anesthesia, the central back of the mouse was shaved and opened for implantation in sterile fashion. Four pieces of ECM scaffolds were overlapped and subcutaneously implanted in the dorsal of the mouse. And only one piece of PLGA or BCSp was implanted. And the mouse was sutured by Nylon suture. The autologous ECM scaffolds were implanted in their respective mice where the fibroblasts were isolated. The allogeneic ECM scaffolds were implanted in the mice different those where the fibroblasts were isolated for ECM scaffold preparation.

3.3.6 Histological analysis

After one week of implantation, the mouse was scarified. The back skin and the sample-harboring tissues were excised and fixed in 10% neutral-buffered formalin solution. The specimens were trimmed and embedded in paraffin, and then sectioned at 7-μm intervals. The tissue sections were stained with hematoxylin and eosin to observe the morphology. Because the ECM scaffolds were invisible after implantation, we excised a large area around the implantation site to ensure the scaffolds are held in the specimens. And we marked the implantation part on the mice. During the sectioning, each sample was labelled for several domains and we checked the representative slices of each domain to locate the implanted materials. The sections in positive domains were used for the further exploration. Unfortunately, we still got several cases that didn't have the materials in the paraffin sections even by this way.

3.3.7 Immunohistochemical analysis

To confirm the cell population in the host response, immunohistochemical staining was carried out with Vectastain® Elite® ABC kit (Vector, USA). The antibodies specific to macrophages, neutrophils and T cells were used. And the MHC II positive cells were also stained. The antibodies were listed in Table 3.1. For the immunohistochemical staining, the slide were deparaffinized and hydrated. A heat-mediated antigen-retrieval technique that included 20-min incubation at 95°C in 0.01 M citrate buffer, pH 6.0 was applied. After cooling down, two separate 5-min washes in Tris-buffered saline (TBS)/Tween20, pH 7.4, followed by one wash in phosphate buffered saline (PBS) were performed. Sequentially, 0.3%H_2O_2 in water was used to quench the endogenous peroxidase and the 2% BSA/PBS were used for non-specific antigen blocking. The tissue sections were incubated with 1st antibody for 30 min followed by 30 min' incubation of biotinylated 2nd antibodies (1:200 in 10% serum/PBS, serum is from the species that developed the 2nd antibody). The ABC reagent was then added for 30 min and DAB (Dako) was used to develop the color for 5 min. Finally the slides was counter-stained by hematoxylin and mounted before observation. All incubations were conducted at room temperature (RT).

Table 3.1 Antibodies used for host tissue response

Epitope	Name	Species reactivity	Company	Dilution	Cell type	2nd antibody
Gr-1	Rat monoclonal RB6-8C5	Mouse	Abcam	1:500	Neutrophil	biotinylated anti-rat IgG (Vector, 1:200)
F4/80	Rat monoclonal CI:A3-1	Mouse	eBioscience	1:500	Macrophage	biotinylated anti-rat IgG (Vector, 1:200)
CD3	Rabbit polyclonal affinity purified	Mouse, human, rat	Abcam	1:500	T cell	biotinylated anti-rat IgG (Vector, 1:200)
MHC II	Rat monoclonal ER-TR3	Mouse	Santa Cruz	1:500	MHC class II molecules	biotinylated anti-rabbit IgG (Vector, 1:200)

Only macrophage numbers were counted manually by a cell counter plugin of the software Image J (Version 1.6). Three views, at a magnification 400×, were randomly selected to count the positive stained and total cell numbers for each sample. And each group has three parallel samples.

The normality of the data was tested by Kolmogorov-Smirnov method and homogeneity of variance was done by Bartlett's Test. If the data fulfill these criteria, we will carry out the parametric test; otherwise the non-parametric test will be used. Therefore, the differences of the average macrophage numbers among all the groups were statistically examined by non-parametric Kruskal-Wallis Test with a Stell-Dwass post hoc test for the pairwise comparison. The differences of macrophage/ total cell percentages were statistically examined by one way analysis of variance (ANOVA) with a Tukey post hoc test for the pairwise comparison. Statistical significance was determined at $P<0.05$. All the analyses were performed with the software Kyplot 2.0.

Because we only got one case that having the materials being sectioned in Triton-treated AESca-mF group, we didn’t carry out the statistically analysis for this group. Because the macrophages in the control group in quite different from other groups, we didn’t carry out the statistically analysis for the macrophage percentages.

3.3.8 Gene expression analysis

To further investigate the host tissue response to the implanted biomaterials, the expression of genes encoding immune relative cytokines was analyzed. Total mRNA of the

connective tissues under the materials (skin is above on the materials) was isolated and their respective cDNA was synthesized.

The harvested connective tissues were frozen in liquid nitrogen and crushed into powder after washing by PBS. The powders were dissolved in 1 mL Isogen (Nippon Gene, Toyama, Japan) and the total RNA was extracted following the manufacturer's protocol. One microgram total RNA was reversely transcribed into cDNA using random hexamer (Applied Biosystems) in 20 μL reaction on GeneAmp 9700 PCR systems (Applied Biosystems).

An aliquot (1 μL) of 10-times diluted cDNA reaction solution was used for each 25 μL real-time PCR reaction together with TaqMan Gene Expression assay kits. Real-time PCR analysis was conducted and monitored using the 7500 Real-Time PCR System (Applied Biosystems). After an initial incubation step of 2 min at 50°C and denaturation for 10 min at 95°C, 40 cycles (95°C for 15 s, 60°C for 1 min) PCR were performed. Reactions were performed in triplicate. GAPDH recombinant RNA levels were used as endogenous controls and gene expression levels relative to 18S were calculated using the comparative Ct method.

In the real-time RT-PCR reactions, TaqMan Gene Expression assay kits (Applied Biosystems) were used for transcript levels of tumor necrosis factor-alpha (TNF-α, reference sequence NM_013693.2), interleukin-2 (IL-2, reference sequence NM_008366.2), interleukin-4 (IL-4, reference sequence NM_021283.1), interleukin-10 (IL-10, reference sequence NM_010548.1), major histocompatibility complex class II (MHC II or H-2, reference sequence NM_010386.3) and GAPDH (reference sequence NM_008084.2). Three samples under each condition were used for measurement to calculate the means and standard deviations (n=3) except the Triton X-100 treated AESca group.

Data are reported as means ± standard deviation. The differences of the above gene expressions were statistically examined by one way analysis of variance (ANOVA) with a Tukey-Kramer post hoc test for the pairwise comparison. Statistical significance was determined at $P<0.05$. All the analyses were performed with the software Kyplot 2.0.

3.4 Results

3.4.1 Mouse fibroblasts-derived ECM scaffolds

Mouse fibroblasts were isolated from mouse ear biopsies. The fibroblasts adhered on the cell-culture flasks and showed spindle-like morphology (Figure 3.2a). The fibroblasts proliferated normally during subculture. After being subcultured twice, the fibroblasts were harvested and seeded in PLGA mesh templates. The cells adhered, proliferated and produced ECM in the PLGA meshes. After 10 days culture, the Cell-ECM-Template complexes were decellularized and the PLGA templates were removed to form the mouse-fibroblasts-derived ECM scaffolds. The ECM scaffolds showed a mesh-like structure that was a replica of the PGLA mesh template.

Figure 3.2 Phase contrast micrograph of mouse fibroblasts (a) and gross appearance of freeze-dried AESCa-mF (b).

The scaffolds were observed by SEM. The scaffold had a mesh-like porous structure (**Figure 3.3**).

Figure 3.3 SEM images of AESca-mF at low (a) magnification and (b) high magnification.

HE staining showed that no cell nucleus was observed in the AESca-mF (Figure 3.4b). The results indicated that the AESca-mF was completely decellularized.

Figure 3.4 HE staining images of AESca-mF before (a) and after (b) decellularization. Scale bar=100μm.

