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**DEVELOPMENT OF TISSUE ENGINEERING GRAFTS
FOR OSTEOCHONDRAL REGENERATION**

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School of Chemical and Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2018

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Wang Dongan

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was the first and/or corresponding author.

Chapter 2 is published as X. Nie, D.-A. Wang, Decellularized orthopaedic tissue-engineered grafts: biomaterial scaffolds synthesised by therapeutic cells, *Biomater. Sci.* (2018). doi:10.1039/C8BM00772A.

The contributions of the co-authors are as follows:

- A/Prof Wang Dongan provided the initial project direction and edited the manuscript drafts.
- I prepared the manuscript drafts.

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SUMMARY

Tissue engineering is the combination of cells, biomaterials and growth factors to stimulate the regeneration of damaged tissue or replace the dysfunctional tissue. Since the first success of proliferation of keratocytes in vitro, tissue engineering grafts have been regarded as a promising alternative method to current allo-, auto- or xeno-graft treatment for loss of osteochondral tissue due to injury. Although widely applied, allo and xeno grafts bears the risk of disease transmission and immune rejection, whereas auto graft faces the challenge of limited source and donor site morbidity. In addition, due to the dense nature of osteochondral tissue, native tissue derived grafts meets the problem of integration with surrounding tissue. Hence tissue engineering grafts are attracting more and more attention as methods to repair osteochondral defects.

Osteochondral tissue consists of two different types tissue, i.e. cartilage and subchondral bone, clapping a transition zone in between. Hence to develop a full-scaled graft with two different phases matching the cartilage and bone respectively and a transition zone in the interface is critical to the success of the field. On the other hand, when entering clinical application, living tissue engineering grafts bring about problems in logistics and cell sources. Therefore, decellularized tissue engineering grafts become popular as an acellular version of osteochondral grafts, where the cells were removed, and the extracellular matrix were retained.

In the first instance, the biphasic grafts with a naturally formed interpenetration network as the transition zone was fabricated by using sintered poly (lactic-co-glycolic acid) microspheres scaffold as the subchondral bone phase and microcavity 3D chondrocytes culture system to generate the cartilage phase. The interpenetration network was formed by the natural cell migration process from the cartilage phase to

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In the second instance, a scaffold-free graft fabricated using a transient scaffold named living hyaline cartilage graft was decellularized to make an acellular hyaline like manmade cartilage graft which broadens the cell source and circumvent the hurdles in clinical regulation. A combination of physical, chemical and biological methods has been applied on the living hyaline cartilage graft to generate decellularized manmade hyaline cartilage graft which was evaluated for the capability to repair both cartilage defect and osteochondral defect in both pig and rabbit animal models. The graft has been shown to regenerate cartilage and subchondral tissue in the defect with the same morphology and composition as native cartilage and subchondral bone tissue. In addition, the thorough integration to the surrounding tissue has been exhibited. These results are due to the dual advantages of genuineness in phenotype, composition and architecture of the graft to native cartilage and high porosity to facilitate cell migration which eventually leads to the recovery of the function of osteochondral tissue, i.e. the mechanical strength.

In summary, the tissue engineering grafts were characterized and the efficacy to repair osteochondral grafts were evaluated. It was found that the biphasic tissue engineering living grafts were able to repair osteochondral defects successfully whereas the decellularized hyaline-like cartilage graft was able to repair both cartilage and osteochondral defects successfully. Collectively, the results implied the sufficiency of using cartilage graft to repair osteochondral defects which requires further detailed study to elucidate the mechanism.

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LIST OF ABBREVIATIONS

μCT	Microcomputed tomography
ACI	Autologous chondrocyte implantation
BMC	Bone marrow cell
BST	Bone marrow stimulation technique
BV/TV	Bone volume to tissue volume
CC medium	Chondrocyte culture medium
CH ₂ Cl ₂	Dichloromethane
CNS	Central nervous system
Col I	Type I collagen
Col II	Type II Collagen
Col III	Type III collagen
COMP	Cartilage oligomeric matrix protein
DAPI	4',6-diamidino-2-phenylindole
dLhCG	Decellularized LhCG
dLhCG+	Decellularized full-scaled osteochondral graft
DMEM	Dulbecco's Modified Eagle Medium
DMMB	1,9-dimethylmethylene blue
DTT	Dithiothreitol
ECM	Extracellular matrix
ECM-LhCG+	ECM block of LhCG+ construct
EDTA	Disodium ethylene diamine tetra acetic acid
FBS	Fetal bovine serum
FE-SEM	Field emission scanning electron microscope
GAG	Glycosaminoglycan
H&E	Hematoxylin and eosin

HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
HHGS	Histological/Histochemical Grading System
IACUC	Institutional animal care and use committee
ICRS	International Cartilage Repair Society
IHC	Immunohistochemistry
IPN	Interpenetrating network
LhCG	Living hyaline cartilage graft
LhCG+	Full-scaled osteochondral graft
MSC	Mesenchymal stem cell
MT	Masson's Trichrome
NEAA	Non-essential amino acids
OATS	Osteochondral autograft transfer system
OCT compound	Optimal cutting temperature compound
PBS	Phosphorous buffered saline
PCL	Poly (ϵ -caprolactone)
PGs	Proteoglycans
PLA	Poly lactide
PLGA	Poly (lactic-co-glycolic acid)
ROI	Region of interest
Saf O	Safranin O
SDS	Sodium dodecyl sulphate
SDSC	Synovium derived stem cell
SEM	Scanning electron microscope
SMS	Sintered microspheres scaffold
SMS-LhCG+	SMS block of LhCG+ construct

TGF- β	Transforming growth factor beta
TKS cocktail	Tiletamine, zolazepam, ketamine and xylazine
TMJ	Temporomandibular joint
TV	Total tissue volume

Chapter 1 Introduction

Hydrogel-based scaffolds remain a prevalent choice for regenerative medical treatments because of their capacities of imitating native extracellular matrices. Hydrogel can be classified into either synthetic or natural origins.[1] The natural hydrogels are cell benevolent as they mimic the natural microenvironment of the native tissue.[2] On the other hand, the biocompatible synthetic hydrogel is hydrophilic and can be tuneable for customized application. Because of the high calibre of hydrogels, the hydrogel-based scaffolds have much capacities and applications in regenerative medicine.[3] However even natural hydrogels face the problem of batch to batch variance and risk of allergy reaction.[4] Hence scaffold-free grafts have attracted more attention in tissue engineering field recently. In the fabrication process, scaffold could be used as a transient template to facilitate the cell proliferation and tissue growth and removed after maturation of graft.[5] Ultimately a graft with living cells and ECM secreted by the cells is generated as a tissue engineering product.[6]

In orthopaedic surgery a constant challenge lies in the reconstruction of musculoskeletal defects. Hydrogel in tissue engineering is utilized as a scaffold which is a template for cell proliferation and secretion furthermore as a guidance for neo tissue formation.[7] Extracellular matrix (ECM) is a desirable biological scaffold due to its complex composition and three-dimensional architecture driving the homeostasis and regeneration of tissues.[8] Successfully used in a variety of regenerative medicine applications, ECM could be achieved by decellularization of engineered tissue. Other than using decellularized grafts directly as scaffolds, decellularized grafts could also be coated onto or incorporated into synthetic biomaterials to enhance the biological performance substantially on the integration to the surrounding tissue and the bioactivity for neo-tissue generation.[9] However, at present, widely adopted decellularized

scaffolds are mainly decellularized native scaffolds, which meets the limitation of inherent heterogeneity, fixed shape and insufficient source.[10] Decellularized tissue engineering scaffold is promising to avoid the restrictions and is imminent in the regenerative medicine field.

1.1 Hypothesis and objectives

In this research project our objective is to develop ECM-based hydrogels that can accelerate the tissue regeneration of osteochondral defect. Osteochondral tissue contains both cartilage layer and subchondral bone layer.[11] Hence two different design mechanisms were utilized in the project, which are the monophasic pure cartilaginous phenotype graft and full-scaled gradient grafts mimicking the entire osteochondral tissue including both cartilage and subchondral bone. Due to the advantages of decellularized tissue engineering grafts, including logistic simplicity, clinical regulation obedience, graft life span and broader cell sources, both monophasic and biphasic grafts have been decellularized.[12] The tissue repair efficacy of all grafts was studied by engraftment to osteochondral defects, consisting of cartilage defect and osteochondral defect penetrating to subchondral bone.

To achieve the objective, the research has been further categorized into the following specific aims

1. Investigate the feasibility of decellularization of Living hyaline cartilage graft (LhCG), a prototype graft that has been established in our lab previously
2. Develop of a full-scaled graft (LhCG+) based on the microcavity cell culture method that has been developed previously in this lab and investigate the potential of decellularization on the grafts to fabricate decellularized full-scaled graft (dLhCG+)

3. Investigate the cartilage tissue repair capability of the decellularized LhCG (dLhCG) grafts
4. Evaluate the osteochondral tissue repair capability of the LhCG+ and dLhCG+ graft developed in specific aim 2
5. Investigate the osteochondral tissue repair capability of the dLhCG grafts developed in specific aim 1

We hypothesized that decellularization on LhCG and biphasic grafts were feasible. Moreover, the tissue repair capability is not affected by the decellularization process, namely, the decellularized grafts have the same tissue repair capability as the cellular grafts which is able to repair the cartilage defect and accelerate the osteochondral regeneration process.

1.2 Thesis organization

This thesis is organized into seven chapters. Chapter 1 (the current chapter) introduces the thesis and states the objective, specific aims and the hypothesis of the project as well as the structure of the thesis.

Chapter 2 provides review on current research achievements in tissue engineering osteochondral grafts that were reported or widely used in clinical application. The review on osteochondral tissue engineering grafts describes the rational of using decellularized tissue engineering grafts for different regenerative purposes and details their application in orthopaedic defects repair.

Chapter 3 studies the various decellularization methods to evaluate the efficiency of decellularizing tissue engineering cartilage graft – living hyaline cartilage graft (LhCG). A series of combinations of methods have been applied on LhCG to optimize the decellularization protocol and hence to fabricate an acellular ECM based tissue

engineering graft – decellularized LhCG (dLhCG) whose character and biocompatibility is investigated. In addition, chapter 3 describes the investigation to fabricate a biphasic osteochondral graft (LhCG+) and the decellularized biphasic osteochondral grafts (dLhCG+) whose character was examined too.

Chapter 4 investigates the *in vivo* tissue repair efficacy of dLhCG grafts for cartilage defect in direct comparison to clinically available autologous chondrocyte implantation (ACI) method. Based on the results in the *in vitro* characterization presented in Chapter 3, it is believed that dLhCG is highly biocompatible and hence both *allo* and *auto* dLhCG grafts have been tested in the experiment.

Chapter 5 investigates the capability of biphasic osteochondral grafts – LhCG+ and its decellularized version – dLhCG+ for repair of osteochondral defect. The hypothesis of the study is to repair the osteochondral defect by a biphasic graft containing both cartilage and subchondral layer with a transition zone in between that is imitating the native tissue composition. On the other hand, this study also serves as a comparison being a graft with a subchondral bone substitute to a pure cartilaginous graft like dLhCG and LhCG in Chapter 6 which in turn verifies the necessity of specific repair in subchondral bone layer in osteochondral defect repair.

Chapter 6 investigates the capability of dLhCG and LhCG grafts for osteochondral defect repair. The design of this study owes the hypothesis that osteochondral defect although contains both cartilage and subchondral bone zones, whose repair is manageable with a satisfactory repair at the cartilage layer. In other words, without specific subchondral bone substitute, the osteochondral restoration can be achieved by shielding of cartilage graft and self-regeneration of subchondral bone.

Chapter 7 summarizes and concludes all the findings and suggests the future work to be done.

Chapter 2 Background

Orthopaedic tissue is a type of tissue related to the musculoskeletal system including bone, cartilage, muscle, ligament and tendons.[13] Orthopaedic grafts are commonly adopted when there is a volumetric loss in these tissues due to injury or degenerative diseases.[14] Currently the most frequently used grafts are autologous, allogeneic or xenogeneic grafts. However, the source of these grafts is limited.[15] For the autologous grafts, donor site morbidity is a long-standing risk, especially for cartilage tissue which has a very limited self-repair capability.[16] Whereas for allogeneic and xenogeneic grafts, the risk of rejection, disease transmission and infection, the inability to be customized and the inherent heterogeneity is the restriction.[9] Therefore, people turned into tissue engineering field to find solutions for orthopaedic injuries and diseases.[18]

Tissue engineering products from xenogeneic or allogeneic source are going to induce inflammatory response after implantation due to the cellular components as a main source of antigen.[19] However, the extracellular matrix (ECM) of the tissue is relatively tolerable due to the conservation of the components among different species.[20][20] Hence, the tissue engineered grafts could be decellularized to reduce the immune response which may lead to abnormal remodelling and thus the failure of the graft.[21] On the other hand, the complex structure and delicate architecture of native ECM is very difficult to be imitated by synthetic biomaterials.[22] In addition, ECM contains a unique combination of various biomolecules and signalling cues.[23] The migration, proliferation and differentiation of cells are affected by the ECM not mentioning the function of providing a structure and substrate for cell adhesion.[24] The goal of decellularization is to remove the cellular component maximally and retain the ECM maximally simultaneously.[25]

Other than the biological advantages, decellularization brings about other practical advantages which are critical to successful translation to clinical application. Since typically substantial infrastructure and logistics is a necessity of tissue engineering products, decellularization can reduce the troubles caused by the living nature of the grafts.[26] Decellularized tissue engineering products have the potential to be commercialized and industrialized to become “off-the-shelf” products. This potential not only is a logistic advantage, but also is a benefit to patients clinically, as it could be stored for relatively longer period and turned into a ready-made commodity, thus expand the operation window period for patients.[27][27] All in all, decellularized tissue engineering graft has the possibility of translation from bench to bedside, though until now only limited grafts have become commercially available.[28]

A biomaterial that mimics the functional properties of native tissue ECM, including reparative cell encapsulation, cell proliferation support and ECM production stimulation, assembles the ideal scaffold for tissue repair under the tissue engineering paradigm.[29] The purpose of scaffold is to provide adequate mechanical support for regenerating tissue, to release biochemical cues, to guide cell growth and tissue remodelling and ultimately enhance the repair or replace of damaged tissue.[30] Selection of the most appropriate material is the first consideration in designing scaffolds.[31] Myriad results have been reported in investigation of different types of scaffolds. Polymer scaffolds and biological scaffolds are two main categories under investigation.[32] During past decades, incorporation of ECM into polymer scaffolds or utilization of tissue engineered ECM as biological scaffold has drawn more attention. This chapter provides a comprehensive overview of the decellularized tissue engineering orthopaedic grafts that are either decorated to polymer to improve the overall performance of the biomaterial or adopted as a scaffold synthesized by therapeutic cells.

2.1 Considerations in scaffolds design

Many scaffolds are in various phases of development currently from concept to product. To achieve the goal of tissue engineering and regenerative medicine the capability of the scaffold to create functional artificial tissue or organ is critical.[33] The scaffold is expected to support cell proliferation, differentiation and function and is used as a drug carrier or as vehicles of bioactive factors.[34][34] The characteristic of a scaffold involves biomechanical property, surface characteristics, permeability, biocompatibility and biodegradability.[30]

As the primary function of musculoskeletal system includes body support, the biomechanical property should be comparable to native orthopaedic tissue.[35] At molecular level, the scaffold should be able to resist radial and longitudinal deformation and absorb compressive loads.[36] Macroscopically adequate biomechanical property is required to match the need of handling and the function of the intended site of implantation.[37] Surface characteristics is important since the cell-biomaterial interactions should promote cell adhesion and ECM production. Surface chemistry plays the key role in guidance of cell attachment, proliferation and differentiation, though, the porosity of the scaffold plays a significantly role too.[38] The interconnecting pores with appropriate scale is required *in vitro* to maintain the phenotype of cells encapsulated, to permit transport of sufficient gas, nutrients and waste, small molecule mediators and regulatory factors.[29,39] Moreover, the appropriate porosity *in vivo* facilitates integration of the graft with surrounding tissue, vascularization of grafts and remodelling via cell migration in tissue regeneration, replacement and repair process after implantation.[40]

The biological basis of biocompatibility is the good contact with native tissue compartment.[41] The function of scaffold in the context of orthopaedic tissue relies on the adhesion and integration between the *de novo* ECM and the native surrounding tissue.[42] Therefore, the scaffold must not induce any adverse response.[43] By the end of the reparative period, the scaffold should be able to be absorbed by or integrated to the surrounding tissue.[44] Other than the avoidance of the adverse event after implantation, biodegradation has been revealed to influence the tissue regeneration via the degradation product.[45] The degradation product of ECM has recently been revealed to mediate important components of constructive host tissue response.[46] Naturally, after tissue damage, leukocytes degrade ECM to release factors that resist bacterial infection, stimulate angiogenesis and attract host cells that can repair and reconstruct damaged tissue.[47] Hence other than the signalling factors and adhesive proteins on the intact ECM, the degradation of ECM induces spatially and temporally localized biological effects.[48]

2.2 ECM-based scaffolds as implants in regenerative medicine

Each type of tissue if examined in detail has its own specific ECM composition, which is secreted by the corresponding cells in the tissue and in turn influences the behaviour of cells inside or transplanted.[49–51] The influence is caused by the complex bioactive network containing proteins and polysaccharides.[52] The interaction between cells and ECM implies that ECM is a dynamic meshwork instead of static.[53,54] The functions of various types of orthopaedic tissue depend upon the organization of ECM and cells to but not limited to withstand or produce forces.[55] Hence, much research work focuses on decellularized ECM as a scaffold.