FTIR spectra shows that no PLGA-derived ester bonds were detected (Figure 3.5), which indicats that the PLGA was removed from the scaffolds. Immunohistochemical staining showed that the ECM scaffolds were mainly composed of collagen I, collagen III, vitronectin, fibronectin and laminin.

Figure 3.5 ATR-FTIR spectra shows PLGA was completely removed from the ECM scaffolds.

Immunohistochemical staining showed that the ECM scaffolds were mainly composed of collagen I, vitronectin, fibronectin, and other molecules, such as collagen III, laminin, decorin, versican and aggrecan (Figure 3.6). No collagen II was detected by the fluorescent immunostaining.

Figure 3.6 ECM molecules composing the ECM scaffolds derived from mouse fibroblasts. Scale bar = 100μm.

3.4.2 Gross appearance of implanted scaffolds

After implantation, the AESca-mF were completely integrated with the surrounding tissues and it was difficult to distinguish the ECM scaffolds from the surrounding tissues. So it became difficult to harvest all the samples. We could not harvest all the samples containing the ECM scaffolds. And some mice died during operation and the feeding. The sample number was listed in Table 3.2.

Table 3.2 Harvested samples

Group	Harvested samples	Scaffolds-harboring samples	Real-time samples
AESca	4	3	3
AESca (Triton)	2	1	2
HESca	5	3	3
BCSp	4	3	3
PLGA	4	4	3
Control	3	3	3

The PLGA and BCSp could be clearly observed and located (Figure 3.7). The tissue colors in AESca and control groups were lighter than those of other groups, which might indicate less inflammation in the autologous ECM scaffolds.

Figure 3.7 Gross appearance of the harvested samples. The marked areas are the scaffold containing parts.

3.4.3 HE staining results

The AESca-mF was implanted into the subcutaneous pocket of the ICR mice. From the HE staining (Figure 3.8 and Figure 3.9), there was no dense fibrous tissue was found in the control group. The AES-mF was penetrated with host cells composed of macrophages and fibroblasts. The Triton X-100 decellularized AES-mF showed similar results as AES-mF do. There was no obvious difference between the AESca-mF and HESca-mF from HE staining. The bovine collagen sponges were surrounded by dense fibrous layers, and there were only a few cells that penetrated into the inner part of the collagen sponge. The PLGA fibers were surrounded by fibrous tissues. And foreign body giant cells were observed (Figure 3.9). With high possibility, the BCSp and PLGA will be isolated by host tissue instead of being remodeled.

Figure 3.8 HE staining of mouse skin and the materials. Mu: muscle layer, Ep: epidermis, arrows: implanted materials. The area between two dotted lines indicated the areas of the ECM scaffolds. Scale bar=200μm.

Figure 3.9 High magnification of the HE staining. Mu: muscle layer, Ep: epidermis, arrows: implanted materials, arrow heads: foreign body giant cells. The area between two dotted lines indicated the areas of the ECM scaffolds. Scale bar=100μm.

Furthermore, blood-vessel-like structures were observed in the AESca and HESca (Figure 3.10). The results indicated that ECM scaffolds might induce angiogenesis after one week implantation.

Figure 3.10 Blood vessel-like structure observed in the ECM scaffolds. Scale bar=100μm.

3.4.3 Immunohistochemical staining results

Macrophages were stained by F4/80 antibody. Figure 3.11 shows macrophages stained by mAb F4/80 with Vectastain ® Elite ® ABC kit (Vector, USA). Macrophages were detected in all the mice. The control showed the least macrophages. The macrophages number increased in the order of AESca, HESca, PLGA or BCSp.

Figure 3.11 Macrophages immunostained by F4/80 antibody with Elite ABC kit. Mu: muscle layer, Ep: epidermis, arrows: implanted materials. The area between two dotted lines indicated the areas of the ECM scaffolds. Scale bar=100μm.

MHC class II positive cells were only observed in HESca and bovine collagen sponge (BCSp) implantation groups, which indicates that HESca and BCSp were recognized by the hosts as exogenous biomaterials. On the other hands, AEsca and PLGA scaffolds didn't show MHC II positive cells. The HESca and BCSp groups might induce chronic inflammation.

Figure 3.12 MHC II staining by MHC II specific antibody with Elite ABC kit. The red cycles represent the positively stained cells. Scale bar=100μm.

No T cells were detected in either group. Only a few neutrophils were observed in the PLGA, BCSp and HESca groups (Figure 3.13). It indicated that there might be acute inflammation in these groups.

Figure 3.13 Neutrophils stained by Gr-1 antibody with Elite ABC kit. The red cycles indicated the positively stained cells. Scale bar=100μm.

3.4.4 Quantification of macrophages

The macrophage numbers are shown in Figure 3.14. Control group had very few macrophages, which might be related to the wound healing. AESca and HESca induced significantly ($p<0.05$) less macrophages than did the bovine collagen sponge and PLGA. The AESca induced fewer macrophages than did the HESca. But there was no significant difference between these two groups.

Figure 3.14 Macrophage number per view of each group. Data represent means ± SD (n=3). *, $p<0.05$; * *, $p<0.01$.

The percentages of macrophages/total cell in different groups are shown in Figure 3.15. AESca caused the mildest inflammation. The inflammation of HESca group was higher than the AESca ($P<0.001$), but still significantly lower than be the bovine collagen sponge and PLGA knitted mesh groups ($P<0.001$). However, there is no significant difference between bovine collagen and PLGA knitted mesh groups.

Figure 3.15 Macrophage/total cell percentages of each group. Data represent means ± SD (n=3). * * *, $p<0.001$.

3.4.5 Real-time PCR analysis

The interleukin 10 (IL-10) gene expressions were significant lower in the AESca and control groups than be those in the HESca, BSCp and PLGA groups (Figure 3.16). Although the high expression of the IL-10 indicates an accommodating trend of the host to implanted materials and down-regulation of the inflammation, it was relative to the recruitment of macrophages at the beginning. IL-2 and IL-4 gene expression wasn't detected. It coincided with the results no T-cell was detected in the implanted samples because IL-2 and IL-4 are mainly secreted by T cells. There was no obvious difference of the TNF-α and MHC II gene expression in all samples although their expression in ASEca and HSEca groups was lower than the other groups.

Figure 3.16 The IL-10 gene expression in the humoral response. The gene encoding IL-10 expressed obviously higher in the BCSp group than did the other groups. Data represent means ± SD (n=2 in AESca-Triton group and n=3 in other groups). *, P<0.05.

Figure 3.17 The TNF-α, MHC II, IL-2 and IL-4 gene expression in the immune response. There is no

significant difference among these groups in TNF-α and MHC II gene expression. No IL-2 and IL-4 expression was detected in this study. Data represent means ± SD (n=2 in AESca-Triton group and n=3 in other groups).

3.5 Discussion

The biocompatibility of the biomaterials is one of the most important issues in the tissue engineering. The better understanding the biocompatibility and the mechanisms of autologous ECM scaffolds are very important for the success application of ECM scaffolds. In this study, the autologous ECM scaffolds fabrication combined 3D cell culture, decellularization and PLGA template leaching techniques. All the processes might influence the *in vivo* performance of the ECM scaffolds. Therefore, the investigation of the host response to the ECM scaffolds is useful for promotion of the fabrication techniques.

Many researchers have developed a lot of methods to omit the immune reaction of the scaffolds. The scaffold-free engineering for skin [6,7], bone [8]and cartilage [9] have been reported. Furthermore, cell sheet engineering have been studied allover the world [10,11] . But it's difficult to generate 3D structures. And the mechanism strength is not ideal. But the advantages of using a scaffold are attractive for the field of tissue engineering. Hydrogel template leaching technique has been developed for tissue engineering [12]. Clotted autologous serum and plasma have been used as autologous scaffolds for tissue engineering [13,14] . However, the clotted serum or plasma couldn't mimic the in vivo microenvironment for the cells. Jin et al [15] have reported to use chondrocyte-derived ECM as scaffolds, but they didn't induce the autologous cells to produce the autologous ECM. And the porosity and interconnectivity were not ideal.