In the past decades, the advancement in the field has been able to put certain products into the market, but the wide application to patients is still limited. Currently the main products can be categorized into skin, cartilage, bone, cardiovascular and central nervous system (CNS) products. [56][56] Due to the scope of this review, the cartilage and bone tissue engineering product is the focus of the discussion.[57] Currently, the commercially available tissue engineering cartilage graft is only autologous chondrocytes implantation product, which aims to repair hyaline cartilage.[58] Autologous chondrocytes implantation (ACI) is the surgery procedure that was invented to treat cartilage defect first published in 1994.[59] Briefly, the chondrocytes harvested from a non-loading region in the knee were expanded *in vitro* before injection into the defect under the cover of a periosteal flap.[60] For bone grafts, currently available products in the market are still mainly allogeneic or autologous graft developed by decellularization of native bone or biomaterials and synthetic bone fillers.

The biochemical composition of ECM scaffold enables on site delivery of growth factors and cytokines, thus affects the activity of macrophages and subsequently guides cell migration and recruitment.[30][30] The tissue specific property of ECM scaffold is manifested by the large variety of matrix macromolecules and their corresponding structure.[61] Despite the variance among different types of tissue, the major constituents of ECM are highly acidic and hydrated glycosaminoglycan (GAG) and fibrous-forming proteins, such as collagens, elastin, fibronectin, laminins, glycoproteins, proteoglycans (PGs).[62][62] Majority of the extracellular interstitial space within the tissue is filled with PGs in the form of hydrogel. The function of PGs ranges widely from buffering, hydration, binding to force-resistance properties.[63] On the other hand, the function of GAGs is more specific, including retention of water and binding growth factors and cytokines.[46] Cells constantly interact with the surrounding ECM by

modifying the ECM scaffold using proteinases and reciprocally ECM scaffold steers the metabolic and secretory activity of cells via functional biological cues, i.e. growth factors and cytokines, incorporated in the scaffold.[24]

Other than the biochemical cues, ECM scaffold provides a three-dimensional structure that supports cell growth and regulates cell phenotype.[64] For instance, to initiate chondrocytes differentiation a nonporous scaffold that could maintain the cells in rounded shape is preferred over porous scaffold which induces the production of fibrous or fibrocartilaginous tissues.[65] As mentioned previously, ECM is critical in tissue engineering as it is the environment that cells interact with. The interaction is not only affected by the biochemical composition, but also is influenced by the architecture and topography.[66][34] Healthy osteochondral tissue owes an inherent gradient structure.[67][67] By decellularization of orthopaedic tissue the ultrastructure and 3-D architecture can be largely preserved by avoiding harsh chaotropic agents in the process.[68] In addition to inducing tissue specific cell phenotype, microscopic and ultrastructure features of matrix is important in modulating behaviours of cells that are in contact with the ECM scaffold by controlling the feasibility of cells migrations into the scaffold.[69] Moreover, mostly the 3D structure is formed by collagen fibres whose alignment is associated with the incorporation of growth factors and other functional proteins.[70] Compared to synthetic scaffold, ECM scaffold owes an intact 3D structure and maintains initial geometry and flexibility.[71]

The overall structure of ECM is determined by the composition and the structure of the composition molecules which also affects the biomechanical properties of the formed network.[72] The stiffness of the extracellular matrix provides more than mechanical support for surrounding cells. The cell migration, cell cycle progression, and cell fate decision were impacted by the biomechanical property too.[72] A main structural

element of the ECM, collagen provides tensile strength, regulates cell adhesion, supports chemotaxis and cell migration, and directs tissue development ultimately.[73] The biomechanical environment dictated largely by the ECM scaffold can have a great influence on cell differentiation. For example, stiff biomaterials induce osteogenic differentiation of mesenchymal stem cells (MSCs) whereas soft biomaterials induce neuronal differentiation of MSCs.[74] Further more tensile strength influences the ECM secretion of cells which has been shown by upregulation of Type II Collagen (Col II) expression under proper and cyclic tensile strain.[75][75] The alignment and organization of collagen fibre in ECM is dependent on tissue type especially in orthopaedic tissues, for example, in tendon and ligament, the fibre is highly aligned along the axial orientation of the tissue to provide the greatest resistance to strain in the load-bearing condition.[76] On the contrary, Articular cartilage owes complex mechanical properties that rely on the anisotropy of the tissue with a zonal structure which each has a unique ECM composition and organization.[77] The difficulty in replication of the organization and alignment of the collagen fibre and further the mechanical property of tissue makes the ECM scaffold derived from the same type of tissue a wise choice for orthopaedic tissue repair.[78]

2.3 Improving scaffold property by hybridization with ECM

Scaffolds are typically used as cells or drug delivery vehicles and could be made of synthetic and natural biomaterials.[79] Synthetic scaffolds are characterized by reproducible and controllable mechanical and chemical properties, while ECM-based natural biomaterial is characteristic of being bioactive and inducing constructive host tissue response. Therefore, to prevent the formation of fibrous encapsulation on synthetic scaffolds after implantation *in vivo*, ECM as a natural biomaterial has been hybridized into synthetic scaffold to improve its performance. [80]

Table 2-1 Different ECM hybridization mechanisms and their respective advantages and disadvantages

ECM hybridization mechanism		Pros	Cons
Incorporation	Physical mix	Minimally invasive delivery; Maintenance of 3D structure	Limited source and difficulty to scale up
	Chemical cross-linking	within ECM fragments; Bioactivity and constructive	Loss of bioactivity due to the chemical modification
	Biological digestion	host tissue response induction; Prevention of fibrous encapsulation formation	Loss of 3D structure after enzyme digestion
Surface modification	Physical adsorption	Sample fabrication process	Non-ideal <i>in vivo</i> remodelling due to the contact with synthetic material after cell infiltration;
	Biological deposition	Retention of more intact microstructure; improvement on integration to surrounding tissue;	Difficulty to scale up and generate 3D graft

2.3.1 Incorporating ECM fragments in scaffold

ECM contains various sizes of proteins. ECM powder or particulate form is commonly transformed from lyophilized sheets of ECM. Lyophilization is a process to remove water by sublimation at low temperature and low pressure.[81] ECM in particle form facilitates the delivery in minimally invasive manner. Within the particles or fragments, the 3D structure is maintained.[82][82] Other than dissolved as suspension, ECM fragments could also be incorporated with synthetic materials to make hybrid ECM-based scaffolds.

2.3.1.1 Physical mix

ECM fragments achieved from decellularization on native or tissue engineered grafts combined with degradable biomaterials have achieved positive clinical results.[83] Yang et. al. reported a composite scaffold consists of silk fibroin and cartilage ECM fragments as a sequential delivery vehicle of transforming growth factor beta (TGF- β)

to enhance chondrogenesis.[84][84] Although it is claimed within the particles that commonly have a size in micrometre scales there is a 3D structure, the biochemical composition only in the ECM has been reported to upregulate *Col2A1* gene and show a region-specific property.[85,86] Other than EMC fragments in the solid form, ECM particles dissolved in water produces hydrogel that can be administered minimally invasive to treat cartilage defect.[87] However, the microstructure of scaffold is critical to the tissue repair efficacy of the scaffold. Therefore, a freezing method has been reported to be effective when applied on decellularized cartilage powder generated hydrogel to fabricate a hemispherical shaped scaffold with aligned pores to facilitate infiltration of MSCs and matrix deposition.[88] Not only ECM fragments derived from decellularized healthy cartilage, osteoarthritic cartilage fragments after freeze-thaw cycles wrapped in fibrin glue has been reported to promote chondrogenic differentiation of mesenchymal stem cells (MSCs).[89]

Other than cartilage tissue engineering scaffolds, bone tissue engineering scaffolds are enhanced by incorporation of cell-free ECM fragments obtained from decellularization or demineralization of bone tissue.[90] The bone matrix fragments have been incorporated into both Polylactide (PLA) beads and Poly (lactide-co-glycolic acid) (PLGA) matrices.[91] It was reported that hybrid PLA bone matrix beads were able to differentiate MSCs into osteoblasts.[92] Interestingly PLGA scaffolds incorporated decellularized bone matrix fragments were able to achieve better mechanical properties compared to PLGA scaffolds incorporated with bone tissue fragments.[93] Inspired by the natural endochondral ossification process in growing skeletal system, cartilage derived matrix fragments mixed with MSCs encapsulated by gelatine methacrylamide hydrogel have been reported to be remodelled into mineralized bone tissue subcutaneously in rats.[94] Although not frequent, demineralized bone matrix has been

reported to be mixed with chitosan hydrogel to generate a hybrid scaffold to treat cartilage defects too.[95][95] However a simple mix of fragmented ECM is not sufficient due to the loss of 3D structures in a scale comparable to defect size.[96] In addition, the ECM particles are still derived from native tissue with limited source.