Subcutaneous antigen encounter favors the induction of an immune response because of the high density of dendritic cells in the skin [16]. And subcutaneous implantation is relatively easy for the operation. So we implanted the ECM scaffolds subcutaneously. Dermal fibroblasts are an important cell source in tissue engineering. Dermal fibroblasts may be easily isolated and used up to passage 14 without a decrease in collagen synthesis or a reduction in growth rate. The fibroblasts have been widely used for built skin equivalents [7] and other tissues [17]. The fibroblasts could produce ECM molecules, such as collagens, glycosaminoglycans and glycoproteins [6,18-20]. Therefore, we tried to use fibroblasts to fabricate ECM Scaffolds.

Autografts is considered to be the good standard because they won't induce immune reactions which were potent to lead to failure of tissue engineering[21]. To develop the autologous ECM scaffolds is a greet challenge for tissue engineering because of the lack of autologous materials. However, even the autograft will induce inflammation after the implantation. After implantation, autograft tendons undergo a process of remodeling that includes necrosis, revascularization, cellular repopulation, collagen remodeling, and maturation [22,23]. Therefore, the most moderate inflammation in AESca group might be helpful in the tissue remodeling.

The complete removal of all the cellular components seems to be impossible even with the most rigorous processing methods [24]. The remnant DNA was implicated as the cause of inflammatory reactions following the implantation [25]. However, despite the universal presence of

DNA remnants in ECM scaffolds, the clinical efficacy of these devices for their intended application has been largely positive [24,26]. Currently, the U.S. Federal Drug Administration does not regulate the limits for DNA in biological scaffold materials.

We have been using fetal bovine serum for the 3D cell culture to fabricate the ECM scaffolds. The fabricated ECM might contain bovine fibronectin and vitronectin which is existed in bovine serum. The containing of these bovine proteins might cause an inflammation. The Usages autologous serum or plasma has been tried by many researchers. Autologous bone marrow-derived plasma has been used to culture hMSCs successfully instead of the commonly used fetal bovine serum [27]. The chemical defined medium culture is also prospective [28]. We also hope to take place of bovine serum with other more prospective methods.

Cytokines play important role in the host tissue response [29] . TNF-α, IFN-γ, and IL-1 are considered to as typical “T helper 1 type” (Th1) cytokines. They lead to macrophage activation, and are believed to play a central role in the initiation of the pro-inflammatory cascade. Activation of this way is associated with both allogeneic and xenogeneic transplant rejection [30,31]. In contrast, IL-4, IL-10 and TGF-β are the Th2 cytokines. Following organ implantation, activation of the Th2 pathway coincides with graft acceptance [32,33]. To understand the cytokines types and production would benefit the application of ECM scaffolds. Although the high expression of the IL-10 indicates an accommodating trend of the host to implanted materials and down-regulation of the inflammation, it was relative to the recruitment of macrophages at the beginning.

Macrophages are one of the most important cells involved in the inflammation. Phenotypic and functional polarization of the mononuclear macrophage population has recently been described [34-37]. The pro-inflammatory, cytotoxic macrophage phenotype, signified as M1, is characterized by cells that promote pathogen killing and cells that are associated with classic signs of inflammation, especially chronic inflammation. The anti-inflammatory macrophage phenotype, signified as M2, promotes immunoregulation, tissue repair, and constructive tissue remodeling. Although morphologically indistinguishable by routine methods of histological examination, mononuclear macrophages from these two pathways can be identified and distinguished by their cell surface markers and by their cytokine and gene expression profiles. A further work to distinguish the subpopulation of macrophages is needed for the autologous ECM scaffolds.

In tissue engineering, cells are usually seeded and cultured in the scaffolds to develop proper tissues in vitro before implantation. Just investigating the immune response to the scaffolds is not enough. The implantation of cell-reseeded constructs is requested in the future work.

3.6 Conclusions

The biocompatibility of autologous ECM scaffolds was evaluated by using subcutaneous implantation test. The evaluation techniques included histological analysis, immunohistochemical staining for the immunocyte classification and real-time PCR for the cytokine gene expression. The autologous ECM scaffolds induce the least inflammatory and immunoreactions. The autologous ECM scaffolds were completely integrated with the host tissues. The excellent biocompatibility would facilitate the application of autologous ECM scaffolds in the tissue engineering.

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Chapter 4

Cartilage tissue engineering using ECM scaffolds

4.1 Summary

In this chapter, ECM scaffolds were used to examine their potential for cartilage tissue engineering. Human mesenchymal stem cells (MSCs) were seeded on the ECM scaffolds. Two ECM scaffolds were used: one was MSC-derived ECM scaffold and another was chondrocyte-derived ECM scaffold. The MSCs attached, proliferated and showed high viability in both MSC- and chondrocyte-derived ECM scaffolds. There was no significant difference between these two scaffolds. The size, GAG content and dry weight of engineered tissue regenerated in the ECM scaffolds were higher than those of tissue regenerated by conventional pellet culture. The gene expression of genes encoding collagen I, collagen II, aggrecan, collagen X, and sox 9 was examined by real-time PCR. The expression of these genes was up-regulated when MSCs were cultured in the ECM scaffolds in comparison to pellet-cultured MSCs. The gene expression level increased with culture period. Hematoxylin and eosin staining indicated that the cells showed round morphology. Safranin O-positive staining indicated the existence of GAG. Toluidine blue staining demonstrated the typical metachromasia of cartilage. Immunohistochemical staining with antibodies to type I and type II collagen and cartilage aggrecan showed that the type I collagen, type II collagen and cartilage aggrecan were positively stained. These matrices surrounded the cells. These results indicate that cartilage-like tissue was regenerated after MSCs were cultured in the ECM scaffolds derived from MSCs or chondrocytes.

Figure 4.1 The illustration of cartilage tissue engineering by using ECM scaffolds. (A) Engineered cartilage-like tissues. (B) Toluidine blue staining of cartilage-like tissue.

4.2 Introduction

Articular cartilage is avascular and consists of sparsely embedded chondrocytes in an organic matrix primarily consisting of type II collagen and proteoglycan. It possesses limited capacity for intrinsic repair or regeneration, and even minor lesions or injuries may lead to progressive damage and joint degeneration. Although many methods including subchondral drilling, osteochondral allografting, and periosteal or perichondral tissue grafting have been tried in attempts to repair defects in articular cartilage, these techniques cannot reproduce the reparative tissue characteristics of hyaline cartilage or optimal filling of the defects [1-5] . In rescent years, tissue engineering has been rapidly developed and been proved to be one of the most promising alternative therapies for articular cartilage defects[6-8]. This approach consists of an interactive triad of responsive cells, a supportive matrix, and bioactive molecules promoting differentiation and regeneration.

Numerous scaffolds and matrices have been explored for cartilage tissue engineering including both natural (collagen, chitosan, hyaluronic acid, alginate, etc.) and synthetic polymers (PLA, PGA, PLGA, PEG, etc). But they have respective limitations. For example, although polyester scaffolds have been widely used, they too have drawbacks such as poor cell attachment, difficulty in molding, and mild inflammatory reaction once implanted in vivo.

Chondrocytes, the major cell type that is present in differentiated cartilage, is the most obvious cell option for cartilage tissue engineering. But chondrocytes have some drawbacks that limit their applications in cartilage regeneration. Mesenchymal stem cells (MSCs) are a prospective cell source for the engineering of cartilage tissue. They are relatively easy to obtain from a small aspirate of bone marrow and have multiple potentials to differentiate into different cell lineages such as osteoblasts, chondrocytes, adipose cells, ligament cells, and neural cells [9,10] . The applications of MSCs in cartilage tissue engineering have achieved great success.