2.3.1.2 Chemical cross-linking

Chemical cross-linking modification, such as ultraviolet irradiation, carbodiimide, glutaraldehyde and genipin, can improve the mechanical property and prevent cell mediated material contraction. Fibrinogen as a natural bioglue could form stable hydrogels in the presence of thrombin with addition of cartilage matrix particles up to 2% (w/v).[97] Simultaneously the fibrin hydrogel delivers TGF- β 3 and increases production of GAGs *in vivo*. [98] ECM functional bioactive molecules, mainly collagen and GAGs, rather than ECM particles derived from native tissue have been cross-linked with or without biocompatible materials including chitosan[99] and gelatin[100] which[101][101] have been reported to be beneficial to the proliferation of chondrocytes[102], differentiation of MSCs residing in the scaffold[103] and production of cartilage tissue due to the increased compressive stiffness, decreased pore size[104] and improved bioadhesion. However, it has been reported that chemical cross-linker in comparison to dehydrothermal physical cross-linking and irradiation cross-linking when applied to native decellularized cartilage matrix fragments achieved inferior chondrogenic differentiation induction effect as shown by worse cell adhesion and lower GAG secretion.[105]

When ECM fragments were solubilized, hydrogel could be formed as a functionalized scaffold to treat cartilage defect, however the mechanotransduction is critical for *de novo* cartilage tissue formation especially *in vivo* in the early stage of tissue repair.[106] To overcome the limitation of hydrogel in application to treat defects of loading-bearing

tissue, such as cartilage, methacrylation and UV radiation has been applied to hydrogels fabricated from decellularized cartilage fragments, which has been reported to approach the compressive modulus of native articular cartilage.[107] Similarly, decellularized bone matrix has also been dissolved in hexafluoro-2-propanol, and trifluoroacetic acid blended solvent and electrospun to fabricate cytocompatible and tensile nanofibers with Young's Modulus of 3.37 MPa.[102] However, the chemical cross-linking modification such as methacrylation bears a risk of change in chemical property of the ECM fragments cross-linked in the scaffold.

2.3.1.3 Biological digestion and solubilization

The potential of ECM fragments to facilitate constructive and functional tissue remodelling has been broadened with the discovery of the possibility of ECM being digested and solubilized to form hydrogel.[108] Although the mechanism is not fully understood, ECM-based hydrogel has been used as functionalized scaffold *in vitro* and to treat irregular defects *in vivo*. Currently some mechanism has been proposed to explain the phenomena, including structural and signal cues retained within ECM-based hydrogel since an ideal gel process could prevent the destroy of active growth factors or low molecular weight peptides and the hydrogel could be designed to have a rheology and viscosity that matches the native interested tissue.[109] Not only to deliver cells but also therapeutic drugs such as heparin has been successfully achieved by hydrogels from pepsin digested pericardium ECM fragments.[110]

Enzyme digestion is a popular method adopted to generate ECM-based hydrogel, at the same time, it is a method of decellularization. An interesting system consists of trypsin and ethylenediamine tetraacetic acid decellularized bone ECM-based hydrogel incorporating demineralized bone matrix fragments fabricated by pepsin digestion has

been developed and demonstrated to enhance proliferation of mouse primary calvarial cells.[111] Pepsin has also been used to digest cartilage which has shown *in vitro* chondrogenic induction capability of human MSCs.[112][112] As a special type of cartilage, meniscus has also been digested by pepsin and gelled by increasing and stabilizing pH using sodium hydroxide and phosphate buffered saline (PBS) which works as a microenvironment to elongate retention of encapsulated human MSCs after *in vivo* transplantation and contributes to tissue regeneration.[113] Several other ECM hydrogels have been reported to have *in situ* gelation and tunable compression and initial modulus to improve meniscus tissue repair efficacy.[114–116] Although the administration and customization of scaffold shape has become much easier after digestion, the 3D structure has been more severely destroyed after digestion even compared to physical fragmentation.[117]

Recently 3D printing has become a trend in fabrication of functionalized scaffolds some of which uses ECM-based hydrogel as a bioink. Modified biopolymers presented in cartilage ECM, i.e. gelatine methacrylamide, chondroitin sulfate amino ethyl methacrylate and hyaluronic acid methacrylate loaded with MSCs have been used in combination with alginate as photocurable bioink to form stable 3D printed fibres.[118] The MSCs encapsulated have shown increased cell viability and chondrogenic differentiation.[119] A limitation, however, is that alginate derived bioinks are significantly mechanically inferior compared to native tissue which affects the *in vivo* remodelling. Moreover, general problems of 3D printing using biomaterials such as poor printing resolution and slow speed need to be addressed.[120]

2.3.2 Coating ECM on scaffold surface

In the context of orthopaedic tissue engineering, tissue regeneration outcomes can be enhanced by decoration of ECM on the surface of biomaterials and stem cell fate can be directed by *in vivo* niche provide by the ECM.[121] Synthetic scaffolds provide advantages due to the character of uniform and reproducible mechanical and material properties yet promoting cell adhesion and presenting instructive cues to relevant cells could improve their therapeutic potentials, such as enhancement of survival and function of relevant cells. By synergizing the benefits of both instructive ECM and synthetic materials, the capacity of ECM-coated scaffold to promote cell persistence and enhance tissue regeneration is anticipated.

2.3.2.1 Physical absorption

To coat ECM onto scaffold, directly cell-secreted ECM deposition onto porous scaffold is widely adopted due to the simplicity in handling.[122] Leach et. al. has reported a human MSCs deposited ECM which has been homogenized into solution and deposited by micropipette directly onto scaffold followed by a gentle vacuum to ensure the coating of ECM to significantly increase vascularization and enhance cellularization and MSCs osteogenic differentiation after subcutaneous implantation.[123,124] Other than homogenized ECM, cell-derived ECM cell sheets have been wrapped directly around bone marrow stromal cells engineered cartilage graft to maintain the chondrogenic phenotype after subcutaneous implantation. However, the efficiency of decellularized cell sheet in the same scenario is worth studying to avoid adverse event caused by xenogeneic cells.[125] Other than dropping gel status ECM onto the scaffold, the ECM could also be adsorbed onto the surface of the scaffold by physically soaking the entire scaffold into an ECM hydrogel.[126] As reported by Thai et. al. silk fibroin scaffold after coating by fibronectin/decellularized pulp ECM induced the formation of new bone with low inflammation and high vascularization in maxillofacial surgery.[127] Physical

adsorption of ECM no matter cell sheets or solution has only modified the surface chemistry of the scaffold.[128] In the *in vivo* remodelling process, the infiltration of cells into the scaffold is critical to the success of the scaffold. In that sense, the modification on surface only is still not ideal to eliminate the inflammation caused by synthetic materials.