In this chapter, MSC-derived ECM scaffolds (AESca-M) and chondrocyte-derived ECM scaffolds (AESca-C) were used for the culture of MSCs because the MSCs and chondrocytes are the most commonly used cells types for cartilage tissue engineering.

4.3 Materials and methods

4.3.1 Preparation of AEsca-M and AESca-C

AEsca-M and AESca-C were prepared from mesenchymal stem cells and articular chondrocytes as described in Chapter 2.

4.3.2 Cell seeding and chondrogenic differentiation of MSCs in AESca

MSCs (P5) were collected by Trypsin/EDTA treatment. Cells were washed once by serum-free medium and seeded in the scaffolds at a cell density of 1×10^6 cells/mL (100μl/scaffold). The scaffolds were spread in 60mm tissue culture dish (Falcon) and covered with sterile glass

rings (inner Φ=5mm, outer Φ=7mm) to avoid cell leakage. The rings were removed after culture for 3h. The constructs were then transferred into 50mL conical polypropylene tubes containing in chondrogenic induction medium composed of Dulbecco's modified Eagle's medium (high glucose, Sigma) supplemented with 4mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.1 mM nonessential amino acid, 0.4 mM proline, and 50 μg/mL ascorbic acid, 100 nM dexamethasone, 1% ITS+1, and 10 ng/mL transforming growth factor β3 (TGF-β3). The cells were cultured for 4 weeks. The polypropylene tubes were used to reduce the adhesion of TGF-β3.

The cell adhesion in the scaffolds was observed by a scanning electron microscope. After culture for two hours and 1 week, the scaffolds were washed by PBS, fixed by 0.1% glutaraldehyde/PBS. After freeze-drying, the scaffolds were coated with Pt and observed by SEM.

And the conventional pellet culture of MSCs was carried out for the comparison. One hundred microliter cell suspension (1×10^6 cells/mL) was added to 3mL serum-free medium in 15mL conical polypropylene tubes and centrifuged at 500×g for 5min. The cell pellet was detached by gently pipetting after an incubation of 12h. Then the cell pellets were cultured by the same method that used for cell culture in ECM scaffolds.

4.3.3 Cell seeding efficiency

The cell seeding efficiency was investigated by counting the unattached cells in the cell culture dishes. The unattached cells were collected from the dishes after moving the scaffolds to conical polypropylene tubes by trypsin/EDTA and counted by a hemocytometer. The attached cell number was calculated by subtracting ht unattached cell number form the seeded cell number. The cell seeding efficiency was calculated by dividing the attached cell numbers with the total seeding cell number. Eleven samples were used to calculate the mean and standard derivation.

4.3.4 Viability of MSCs in AESca

After being cultured for 4 weeks, the cell viability was examined by a Cellstain live-dead double staining kit (Dojindo, Tokyo, Japan). The constructs were washed by PBS for 3 times and incubated in 2 μM Calcein-AM (stain live cells) and 4μM Propidium Iodide (PI) (stain dead cells) in serum-free DMEM for 40 min. And then the constructs were immersed in serum-free medium in a 35mm tissue culture dish after being rinsed by PBS. The cells were observed under a laser confocal scanning microscope.

4.3.5 GAG amount of engineered tissues

The engineered tissue samples were washed with PBS and MilliQ water, and freeze-dried. The dry weight of the samples was measured using an electronic balance(AG135, Mettler Toledo, Switzerland) and the GAG amounts were measured as the same method described in Chapter 2. The constructs were digested in 100μl papain solution at 60°C for 6h. Papain (Sigma) was dissolved at 400μg/mL in sterile 0.1M phosphate buffer, pH 6.0, with 5 mM cystein·HCl and 5mM EDTA. The sulfated-glycosaminoglycan (sGAG) amount was measured by Blyscan™ sulfated

sGAG assay kit (Biocolor) according to the manufacturer's manual. The specimen lysate was mixed with Blyscan™ dye to bind the sGAG. And the sGAG-dye complex was collected by centrifugation. After removing the supernatant and draining the tube, the dissociation reagent was added. Finally 200μl solution was transferred into each well of a 96-well plate. The absorbance against the background control was obtained at a wavelength of 656 nm by a Benchmark Plus™ microplate spectrophotometer (Bio-Rad) and the GAG amount (n=3) was calculated by using a standard curve obtained with the standard sGAG supplied by the kit.

4.3.6 Histochemical analysis

The engineered tissue samples were washed by PBS and fixed with 10% neutral buffered formalin. They were embedded in paraffin and sectioned in 7μm thickness. The sections were dyed with HE staining for the morphology and stained with safranin O/fast green and toluidine blue for visualization of the extracellular GAG.

4.3.7 Immunohistochemical staining

Immunohistochemical analyses of type I collagen, type II collagen and aggrecan were performed with a kit named Dakocytomation Envision+ System-HRP (DAB) (Dako) according to the instructions. The deparaffinized sections were incubated with proteinase K for 10min for antigen retreaval, and blocked with peroxidase blocking solution for 10min and 10% goat serum solution for 30min. The sections were incubated with 1st antibodies for 1h followed by an incubation with peroxidase labelled polymer-conjugated 2nd antibodies for another 30 min. The sections were further incubated with 3,3' diaminobenzidine (DAB) chromogen solution for 10min to visualize the bound antibodies. The nuclei were counterstained with hematoxylin. All incubations were conducted at room temperature (RT). The antibodies were listed in Table 4.1.

Table 4.1 The antibodies used for the immunohistochemical staining

Name	Inoculated animal	Species Reactivity	Company	Dilution	2nd antibody
Anti-collagen I (affinity purified)	Rabbit	Human	Monosan	1:400	Polymer conjugated rabbit anti-rabbit IgG (Dako, 1:200)
Anti-collagen II (monoclonal 6B3)	Mouse	Human, mouse, chicken, salamander	Neomarker	1:200	Polymer conjugated rabbit anti-mouse IgG (Dako, 1:200)
Anti-Aggrecan (monoclonal)	Mouse	Human, bovine, rabbit, canine, chicken	Chemicon	1:1500	Polymer conjugated rabbit anti-mouse IgG (Dako, 1:200)

4.3.8 Gene expression analysis

The chondrogenesis relative gene expression was analyzed by real-time PCR reaction. Firstly, the cultured constructs were frozen in liquid nitrogen and crushed into powder after washing by PBS.

The powders were dissolved in 1mL Isogen (Nippon Gene) and the total RNA was extracted following the manufacturer's protocol. 1 μg total RNA was reversely transcribed into cDNA using random hexamer (Applied Biosystems) in 20 μL reaction on GeneAmp 9700 PCR systems (Applied Biosystems). An aliquot (1 μL) of 10-times diluted reaction solution was used for each 25 μL real-time PCR reaction together with 300 nM forward and reverse primers and 150 nM probes and qPCR Master Mix (Eurogentec). Real-time PCR analysis was conducted and monitored using a 7500 Real-Time PCR System (Applied Biosystems). After an initial incubation step of 2 min at 50°C and denaturation for 10 min at 95°C, 40 cycles (95°C for 15 s, 60°C for 1 min) PCR were performed. Reactions were performed in triplicate. GAPDH recombinant RNA levels were used as endogenous controls and gene expression levels relative to 18S were calculated using the comparative Ct method. Three samples under each condition were used for measurement to calculate the means and standard deviations. The primer and probe sequences were according to Martin et al [11]and Schaefer et al [12] (Table 4.2).