2.3.2.2 Biological deposition

Integrated ECM is believed to be in advantage compared to fragmented ECM because of more complete architecture retention.[129] Therefore, ECM coating achieved thorough culturing therapeutic cells seeded in the scaffold to secret ECM becomes an attractive choice. For the orthopaedic tissue repair, the *in-situ* recruitment of endogenous marrow MSCs or other potential cells within the joint determines the success of the tissue regeneration mediated by a cell-free scaffold. Therefore, a biocharacter of the scaffold to allow attachment, growth, and differentiation of the host cells is vital.[130]

The profit of coating biomaterials with cell-derived ECM is the improvement on the integration to the surrounding tissue and the bioactivity for neo-tissue generation.[131] Liao et. al. has reported an electro spun poly (ϵ -caprolactone) (PCL) coated with ECM secreted by chondrocytes seeded on it under dynamic conditions. After decellularization the ECM showed chondrogenic differentiation induction on MSCs.[132] On the contrary, Thakkar et. al. reported that compared to ECM secreted by chondrocytes, ECM secreted by MSCs has better chondrogenic differentiation effect.[133] According to Pei M. et. al. synovium derived stem cells (SDSC) when expanded on decellularized stem cell matrix have manifested a three-dimensional manner of growth and when injected *in vivo* into partial thickness defect in knee cartilage of pigs, these SDSCs enhanced the

Col II and GAG content in the regenerated tissue compared to SDSCs expanded on plastic flasks.[134]

When coated onto scaffold, tissue engineered ECM improves the bioactivity of the scaffold and guides the differentiation of stem cells and maintains and expands osteoblasts.[135] Marrow stromal cell is a major type of stem cell that is used in bone tissue engineering. Under flow shear stress the rat marrow stromal cells deposited bone-like ECM on Titanium scaffold. When the marrow stromal cells were seeded on the ECM under fluidic shear, the osteogenic gene was upregulated while the chondrogenic gene was downregulated compared to the plain titanium scaffold, which showed the osteoinductive effect of the ECM.[136][136] Different from the complicated fabrication process, Martin Decaris et. al. reported ECM secreted by MSCs simply on culture plate well.[137] After decellularization the coated well worked as a scaffold to accelerate naïve human MSCs osteogenic differentiation as demonstrated by alkaline phosphatase and calcium production increase and osteogenic marker upregulation.[138] As *in vivo* and *in vitro* situation could be very different, the *in vivo* test on an *in vitro* successful graft is necessary. Nasser Sadr et. al. fabricated a poly-ester urethane scaffold coated by mineralized ECM secreted by human MSCs under perfusion and in osteogenic medium.[139] After decellularization in the subcutaneous implantation in nude mice the scaffold showed formation of mineralized bone matrix containing bone sialoprotein and even resembled the morphology of an immature osteoid tissue.[140][140] In addition, Deutsch and Gulberg reported ECMs secreted respectively by MSCs and amniotic fluid stem cells. When the scaffold was used to repair rat femoral segmental defect, increased bridging rate was observed for both types of ECM compared to scaffold simple coated by collagen.[141]

Up to date, seldom successful application of tissue engineering grafts of ligament and skeletal muscles was reported.[142] One important reason is the inability of proliferation of mature cells and the de-differentiation of cells after cell passages *in vitro* in these tissues.[143] Thus, an *ex vivo* system to remain the morphology and improve the proliferation of cells is needed. Furthermore, the limited quantity of healthy available cells in organism makes *ex vivo* expansion a must, not only mature cells, but also stem cells. Large number of passages leads to the diminish of differentiation and proliferation ability of the MSCs.[144] Hence, bone marrow cell (BMC) derived matrices were studied to preserve the differentiation and proliferation capability of MSCs by providing a 3D microenvironment. Lai et. al. reported BMC-derived ECM used as scaffold for MSCs residence. Significant difference in bone formation after 7 passages was observed between MSCs cultured on BMC-derived ECM coated flask and plastic flask.[145] Other than proliferation, rejuvenation is also possible using ECM derived from young MSCs.[146] Based on the work of Sun et. al. although being able to proliferate, the bone formation capacity is lost in old MSCs however the scenario is reversible after culture on ECM derived from young MSCs but not from old MSCs. In addition, fetal MSCs derived matrix coated culture wares have been shown to enhance the proliferation and multipotency of adult MSCs compared to common culture wares.[147] However, an *in vitro* niche prepared from stem cells seems to be tissue-specific. According to He et. al. when SDSCs were seeded on SDSC-derived matrix coated flasks, the proliferation and differentiation ability was significantly higher than SDSCs plated on plastic flasks. Nevertheless, the differentiation was only towards synovial cells but neither towards adipogenicity nor osteogenicity.[148][148]

Chondrocytes having stronger proliferation ability than myocytes though, face the problem of de-differentiation after 3 passages *in vitro*. [149] Therefore, the ECM that is

mimicking the chondrocytes *in vivo* niche was studied to prevent the de-differentiation of chondrocytes.[150] Like the young and old MSC secreted ECM tendency, ECM deposited by primary passage compared to passages 2 and 6 no matter on plastic wares or as coating on biomaterials delayed the de-differentiation of chondrocytes cultured on it.[151] On the other hand, the tissue specific property of ECM in the differentiation potency maintenance of MSCs was not bared by ECMs to prevent de-differentiation of chondrocytes. According to Cha et. al. ECM derived from various cells including fibroblasts, pre-osteoblasts and chondrocytes were all able to maintain the phenotype of the chondrocytes up to P5.[152] Currently the ECM engineered by secretion from living cells onto biomaterials are still limited to *in vitro* experiments or utilized as *ex vivo* niche to enhance cell proliferation and guide cell differentiation.[153] The ability to be scaled up and piled up into tissue level grafts needs to be studied to make the methods clinically applicable.

2.3.3 Coating material on ECM

A long-lasting problem of decellularized ECM is the inferior mechanical property mainly caused by the deterioration of collagen in the decellularization process. Hence one research group has put in effort to improve the mechanical property by deposition of TiCaPCON film onto the surface of the ECM. Although the stiffness was increased, but the flexibility is reduced.[154] More important, this coating defeats the purpose of using ECM as substrate to improve the bioactivity of scaffolds.[155] Since the ECM has shown a tissue specific character, the ECM not from the source tissue has been decorated with source tissue specific peptides to improve the microfracture surgery by improving the cell recruitment to the scaffold and led to a superior mechanical performance compared to microfracture alone.[156]

2.4 Decellularized tissue engineering graft

Hybrid scaffolds may capture the advantages of both types of material, namely, on one hand the mechanical and material properties manipulated from the synthetic component and on the other hand the bioactivity provided by the ECM. Nevertheless, the disadvantages of both materials may be manifested simultaneously also, namely, the host inflammatory response to many synthetic materials and the batch-to-batch variance of tissue engineered ECM.[157] Hence synthetic material free decellularized tissue engineering grafts become promising alternative scaffolds to totally avoid the host tissue destructive response and fully utilizing the *in vivo* tissue remodelling capability to finally achieve the ideal integration to native surrounding tissue and stimulant tissue regeneration for defect repair.

In the fabrication process synthetic biomaterials could be used at the beginning as a scaffold, but the degradation profile is well managed such that the graft becomes scaffold-free after the maturation or the synthetic material could be removed after a certain period to make the ultimate graft scaffold-free whose general steps are shown in Figure 2-1.[158,159]

Figure 1 General preparation process of intact decellularized tissue engineering grafts. Firstly, the cells are seeded on a scaffold to secrete ECM that could be used as a coating on the biomaterials to improve the bioactivity of the scaffold. Secondly, a transient scaffold that will be removed after graft maturation could be utilized to achieve a tissue engineering graft as a functionalized scaffold synthesized by therapeutic cells. Based on the integrity and mechanical property of the mature graft, either cells or scaffold will be removed first and followed by another. Finally, the decellularized tissue engineering grafts are fabricated which have shown high biocompatibility and bioactivity as an *ex vivo* biological niche for the expansion of cells and good tissue integration and constructive response induction as an *in vivo* implant for tissue repair.

A scaffold-free graft is difficult to fabricate but is closer to the natural developmental process, which starts from high concentration of cell suspension and triggers the cells to secrete ECM in a confined space within a short period of time.[160] Without synthetic material the cells can fuse and mature faster.[160] After transplantation the variance caused by immunogenicity and other risk factors brought about by materials are minimized too.[161] Lu et. al. prepared an autologous tissue engineering ECM using mouse fibroblasts seeded on a temporary PLGA mesh to achieve a so-called “full autologous tissue engineering” graft and has shown significant reduction of immune rejection compared to allogeneic or synthetic materials.[162]

2.4.1 Cartilage

Currently, the commercially available tissue engineering cartilage graft is only autologous chondrocyte implantation product, which aims to repair hyaline cartilage defect.[163] Injury is a main cause of damage to hyaline cartilage, without proper treatment it may progress on to osteoarthritis and severely affects the life quality of patients. Autologous chondrocyte implantation (ACI) is the surgery procedure that was invented to treat cartilage defect first published in 1994.[59][59] Later, more advanced generations of this treatment have been derived. The original generation of ACI is the implantation of the autologous chondrocytes which are expanded *in vitro* to the defect site under the cover of the periosteal flap. The second generation uses a synthetic membrane instead of the periosteal flap as the cover. The third generation uses a biodegradable material as a scaffold for chondrocytes implantation into the defect. Due to the liquid nature of the autologous chondrocyte implantation the loss of the chondrocytes severely affects the quality of the repair. In addition, overgrowth and secondary damage caused by suturing of membrane onto cartilage are frequently reported complications of ACI.