Table 4.2 Primes and Probes used for real-time PCR analysis

Gene	Primer 5'→3'	Probe 5'→3'
18S	F: GCCGCTAGAGGTGAAATTCTTG	CCGGCGCAAGACGGACCAGA
	R: CATTCTTGGCAAATGCTTTCG	
GAPDH	F: ATGGGGAAGGTGAAGGTCG	CGCCCAATACGACCAAATCCGTTGAC
	R: TAAAAGCAGCCCTGGTGACC	
Type I Collagen	F: CAGCCGCTTCACCTACAG	CCGGTGTGACTCGTGCAGCCATC
	R: TTTTGTATTCAATCACTGTCTTGCC	
Type II Collagen	F: GGCAATAGCAGGTTCACGTACA	CCGGTATGTTTCGTGCAGCCATCCT
	R: CGATAACAGTCTTGCCCCACTT	
Aggrecan	F: TCGAGGACAGCGAGGCC	ATGGAACACGATGCCTTTCACCACGA
	R: TCGAGGGTGTAGCGTGTAGAGA	
Sox 9	F: CACACAGCTCACTCGACCTTG	CCCACGAAGGGCGACGATGG
	R: TTCGGTTATTTTTAGGATCATCTCG	
Type X Collagen	F: CAAGGCACCATCTCCAGGAA	TCCCAGCACGCAGAATCCATCTGA
	R: AAAGGGTATTTGTGGCAGCATATT	

4.3.9 Statistical analysis

All results are presented as the mean ± standard deviation (SD). All the data fulfill the criteria of normal distribution (Kolmogorov-Smirnov test) and equal variance (Bartlett's Test). A two-tailed unpaired *t*-test was used to determine the cell seeding efficiency. For the other data, a one-way analysis of variance (ANOVA) was used for statistical analysis of the difference between groups in each time point. If overall ANOVA was significant ($P<0.05$), multiple comparisons were performed by the Tukey's post hoc test. Statistical significance was determined at $P<0.05$.

4.4 Results

4.4.1 Cell seeding

MSCs were seeded in the AESca-M and AESca-C. The cell seeding efficiency in the AESca-M and AESca-C were 82.1 ± 4.5% and 81.0 ± 6.5% (n=11), respectively. The ECM scaffolds showed high cell seeding efficiency. Observation with optical microscope and SEM demonstrated that the MSCs well attached to the AESca-M (Figure 4.2) and AESca-C (Figure 4.3). After cultured for 1 week, the MSCs proliferated to occupy the space in the ECM scaffolds (Figure 4.4). But there was no obvious difference between the AESca-M and AESca-C in the morphology and cell seeding efficiency.

Figure 4.2 Phase contrast micrographs (a, b) and SEM images (c, d) of MSCs after culture on the AESca-M for 2h.

Figure 4.3 Phase contrast micrographs (a, b) and SEM images (c, d)of MSCs after culture on the AESca-C for 2h.

Figure 4.4 SEM image of MSCs culture in AESca-M for 1 week. Scale bar=50μm.

4.4.2 Cell viability

The cell viability after culture in the scaffolds for 4 weeks was checked by live-dead staining (Figure 4.5). Live cells were stained green and dead cells red. Green living cells were observed while no red dead cells were detected. These results indicate that the cells showed high viability when culture in the ECM scaffolds.

Figure 4.5 Cell viability of MSCs after being cultured for 4 weeks. The image was obtained from laser scanning confocal microscope. Scale bar=100μm.

4.4.3 The cartilage-like tissues

The gross appearance of engineered tissues after culture for 1, 2 and 4 weeks in the ECM scaffolds and pellet culture was shown in Figure 4.6. The engineered tissues were glisteningly white like the native cartilage. The tissues became larger during the culture. The size of the tissues formed in the AESca was larger than that of pellet cultured MSCs.

Figure 4.6 Gross appearances of engineered tissues after 1, 2 and 4 week culture. M: AESca-M, C: AESca-C, P: pellet culture. Scale bar=5mm.

GAG content and dry weight of engineered tissues in the ECM scaffolds were higher than those of tissue regenerated by conventional pellet culture (Figure 4.7). The GAG content and dry weight increased with the culture time. There was no obvious difference between AESca-M and AESca-C. However, the GAG content and dry weight of pellet-cultured MSCs increased very slowly. These results indicate that the ECM scaffolds could promote the secretion of ECM.

Figure 4.7 GAG amount and dry weight of the engineered tissues after 1, 2 and 4 week culture. M: AESca-M, C: AESca-C, P: pellet culture. Data represent means ± SD (n=3). *, $P < 0.05$; * * *, $P < 0.001$.

4.4.4 Histological analysis

HE staining showed that the cells showed round morphology (Figure 4.8).

Figure 4.8 HE staining of engineered tissues after culture for 2w and 4w. Scale bar=500μm.

Safranin O/fast green staining showed the centered areas were positively stained by Safranin O (Figure 4.9). The Safranin O-positive staining indicated the existence of GAG. The GAG mainly existed in the center of the constructs.

Figure 4.9 Safranin O/Fast green staining of engineered tissues after culture for 2w and 4w. Scale bar=500μm.

Toluidine blue staining demonstrated the typical metachromasia of cartilage (Figure 4.10). The Safranin O and Toluidine blue stainings demonstrated the existence of cartilaginous ECM in the engineered tissues. The tissues formed in the ECM scaffolds were more strongly stained than

be those formed by pellet culture.

Figure 4.10 Toluidine blue staining of engineered tissues after culture for 2w and 4w. Scale bar=500μm.

4.4.5 Immunostaining

Immunohistochemical staining with antibodies to type I collagen, type II collagen and cartilage aggrecan showed that the type I collagen, type II collagen and cartilage aggrecan were positively stained. These matrices surrounded the cells (Figure 4.11). These results demonstrate that the engineered tissues have the cartilage specific ECM composition

Figure 4.11 Immunohistochemical staining of engineered tissues after culture for 4w. Scale bar=500μm.

4.4.6 Real-time PCR

The expression of genes encoding collagen I, collagen II, collagen X, aggrecan, and sox 9 was examined by real-time PCR (Figure 4.12). The expression of these genes was up-regulated when MSCs were cultured in the ECM scaffolds in comparison to pellet-cultured MSCs. The gene expression level increased with culture period. The gene expression results, together with the histological and immunohistochemical staining results, indicate that cartilage-like tissue was engineered when MSCs were culture in the MSC- and chondrocyte-derived ECM scaffolds. Both the MSC- and chondrocytes-derived ECM scaffolds promoted chondrogenic differentiation of MSCs. Furthermore, MSC-ECM promoted the chondrogenic gene expression than did the NHAC-ECM. It might be caused by the composition differences between the scaffolds. Therefore, the studies to investigate the differences of the ECM scaffolds derived from various cell types are necessary.

Figure 4.12 Real-time PCR of cartilage tissue engineering. Control means the MSCs used for cell seeding. Data represent means ± SD (n=3). *, $P < 0.05$; * *, $P < 0.01$;* * *, $P < 0.001$.

4.5 Discussion

Adult articular cartilage is an avascular tissue with limited self-repair capacity. Autologous cell-based cartilage tissue engineering provides a promising option for the repair of severe cartilage damages caused by trauma or aging-related degeneration such as osteoarthritis. Successful cartilage tissue engineering requires cells capable of undergoing chondrogenic differentiation upon treatment with appropriate biochemical factors and a three-dimensional (3-D) porous scaffold capable of providing a favorable environment for chondrogenic cell growth and new cartilage-specific ECM formation.