Table 1-2 The summary of fabrication methods and applications of various design in tissue engineering decellularized cartilage graft

Tissue engineering	Brief fabrication methods	Application
decellularized cartilage graft design		
Cell sheets	Juvenile chondrocytes cultured as monolayer first, after confluence the entire sheet is	Chondrogenic induction of MSCs;

	lifted from the substrate and layered, rolled over or draped around rods to fuse into an integrated tissue.	Bone marrow stimulation technique improvement
Cell aggregates	culturing cells under a rotation in suspension or in a non-adherent environment	Decellularization efficiency test
Cell assemblies	Firstly, the cells were seeded on a non-cell-adhesive surface/scaffold. Secondly the cells migrate and aggregate. Thirdly the cells produce the ECM and mature into a graft.	Engineering of articular cartilage, meniscus and temporomandibular joint disc; Work as <i>ex vivo</i> system for cell proliferation and differentiation and models for study

Cartilage is especially suitable for scaffold-free tissue engineering paradigm since it is a tissue with low vascularity and cellularity.[164] Cell sheet engineering has been adopted as a self-organized scaffold-free method. The juvenile chondrocytes are cultured as monolayer first, after the confluence, the entire sheet is lifted from the substrate and layered, rolled over or draped around rods to fuse into an integrated tissue like the native developmental process.[165] Mitani et. al. has developed a cell sheet from chondrocytes which maintains a chondrogenic morphology evident by the upregulation of chondrogenic markers and cumulation of cartilage compositional proteins.[166] However due to the mechanical inferiority, the cell sheets were not decellularized. To resolve this problem, Lu et. al developed a decellularized scaffold-free ECM using a temporary scaffold consisting of PLGA. Briefly chondrocytes were cultured on PLGA scaffold first, after culture for 6 days the entire graft together with PLGA scaffold was decellularized followed by removal of PLGA using sodium phosphate solution. The acquired ECM has shown a significantly better chondrogenic

induction effect on MSCs compared to pellet culture.[167] Moreover, Li et. al. has utilized decellularized cell-derived matrix to improve the bone marrow stimulation techniques (BST).[168] A main limitation of BST is the formation of blood clot at the defect site instead of cartilage like tissue. The decellularized cell-derived matrix was developed from layering decellularized porcine chondrocyte cell sheets, which has been shown to prevent blood clot loss in rabbit model and promote BST in beagle model in terms of histology, biochemical analysis and International Cartilage Repair Society (ICRS) scores. Pre-clinically, a periodontal ligament cell sheet has been used to repair periodontal defect in beagle dogs and found to be able to regenerate bone, periodontal ligament and cementum in three out of five subjects. In this experiment autologous cells were used and implanted into the defect together with the ECM. Cell sheet technique first realized the scaffold-free design of grafts however the handle of cell sheet requires extraordinary patience and carefulness. The application clinically is hence limited. Furthermore, the thickness that is achievable by cell sheet is limited by the passive diffusion of oxygen, nutrients and metabolic wastes.[169]

Other than cell sheet technique, cell aggregates is another widely adopted method, which is achieved by culturing cells under a rotation in suspension or in a non-adherent environment.[170] The tissue formed under aggregates is closest to native cartilage tissue and resolves the common problem of diffusion compared to other 3D cell culture methods. Moreover, aggregation culture further owes potentials to differentiate even re-differentiate cells into chondrocyte phenotype. Ming Pei et. al. reported a matrix deposited by SDSCs on which the chondrocytes cultured for 6 passages were able to be re-differentiated when cultured in cell aggregates treated by TGF- β 1.[171] However, the cell aggregates are integrated due to the existence of cells, decellularization methods are seldom applied.[172] But for cartilage repair, there are already products using

aggregates or spheroids in Phase III clinical trials in Europe called Chondrospheres.[173] Nevertheless, it is more like a modification on autologous chondrocyte implantation with the replacement of chondrocytes into chondrocyte aggregates. The original problems with ACI remain unsolved. However, a scaffold-free tissue engineering chondrocyte aggregates has been utilized by Benjamin Elder et. al. to test the decellularization efficiency of various method.[174] It was believed based on their results that 2% sodium dodecyl sulphate (SDS) treatment for 1 to 2 hours achieved the maximal removal of cellular component with the minimal damage to ECM.[22]

Last type of scaffold-free graft is fabricated using cell-assemble, whose mechanism can be separated into three steps. Firstly, the cells were seeded on a non-cell-adhesive surface/scaffold to ensure the behaviour of cells are regulated by cell-cell interactions dominantly.[175] Secondly the cells migrate and aggregate due to a thermal dynamic reason of minimizing free energy. Thirdly the cells produce the ECM and mature into a graft.[152] In the process, biomaterials could be used as a transient scaffold and provide the trigger for proper cell migration by designing structures with expected free energy minimization path.[176] Wang et. al. has established a microcavity structure which guides the cell migration and proliferation on the edge between the alginate hydrogel phase and empty space.[160] It is currently being studied for decellularization to become an off-the-shelf product. Although self-assemble is developmentally related to cell aggregation, it can be scaled up to a distinctive size and gross appearance comparable to native tissue.[177] Although no product entered clinical trials or was commercialized, there has already been reported successful engineering of articular cartilage, meniscus and temporomandibular joint (TMJ) disc with physiological functions in the scale of native cartilage.[178–180][178–180] Henrique et. al. reported a tissue engineering self-assemble decellularized ECM using human bone marrow stem cells which achieved a

similar level of GAG synthesis from seeded infrapatellar fat pad stem cells under the aid of TGF- β releasing gelatin microspheres.[181][181] Other than working as *ex vivo* system for orthopaedic tissue cells proliferation and differentiation, the decellularized tissue engineering matrices could also be used as *ex vivo* models for various study.[182] An ECM derived from osteoblasts was developed to investigate the bone metastasis of prostate cancer, which induced cells to lose the epithelial phenotype and upregulated the genes related to attachment, migration and even invasive potential.[183][183] Other than cancer, craniosynostosis, the premature ossification of cranial sutures was also investigated using ECM derived from osteoblasts obtained from patients and healthy individuals in parallel. TGF- β 1 a growth factor that has been shown to decrease in craniosynostosis mice model, had a stronger affinity to ECM derived from osteoblasts of patients which demonstrated that ECM plays a vital role in the development of the premature fusion of the cranial sutures.[184]

Although cell-derived matrix is more difficult to be achieved technically, it has extra benefit compared to tissue derived matrix especially for cartilage tissue.[185] Decellularization is commonly realized through physical, chemical and biological process. Other than the traditional freeze-thaw cycle, an electroporation process has recently been reported as effective in removing cellular component especially when taking advantage of innate immune system to complete the decellularization process.[186,187] Ionic solution and detergent are often used to disrupt cell membrane and separate cellular components from ECM. Enzymes facilitate the cellular component washing away coupled by agitation throughout the decellularization process in solution.[188] Cartilage as a compact tissue is hard to decellularize thoroughly following standard whole organ decellularization protocol.[189] Tissue engineering cartilage graft is comparatively easier to decellularize due to the less dense nature and

higher porosity. Nevertheless, decellularization protocol needs to be established and optimized before the clinical application.