Developmentally, skeletal cells [13-17] like osteoblasts and chondrocytes are derived from a common cell source, mesenchymal stem cells (MSCs) [18]. MSCs provide an attractive cell source for cartilage tissue engineering in vitro and in vivo [19-33]. To fully emulate chondrogenesis in vivo, in vitro chondrogenesis of bone marrow-derived MSCs requires cells to be cultured at high density

in defined serum-free medium in a 3-D environment that favors cell condensation and cell–cell interactions analogous to that which occurs during embryonic skeletal development [34-37]. The inherent limitations associated with the conventional cell pellet culture such as weak mechanical properties, inadequate size, uncontrollable morphologies, and poor processability make this culture model impractical for cartilage repair. A broad band of natural and synthetic biomaterials in various forms have been used to support chondrogenesis of MSCs in vitro and/or in vivo [38-40].

The cell adhesion to the ECM scaffolds, which name "3D-matrix adhesion", could enhance cell seeding efficiency, migration, proliferation and other cellular functional activities.[41] In this study, the ECM nano-structure and ECM component such as collagen and fibronectin will benefit the MSC adhesion on the scaffolds. It has been reported that aggrecan improve the chondrogenic differentiation of MSC and be use for to inhibit the chondrocytes dedifferentiation. Our ECM scaffolds contained certain amount of aggrecan. Furthermore, the other ECM molecules could improve the cell proliferation and upregulate the differentiation. Thus, the differentiation and proliferation of MSCs in the ECM scaffolds are promoted compared with pellet cultured MSCs.

Another major cell source used to generate engineered cartilage tissue is chondrocytes, commonly isolated from articular cartilage tissues [42,43]. Freshly isolated MSCs and chondrocytes are normally present in limited numbers. The cells have to be expanded in vitro in order to obtain enough cells for cartilage tissue engineering applications or for related cell therapeutic strategies. The expansion of MSCs can be achieved by culturing the cells in the presence of basic fibroblast growth factor (bFGF) without significantly losing their multi-lineage differentiation potential[44,45] . In contrast, the in vitro expansion of freshly isolated chondrocytes in monolayer culture tends to induce their dedifferentiation, even in the presence of chondrogenic factors like transforming growth factor β (TGF-β1) [46-48]. These dedifferentiated chondrocytes lose the spherical morphology that chondrocytes possess in the physiological environment and acquire a fibroblast-like morphology [46,49]. The dedifferentiation of chondrocytes during expansion in monolayer culture is also characterized by a dramatic decrease in the synthesis of cartilage-specific ECM molecules like collagen type II and aggrecan. These dedifferentiated chondrocytes can be induced to redifferentiate by culturing the cells in a 3-D environment favoring a spherical morphology and the synthesis of cartilage-specific ECM [46,50,51]. Culture expanded autologous articular chondrocytes have been used to treat full-thickness cartilage defects [42,43]. Culture-expanded chondrocytes are not as multipotent as MSCs; therefore, they are less likely to generate unwanted cell types when used for the repair of severely damaged cartilage tissue. Therefore, in the present study, chondrocytes were not used to generate cartilage-like tissues. But it should be interesting to applied ECM scaffolds with chondrocytes for cartilage tissue engineering.

Synthetic and natural polymers are the primary scaffold materials used to create the 3-D environment for cartilage tissue engineering applications [19-33]. However, little research has investigated cartilage ECM-derived biomaterials for use as scaffolds for cartilage tissue engineering. Also, cartilage decellularization is difficult, because cartilage is too compact to allow efficient penetration of solutions used in the decellularization procedure. It has been reported that xenogenic cartilage ECM scaffolds were developed [52]. But ECM was reconstructed and crosslinked. In our work, the ECM scaffolds is away from the similarity to the native cartilage

ECM. Therefore, it is necessary to mimic a more similar environment of the native cartilage by the ECM scaffolds for tissue engineering.

4.6 Conclusions

The ECM scaffolds derived from MSCs and chondrocytes supported cell adhesion and proliferation of MSCs. The ECM scaffolds promoted chondrogenic differentiation of MSCs. The two ECM scaffolds showed similar effects. Cartilage-like tissues were formed when MSCs were cultured in the ECM scaffolds. It's prospective that the application of MSCs- and chondrocyte-derived ECM scaffolds in cartilage tissue engineering.

4.7 References

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Chapter 5

Skin tissue engineering using ECM scaffolds

5.1 Summary

In this chapter, ECM scaffolds were used for the culture of human skin fibroblasts to examine their potential for skin tissue engineering. Fibroblasts were seeded on the ECM scaffolds derived from fibroblasts. After being cultured for certain time, the cells, newly-produced ECM and the ECM scaffolds, built dermis-like constructs.

When fibroblasts were cultured in the fibroblast-derived ECM scaffold, the cells were viable in the scaffold. SEM observation showed that fibroblasts proliferated and filled the pores and space in the scaffold to form layered structure. Histological staining of the cultured fibroblast/ECM implants indicated that the fibroblasts distributed homogenously throughout the scaffold and formed a uniform layer of dermal tissue.

Figure 5.1 Illustration of skin tissue engineering by using ECM scaffolds. (A) The engineered dermal tissue. (B) The viability of fibroblasts in the engineered tissue.

5.2 Introduction

The engineering of skin tissue has been at the forefront of tissue engineering for many years and has now yielded some of the first medical products to come from tissue engineering [1-5]. Today, over 200,000 patients have been treated with tissue-engineered skin products. This is the result of work over the last 30 years in the areas of skin cell biology, extracellular matrix biology, collagen scaffolds, polymer scaffolds, and tissue equivalents. The repair of skin using tissue engineering has taken on many forms, from simple to complex.

The use of cultured fibroblasts to treat skin defects, such as wound trauma or burn injury, has been developed as a challenging method of wound healing [2,3,6]. A temporary scaffold serves as a support for culturing the fibroblasts to construct a three-dimensional skin architecture [7].

Numerous scaffolds have been developed from naturally derived biodegradable polymers for culturing human fibroblasts [4,8-14]. Collagen-based porous scaffolds have been the most popular for skin tissue engineering [15-21]. Combination of a few kinds of isolated extracellular matrices such as collagen and glycosaminoglycans has been reported to reconstruct the biomimetic scaffolds for skin tissue engineering. However, in vivo extracellular contains many kinds of molecules and have very complicated structures. Although isolated ECM components or their combination may mimic *in vivo* ECM to some degree, it has been difficult to reconstruct an *in vivo* ECM structure by conventional physical and chemical methods. Therefore, *in vitro* cultured cells have been used to reconstruct *in vivo* ECM [22-24]. For example, Matrigel™, which is an extract of basement membrane secreted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma cells, has been developed as a substratum for cell culture. Cholangiocytes can form a bile duct-like structure in Matrigel™ [22]. Extracellular matrices synthesized by mesenchymal stem cell-derived osteoblasts on a Ti surface have been used in the culture of mesenchymal stem cells to promote their osteogenic differentiation [23]. Primary hepatocytes can maintain their viability and liver-specific functions for a longer time on reconstructed basement membrane formed by alveolar epithelial cells than they can on type I collagen and Matrigel™ [24].

The dermis is a layer of connective tissue and serves as the base for the epidermis, providing structural support and mechanical strength to the skin. In this study, we used cultured fibroblasts to construct ECM scaffolds. Then we repopulated the ECM scaffolds with fibroblasts to build dermis-like tissues. The engineered dermis-like tissues could be used for skin tissue engineering.

5.3 Materials and methods

5.3.1 Scaffold fabrication

The ECM scaffolds used in this study were prepared from cultured fibroblasts (AEsca-F) as described in Chapter 2.

5.3.2 Cell seeding and cell culture of NHDFs in AESca-F

NHDFs (P5) were detached by Trypsin/EDTA treatment and centrifuge at 200 × *g* for 5 min. The cells were resuspended in Medium 106 at a cell density of 2.5×10^5 cells/mL. Then the fibroblasts were seeded in the ECM scaffolds (100μl/scaffold) and cultured in Medium 106 for 2 weeks. During the cell seeding, the scaffolds were spread in 60mm tissue culture dish (Falcon) and covered with glass rings (inner Φ=5mm, outer Φ=7mm) to avoid cell leakage. The rings were removed after culture for 3h.

To investigate the cell adhesion and distribution in the scaffolds, the fibroblasts cultured in the ECM scaffolds for 30min, 12h and 4 days were fixed with 0.25% glutaraldehyde/PBS at room temperature for 1 h. After being rinsed with PBS and deionized water, the samples were freeze-dried. The samples were coated with gold using a sputter coater and observed by a scanning electron microscope (SEM).

5.3.2 Cell seeding efficiency

The cell seeding efficiency was investigated during cell seeding. After 3h culture, the unattached cells were collected by trypsin/EDTA and counted by a hemocytometer. The cell seeding efficiency was calculated by dividing the attached cell numbers by total seeding cell numbers. Six samples were used to calculate the mean and standard deviation.

5.3.3 Cell viability

The fibroblast viability was examined with a CellStain live-dead double staining kit (Dojindo). The constructs were washed by PBS for 3 times and incubated in 2μM Calcein-AM (stain live cells) and 4μM Propidium Iodide (PI) (stain dead cells) in serum-free medium 106 for 30 min. And then the constructs were immersed in serum-free medium in a 35mm tissue culture dish after being rinsed by PBS. The staining was observed under fluorescent microscope.

5.3.4 Hoechst 33258 staining

The cells cultured in the scaffolds for 12 h and 4 days were washed by PBS and fixed by 10% neutral buffered formalin. The samples were stained by immersing in PBS containing 10μg/mL Hoechst 33258 for 10min. The cells were observed under a fluorescent microscope after washing with PBS.

5.3.5 HE staining

The engineered tissues after culture for 2 weeks were fixed with 10% neutral buffered formalin, embedded in paraffin and sectioned in 7-μm thickness. The sections were stained with hematoxylin and eosin.

5.4 Results

5.4.1 Cell adhesion and distribution in AESca-F

Human dermal fibroblasts, NHDF, were seeded and culture in the fibroblast-derived ECM scaffolds (AESca-F). The cells adhered in the network of the scaffolds (Fig 7a). The cells distributed in the pores and on the walls of the ECM scaffolds. The cell seeding efficiency was 84.7 ± 8.1% (n=6). These results indicated that the ECM scaffolds supported adhesion of fibroblasts.

Figure 5.2 Phase contrast micrographs (a, b) and SEM images (c, d) of NHDFs cultured in AESca-F for 30 min (a, c) and high magnification (b, d).

5.4.2 Cell proliferation

Fibroblast could be obviously observed under optical microscope (Figure 5.3c) 30min after cell seeding. The nuclear staining by Hoechst 3328 also demonstrated the cell adhesion in the scaffolds (Figure 5.3d, e). The cells spread on the scaffolds after 3h culture and proliferated to fill the pros and spaces in the ECM scaffolds after 4 day culture. No cell could be found by optical microscope (Figure 5.3h) but they still existed in the scaffolds (Figure 5.3i, j). Nuclear staining with Hoechst 33258 demonstrated that cells only adhered on the ECM flakes and fiber in the scaffolds after 30 min culture. The cells spread to close up the openings in the mesh-like ECM scaffolds after 3h culture. Cell proliferated to cover all the openings and distributed homogenously.

Figure 5.3 The ECM scaffolds were recellularized by dermal fibroblasts. (a)-(e) 30min post after cell seeding; (f)-(j) 3h after cell seeding; (k)-(o) 4d after cells seeding. Scale bars in (a), (f) and (k) are 100 μm. Scale bars in (b), (g) and (l) are 50 μm. In the other images, scale bar are 200 μm.

5.4.3 Cell viability

The viability of fibroblasts culture in the ECM scaffolds for 12h, 24h and 2weeks were evaluated by Calcein-AM/PI double staining (Figure 5.4). Almost all the cells were stained green and almost no red dead cell was detected. The cells showed high viability in the ECM scaffolds.

Figure 5.4 Calcein-AM/PI double staining of the cells cultured in the ECM scaffolds for 12h (a, b), 24h (c) and 2 weeks (d).

5.4.4 Histology

HE staining (Figure 5.5) showed that the engineered tissue was composed of cells and ECM. The construct was dense and had multiple cell layers. These results indicate that the formation of dermal tissue when fibroblasts were cultured in the ECM scaffolds. Cell distributed homogenously in the scaffolds

Figure 5.5 HE staining of the cross sections of engineered tissues after 2 weeks culture at low (a) and high (b) magnification.

5.5 Discussion

There are several functions for the skin, including protection, regulation of temperature, and sensation. Given the anatomical location and histological structure, the skin serves to protect the internal tissues and organs from injury induced by environmental factors, including chemical toxins, corrosive agents (acids and bases), mechanical forces (stretching, compressing, and shearing), microorganisms (bacteria and viruses), and radiations (UV and X-ray). The skin contains various types of sensory nerve endings, which can sense temperature changes, pressure, mechanical contacting, and chemical corrosion. These sensory structures are critical to the protection of the body from dangerous environmental factors. The skin consists of a rich network of blood vessels, which play an important role in the regulation of body temperature. An increase in the body temperature induces arteriolar dilation, leading to an increase in blood flow to the body surface and thus facilitating heat loss. A decrease in the body temperature exerts an opposite effect. The excretion of sweat is another mechanism that facilitates the loss of body heat and reduces body temperature. The skin participates in the synthesis of vitamin D, a hormone that regulates the metabolism of calcium and phosphate (stimulating the absorption of calcium and phosphate in the intestines, and increasing the level of blood calcium and phosphate). In addition, the skin is an important structure that protects the body from water loss.

The skin loss is one of the oldest and still not totally resolved problems in surgical field. Due to the spontaneous healing of the dermal defects would not occur, the scar formation for the full thickness skin loss would be inevitable unless some skin substitutes are used. In the past decades, many skin substitutes such as xenografts, allografts and autografts have been employed for wound healing. However, because of the antigenicity or the limitation of donor sites, the skin substitutes mentioned above cannot accomplish the purpose of the skin recovery and yet not be used widely [5,25-28]. Therefore, many studies are turning toward the tissue engineering approach, which utilizes both engineering and life science discipline to promote organ or tissue regeneration and to sustain, recover their functions [29-32].

Dermal fibroblasts are an important cell source in tissue engineering. Dermal fibroblasts may be easily isolated and used up to passage 14 without a decrease in collagen synthesis or a

reduction in growth rate. The fibroblasts have been widely used for built skin equivalents [33]and other tissues [34]. The fibroblasts could produce ECM molecules, such as collagens, glycosaminoglycans and glycoproteins [35-38].

The dermal fibroblasts were cultured in the fibroblast-derived ECM scaffolds. The ECM scaffolds promoted the adhesion and proliferation of fibroblasts. The ECM molecules in the scaffolds might function as adhesion-promoting factors to support cell adhesion. Dermal tissue was regenerated after 2 weeks culture. The ECM scaffolds facilitated skin tissue formation and will be useful for skin tissue engineering.

Subcutaneous antigen encounter favors the induction of an immune response because of the high density of dendritic cells in the skin – skin grafts therefore can be expected to be more immunogenic than some other implants [39]. Therefore, it's critical to use low immunogenicity or high biocompatibility scaffolds in the skin tissue engineering. Our autologous ECM scaffolds showed excellent biocompatibility. This property can improve the chance of successful skin engineering.

The ECM scaffolds would provide the similar native structure for the fibroblasts. The ECM components, collagen, fibronectin, laminin improved the adhesion and proliferation of seeded fibroblasts. Furthermore, high interconnectivity will give cells more proliferate space, more cell-cell communication, and more nutrient supply. Another important advantage of ECM scaffolds that they can induce angiogenesis, which are critical for the final success of skin tissue engineering.