2.4.2 Bone

Bone tissue engineering graft is a major type of commercially available product in the market. Although bone has a relatively strong self-repair capability, when the defect size is larger than the critical size, a graft is useful in regenerating the tissue. Due to the problems of natively derived grafts mentioned previously and the lack of osteogenic bioactivity of synthetic grafts, autologous tissue engineering products become focus of research.[190][190] However currently available products in the market are still mainly allogeneic or autologous grafts or biomaterials and synthetic bone fillers.[191]

Compared to cartilage, bone shows a stronger self-repair capability which is able to regenerate via Haversian remodelling even when there is fracture given the remaining healthy parts are aligned.[192] When the defect size increases, the defect could still be self-repaired by endochondral ossification process.[193][193] However, when the defect size is beyond the critical size, it is not able to self-repair. The situation happens when there is traumatic injury of large defect or there is bone tumour. The self-repair capability is also affected by age, health condition or even lifestyle of the individual.[194] The first-line medical treatment for these scenarios is bone graft. The most commonly used graft is autologous. However, complications are documented such as prolonged wound drainage and increased post-operative pain.[195]

Inspired by the native healing process of relatively large bone defect, Cunniffe et. al. hypothesized that decellularized hypertrophic cartilage facilitates endogenous bone formation via endochondral ossification process.[196] The hypertrophy of MSCs seeded in trans well inserts were induced by medium supplemented with L-thyroxin and β -

glycerophosphate.[197] After totally 6 weeks' culture, the hypertrophic cartilage tissue constructs were collected, homogenized, decellularized and cross-linked. The obtained scaffold was implanted into the femoral defect in rats and it was found that full-bridging happened with a 50% chance compared to control as void with 0% full-bridging. It was also revealed that the tissue engineering decellularized hypertrophic cartilage scaffold was capable to acclimate cells that generate bone tissue which was different from empty defect filled up by fibrous tissue.[198] Ma et al. had used cell sheets to engineer large scaffold-free bone tissue which can facilitate bone tissue formation when implanted subcutaneously to nude mice.[199] The decellularization potential of the graft should be further investigated to enlarge the cell source.[200] On the other hand, Qi Xing et al. reported decellularized cell sheets developed from fibroblasts which were able to support the growth of bone marrow derived human MSCs.[201] The decellularized cell sheet was hence believed to be promising in repair bone defect however the 3D graft with a larger size and larger thickness based on the sheet should be further designed and developed due to the need to treat large bone defect that are not self-reparable.[202]

Chapter 3 Development of decellularized scaffold-free ECM based graft (dLhCG) and biphasic osteochondral graft (d/LhCG+)

Summary

The key challenge of lower-limb-joint osteochondral regeneration lies in restoration of the avascular articular cartilage on top of the self-repairable subchondral bone. A quality articular cartilage engraftment is validated by the graft's hyaline cartilaginous phenotype and genuine architecture. In this tissue engineering study, we endeavour to develop a novel decellularized man-made hyaline-like cartilage graft that is produced by 3D cultured porcine chondrocytes in vitro - free of non-cartilaginous constituent. On the other hand, as a complex tissue osteochondral tissue contains both cartilage layer and subchondral bone layer. Hence development of a biphasic graft that matches the two layers respectively in the structure could repair the defect effective. Therefore, another biphasic graft was developed with the cartilage layer fabricated from chondrocytes and the ECM secreted by them and the subchondral bone layer fabricated from sintered poly (lactic-co-glycolic acid) (PLGA) microspheres. This chapter presents the fabrication and characterization of the decellularized man-made hyaline-like cartilage graft and the biphasic osteochondral graft.

3.1 Development and characterization of dLhCG

3.1.1 Introduction

Osteochondral lesions contain lesions in both cartilage and bone tissue. To repair osteochondral lesions, the key challenge lies in regeneration of articular cartilage, as

subchondral bones are capable of self-regeneration given a shield by fine cartilage on top; however, the self-regenerating ability of articular cartilage is significantly compromised due to its avascular and nerveless nature. Therefore, a successful treatment of traumatic osteochondral lesions relies on successful cartilaginous repair.[203]

A quality articular cartilage engraftment is validated by the graft's hyaline cartilaginous phenotype and genuine architecture. In past decades, engrafting efforts have been extensively made with autologous implants of cartilaginous tissues, cells and progenitors, respectively. As a dense tissue with low cellular population and condensed extracellular matrices (ECMs), even despite the sourcing limitation and immune complication, native cartilage transplant, such as “mosaic” osteochondral grafting, encounters great hurdles of on-site integration with surrounding tissues after implantation.[204] Autologous chondrocytes implantation (ACI) has been popularly applied as a second-line therapy; however, the flaws of this treatment remain significant including operational complexity, twice surgeries and variable outcomes in terms of neo-tissue phenotype. Stem cell therapy for osteochondral lesions is believed promising in long-term view, albeit it encounters current bottle neck in practice. The use of mesenchymal stem cells (or marrow stromal cells, namely MSCs) frequently suffers from inadequate regeneration and on-site morbidities with either fibro-cartilaginous or unnatural hypertrophic phenotype, no matter they are availed *in situ* from autologous bone marrow via microfracture, or manually transplanted with or without aids of artificial 3D scaffolds and guided pre-differentiation.[205]

Therefore, successful cartilage tissue engineering is the key point of this project. Cartilage tissue engineering aims and performs to restore cartilage function by developing cartilage substitutes using appropriate cells and biomaterials. As an elegant

connective tissue in musculoskeletal family, cartilage is endowed with compositional purity and structural delicacy, which builds up the bases of cartilage's biomechanical properties and functionalities.[206] In cartilage, parenchyma and stroma are combined; vessels and neurons are void. This feature induces significant challenge on the demand of high compositional purity in engineered cartilage graft. Particularly in articular cartilage graft, two levels of purities are demanded: free of non-cartilaginous constituents and exclusiveness of hyaline or hyaline-like cartilaginous phenotype.[207] Besides the challenge on high purities, there are another two challenges in cartilage tissue engineering: genuine architecture of the graft to match the structural delicacy of native cartilage; and, integration of the graft with the host counterpart after implantation - given the intrinsic obstruction of connection and diffusion between ECM-dense tissues.[208]

Figure 3-1.

Schematic illustration of osteochondral repair using man-made hyaline-like cartilage grafts with a timeline. A). Preparation and implantation of living hyaline cartilage graft (LhCG) and decellularized LhCG (dLhCG). During fabrication, gelatin-based *Microspheres* are used as porogen to create same sized *Microcavities* in *Chondrocytes*-laden hydrogel constructs. As solely cartilage-based grafts, LhCG and dLhCG are respectively implanted to cover the full thickness of osteochondral defects in both articular cartilage (*Cart*) and subchondral bones (*Sub-B*). B) Preparation and implantation of full-scaled osteochondral graft: LhCG+ and dLhCG+, namely LhCG and dLhCG respectively with an integrated subchondral bone substitute made of sintered microsphere scaffold (*SMS*). As full-scaled osteochondral grafts, LhCG+ and dLhCG+ are integrated constructs comprising of two units each: LhCG or dLhCG's *Extracellular matrix*-based "upper" block matching the *Cart* and *SMS* based "lower" block matching the *Sub-B*, in the middle of which there is a transition zone of interpenetrating network (*IPN*) at the junction - mostly on *SMS* side - schematically highlighted in bluish color.

As a solution while addressing all these listed challenges of cartilage tissue engineering, we had engineered a living hyaline cartilage graft (LhCG). As reported before, LhCG is produced by 3D cultured chondrocytes in a transient scaffold made of macro-porous alginate hydrogel *in vitro*. Once abundant cartilage neo-tissues are produced to form interpenetrating network within alginate scaffold, the alginate scaffold is removed instantly, completely and noninvasively by leaching with sodium citrate buffer; the remaining macro-scaled 3D sponge-like network of pure cartilaginous neo-tissues is LhCG (**Figure 3-1**).[209] It contains only living chondrocytes and endogenous ECMs deposited by chondrocytes, hence its ECMs are free of non-cartilaginous constituent and naturally structured with genuine architecture of native cartilage. Moreover, as the entire procedure has always been mediated in non-cell-adhesive hydrogel scaffolds – free of cell focal adhesion, the chondrocytes' phenotype is well preserved against fibrotic de-differentiation. Therefore, hyaline-like cartilaginous phenotype is assured in LhCG. Due to a sponge-like form, LhCG is not fluidic thus free of the leakage problem of ACI. Besides, LhCG is soft and porous, thus diffusion can be facilitated in bulk and integration can be enabled across the interface with surrounding host osteochondral tissues.[210]

Despite all advantages of LhCG, being a tissue engineering product containing living cells, its clinical application encounters significant logistic hassles; more importantly,

concerns and demands of host-graft immune biocompatibility may further confine it to autologous cell sourcing only, which makes additional constraints in operation by twice surgeries, strict window period, and risk of donor site morbidity. We further addressed these drawbacks of LhCG in this study by decellularization to obtain hyaline-like, cartilaginous tissue-specific ECM based acellular product, dLhCG, for engraftment application. (**Figure 3-1**). dLhCG is derived directly from LhCG via decellularization, thus all above listed advantages of LhCG, including the two levels of purities, micro-architectural genuineness, and even the porous sponge-like form, are all inherited.[211] Contrastively, dLhCG only consists of acellular ECMs, free of cellular components, therefore possesses fine immune biocompatibility with the host after implantation. Therefore, dLhCG is superior with dual advantages as a quality articular cartilage graft to perform xeno-engraftment for osteochondral repair. On one hand, dLhCG possesses the superfine architecture of native cartilaginous ECMs because of the naturally secreted ECMs from living chondrocytes in 3D environment that is very difficult to be mimicked by a biomaterial design; on the other hand, dLhCG owns the ease of decellularization to clean cellular component to an extent that native cartilage could not achieved as a matrix-condensed tissue without damage to architecture.[212]