Uniform distribution of cells and extracellular matrices in the engineered skin can also provide some effects to protect tissue contraction during the formation of new tissue. Contraction and scarring are principal concerns during skin regeneration. In the results, homogenous distribution of fibroblasts could be observed. Therefore, the dermal tissue engineered with ECM scaffolds should be useful in the skin tissue engineering.

The skin is a structure that interacts with the external environment at one side and the internal connective tissue at the other side. Thus, a growth model for constructing skin substitutes should provide an environment that mimics the physiological conditions for the natural skin. Such a model can be established by using several necessary components, including epidermal stem cells or keratinocytes [20], fibroblasts (for the formation of dermis-like connective tissue [7]), a matrix scaffold, essential growth-stimulating factors, and culture media. In a skin growth apparatus, a sheet-like matrix scaffold can be constructed with either collagen gel, composite matrix components including collagen, fibronectin, and proteoglycan, or biodegradable polymers. Collected fibroblasts can be seeded in the matrix scaffold with the cells submerged under a culture medium. Epidermal cells or stem cells can be identified, collected, expanded, and seeded on the top of the fibroblast-containing matrix scaffold. Alternatively, host skin specimens can be collected and directly placed on the fibroblast-containing matrix scaffold. In the later case, the surface of the skin specimen should be exposed to the air. The culture medium may be supplemented with desired growth-stimulating factors, such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), and/or fibroblast growth factor (FGF). Other necessary components may also be added, such as insulin (to promote the uptake of glucose and amino acids) and hydrocortisone (to promote cell adhesion and proliferation). The skin constructs can be

cultured under standard conditions (37°C, 5% CO_2 and 95% air). The constructed skin substitutes usually exhibit a skin-like structure and express common epidermal cell markers [40]. The skin construct can be collected and applied to the injured skin. Artificial skin with full layers and functions are necessary for the patients. It will be a great challenge to build up such kind of constructs.

5.6 Conclusions

The fibroblast-derived ECM scaffolds supported fibroblasts adhesion and proliferation of fibroblasts. The fibroblasts showed high viability in the ECM scaffolds. Dermal-like tissue was constructed after fibroblasts were cultured in the ECM scaffolds for 2 weeks. The ECM scaffolds will be useful for skin tissue engineering

5.7 References

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Chapter 6

Concluding remarks and future directions

6.1 Concluding remarks

This thesis summarizes the work for the fabrication of novel biomimetic autologous extracellular matrix scaffolds for tissue engineering. Furthermore, it describes the results of the biocompatibility and applications for cartilage and skin tissue engineering of the fabricated ECM scaffolds.

In **chapter 1**, a general introduction is given to describe the present status of tissue engineering. The basic concept, scaffold materials and cell sources of tissue engineering are reviewed. Furthermore, the objective of this study was defined.

Chapter 2 summarizes the preparation of ECM scaffolds. The ECM scaffolds were prepared by using cultured cells and decellularization method. Decellularization with freeze-thaw cycling plus NH_4OH was the optimal method. And the PLGA template was completely removed. All the MSCs, chondrocytes and fibroblasts can be used to prepare the ECM scaffolds. The ECM scaffolds showed mesh-like porous structures. The ECM scaffolds were mainly composed of type I collagen, fibronectin and other ECM molecules. A certain amount of GAG was reserved in the ECM scaffolds. The composition of the ECM scaffolds changed a little depending on the cell types.

Chapter 3 describes the biocompatibility of ECM scaffolds. The biocompatibility of was evaluated by using subcutaneous implantation test. The evaluation techniques included histological analysis, immunohistochemical staining for the immunocyte classification and real-time PCR for the cytokine gene expression. The autologous ECM scaffolds induced the least inflammatory and immunoreactions. The autologous ECM scaffolds were completely integrated with the host tissues. The excellent biocompatibility would facilitate the application of autologous ECM scaffolds in the tissue engineering.

Chapter 4 summarizes the results of three-dimensional culture of MSCs in the ECM scaffolds for cartilage tissue engineering. The ECM scaffolds derived from MSCs and

chondrocytes supported cell adhesion and proliferation of MSCs. The ECM scaffolds promoted chondrogenic differentiation of MSCs. The two ECM scaffolds showed similar effects. Cartilage-like tissues were formed when MSCs were cultured in the ECM scaffolds. It's prospective that the application of MSCs- and chondrocyte-derived ECM scaffolds in cartilage tissue engineering.

Chapter 5 summarizes the results of culture of fibroblasts in the ECM scaffolds for skin tissue engineering. The fibroblast-derived ECM scaffolds supported fibroblasts adhesion and proliferation. The fibroblasts showed high viability in the ECM scaffolds. Dermis-like tissue was constructed after fibroblasts were cultured in the ECM scaffolds for 2 weeks. The ECM scaffolds will be useful for skin tissue engineering.

6.2 Future directions

This work fabricated the autologous ECM scaffolds for the first time. It is believed that quite a lot of researches should be performed on this field. Firstly, improvement of ECM scaffolds parameters, such as components (both qualitive and quantitive aspects), the structures (macro, micro and nano aspects) and the mechanical strength, are near at hand.

In this work, only four kinds of cells were used to prepare the ECM scaffolds. But human bodies are composed of numerous kinds of cells. Different cells have their unique characters, respectively. The components and structures should be different in the ECM scaffolds derived from different cells types. Even the fabrication methods and conditions would cause certain changes to the ECM scaffolds. Therefore, special concerns should be given to the development of ECM scaffolds when different cells were used. Besides, the mechanisms for interactions between ECM and cells should be elucidated. Thus, further researches should be done to determine the favorite ECM scaffolds for a specific regeneration purpose.

Biocompatibility is an important issue in tissue engineering. Long time and wide range studies should be taken to investigate the in vivo performance of autologous ECM scaffolds, which are critical for the clinical applications. Furthermore, fetal bovine serum was used for cell culture. The bovine origin molecules would cause adverse effect. The researches for chemical defined culture or serum free culture would benefit the solution of this problem.

ECM scaffolds could be applied to engineer more complicate tissues or organs. Although it would like to be a great challenge, the autologous ECM scaffolds are supposed to give a breakthrough in tissue engineering. In conclusion, novel autologous ECM scaffolds were developed by cell culture in a biodegradable porous scaffold and decellularization. The cell-derived ECM scaffolds promoted cell adhesion, proliferation and differentiation, and showed excellent biocompatibility. The autologous ECM scaffolds would have a promising future in tissue engineering.

List of publications

1. Hongxu Lu, Likun Guo, Naoki Kawazoe, Tetsuya Tateishi, Guoping Chen; Effects of poly(L-lysine), poly(acrylic acid) and poly(ethylene glycol) on the adhesion, proliferation and chondrogenic differentiation of human mesenchymal stem cells, Journal of Biomaterials Science: Polymer Edition, in press.

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3. Hongxu Lu, Xiaogang Jin, Naoki Kawazoe, Tetsuya Tateishi, Guoping Chen, Jiang Chang; *In vitro* proliferation and osteogenic differentiation of human bone marrow-derived mesenchymal stem cells cultured with hardystonite ($Ca_2ZnSi_2O_7$) and β-TCP ceramics, Journal of Biomedical Materials Research Part B: Applied Biomaterials, submitted.

4. Takashi Hoshiba, Tomoe Yamada, Hongxu Lu, Naoki Kawazoe, Tetsuya Tateishi, Guoping Chen; Nuclear deformation and expression change of cartilaginous genes during *in vitro* expansion of chondrocytes, Biochemical and Biophysical Research Communications, 374, 688-692 (2008).

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7. Hongxu Lu, Takashi Hoshiba, Naoki Kawazoe, Tetsuya Tateishi, Guoping Chen; Decellularization of three-dimensionally cultured cells in a biodegradable scaffold, in preparation.

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