3.1.2 Materials and methods

Fabrication of LhCG: Unless stated otherwise, all chemicals used were purchased from Sigma Aldrich, Singapore. All cell-culture reagents were purchased from Invitrogen, Life Technologies. The fabrication of LhCG was done according to the previously established protocol. Briefly, gelatin microspheres were produced using a double-emulsion method and sieved to obtain microspheres between 150 and 180 μm in diameter. The microspheres were then sterilized in 1000 units/mL of penicillin and streptomycin each in PBS and stored at 4°C until use. Separately, primary porcine

chondrocytes were harvested and cultured in flasks until they reached 80-90% confluence before being trypsinized and co-encapsulated (1×10^7 cells/mL) with gelatin microspheres (0.3g/mL) in alginate hydrogel. The hydrogel constructs were subsequently cultured on agarose-coated tissue culture plates in chondrocyte culture (CC) medium, which composed of Dulbecco's modified Eagle medium (DMEM) supplemented with 20% (v/v) fetal bovine serum (FBS, Invitrogen), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (HEPES, 0.01 M), non-essential amino acids (NEAA, 0.1 mM), L-proline (0.4 mM), vitamin C (0.05 mg/mL), penicillin (100 units/mL), and streptomycin (100 mg/mL). All the hydrogel constructs were maintained on an orbital shaker at 50 rpm in a humidified incubator at 37 °C with 5% CO₂. After 35 days, the hydrogel constructs were immersed in sodium citrate (55 mM in 0.15 M NaCl solution) for 10 mins at room temperature to remove the alginate hydrogel scaffold and cultured in CC medium for another ten days for maturation. Henceforth, these scaffold-free constructs were named LhCG. The thickness of the graft is 1.5 mm which matches to the depth of the cartilage defect.

Fabrication of dLhCG: Four decellularization methods were applied to decellularized LhCGs. dLhCG G1 was frozen at -20°C for three hours and thawed under room temperature for four hours. The freeze-thaw cycle was repeated three times. Subsequently, the graft was soaked in 1% sodium dodecyl sulphate (SDS) solution diluted by hypotonic solution for 72 hours. dLhCG G2 was frozen at -80°C for three hours and thawed under room temperature for four hours. The freeze-thaw cycle was repeated three times. Subsequently, the graft was soaked in hypotonic solution for 24 hours followed by 1% Triton-X (1st Base) diluted in hypotonic solution for 48 hrs. dLhCG G3 was immersed in hypotonic solution for 48 hours and then in 1% Triton-X in hypotonic solution for 72 hours. dLhCG G4 was immersed in 1% SDS solution

diluted by hypotonic solution for 72 hours followed by 1% Triton-X in hypotonic solution for another 72 hours. Finally grafts in all groups were put into DNase I (0.5 mg/mL) and RNase A (50 µg/mL) solution for 3 hours. Subsequently all grafts were washed by PBS for 24 hours. During the decellularization process by solution methods in all groups agitation of 150 rpm was applied under 37°C. Henceforth, the dLhCG G2 was named dLhCG.

Scanning Electron Microscopy: The LhCG and various dLhCG grafts were fixed in 4% formaldehyde and dehydrated in ethanol followed by freeze-drying. The samples were then sputter coated (Jeol JFC-1600) and observed in Field Emission Scanning Electron Microscope (FE-SEM) (JSM-7600F).

Biocompatibility test of dLhCG: All animal surgeries were performed according to the regulation of care and use of laboratory animals, and the protocols were approved by the Institutional Animal Care and Use Committee (IACUC). 2 female (Sprague-Dawley) rats (8 weeks old) were used for implantation, where the dLhCG grafts were embedded into the great omentum of the rats. Ketamine (75 mg/kg)/Xylazine (10 mg/kg) mixture was dosed intraperitoneally. The upper medial abdomen is the incision site. Xiphoid was the starting point of cut down which went inferior to the lower abdomen. The skin, subcutaneous tissue and parietal peritoneum were cut open layer by layer. The liver and stomach were flipped up to expose omentum. One sample was embedded in the omentum. Saline was used to wash the abdomen cavity and the incision site of the surgery. The incision site was sutured layer by layer.

Biochemical Analysis: Samples were frozen at -20 °C before freeze-drying for 24 h, followed by overnight digestion in papain buffer solution consisting of 0.3 mg/ml papain dissolved in 0.2 mM dithiothreitol (DTT) and 0.1 mM disodium ethylene diamine tetra

acetic acid (EDTA). DNA content was measured by the fluorometric Hoechst 33258 assay. Glycosaminoglycan (GAG) content was measured by 1,9-dimethylmethylen blue (DMMB) dye binding assay while total collagen content was quantified by using L-hydroxyproline assay from acid hydrolyzed samples.

Histology and immune-histology: The harvested samples from biocompatibility test were embedded in Optimal cutting temperature compound (OCT compound). The samples were cryo-sectioned with a thickness of 10 μ m through the center of the defect site, and then stained with Hematoxylin and Eosin (H&E), Safranin-O, Masson's Trichrome, Alizarin red and Picrosirius red according to standard protocols. In addition, the tissue sections were also used for immunohistochemistry with anti-type I (Abcam, USA), anti-type II collagen antibodies, anti-CD3 and anti-CD68 antibodies (Abcam, USA). For *in vitro* samples the slides were incubated with goat anti-rabbit IgG-FITC or chicken anti-mouse IgG (Santa Cruz, Singapore) at 37°C for 1 hour. The nuclei were then counterstained by 4',6-diamidino-2-phenylindole (DAPI) and observed under fluorescence microscope. For *in vivo* samples, the slides were treated according to the protocol of Ultra Vision Quanto Detection System HRP DAB kit (Thermo Scientific) and counterstained by Hematoxylin and observed under bright field.

3.1.3 Results and Discussion

3.1.3.1 Decellularization of engineered cartilage graft: from LhCG to dLhCG

The LhCG samples were produced with porcine chondrocytes (Passage 1) harvested from domestic pig knee articular cartilage as reported before (**Figure 3-1**). Here, the histology, scanning electron microscopy (SEM) and biochemical data of which again validated the tissue engineering product, LhCG, as a porous version of living articular cartilage template (**Figure 3-2a-c**). Four different decellularizing approaches were

applied on LhCG resulting in the products of dLhCG G1, G2, G3 and G4, respectively (**Figure 3-2a-c**). Optimal decellularization of a biological tissue pursues maximal clearance of cellular antigens mainly marked by DNA contents as well as minimal loss and distortion of ECMs. Based on the dual criteria, dLhCG G2 excelled other dLhCG products by the smallest DNA residue and the greatest preservation of glycosaminoglycan (GAG) and Type II collagen (Col II) at the same time (**Figure 3-2c**), plus the finest maintenance of cartilaginous ECM architecture (**Figure 3-2a, b**). The *in vivo* biocompatibility of dLhCG G2 product was tested by implanting it as a decellularized xenograft in rat greater omentum for one and two weeks, respectively. The histology reveals insignificant GAG and collagen degradation and initial angiogenesis alone with recellularization of non-immunocytes taking place in dLhCG G2 implant (**Figure 3-2d**), thus indicates an undetectable grade of acute immunogenicity or other toxicity of the implant to the host. Therefore, the clearance of cellular antigens in dLhCG G2 is validated. As an optimal product of decellularization, throughout all following experiments in this study, dLhCG G2 products were employed as the only executive dLhCG samples, the decellularization approach of which was also employed as the only executive methodology for the same.

Fig 3-2A

Fig 3-2B

Fig 3-2C

Figure 3-2.

Decellularized living hyaline cartilage graft (dLhCG) from LhCG: optimization of decellularization and biocompatibility test. A). Photographies of LhCG and various groups of dLhCG samples achieved from different decellularizing methods, namely, dLhCG G1, G2, G3 and G4 (sample diameter ~8mm, thickness 1~2mm); and histology of them, respectively, by Haematoxylin and eosin (H&E) stain, Safranin-O (Saf O) stain, and immuno-histochemistry (IHC) stain with primary antibody of collagen Type II (Col II). Scale bar: 200 μm . B) Scanning electron microscopy (SEM) of LhCG, dLhCG G1, G2, G3 and G4. Scale bar: 1mm. C). Quantification of DNA residue, glycosaminoglycan (GAG) and total collagen contents in various groups of dLhCG samples: dLhCG G1, G2, G3 and G4 in comparison to LhCG. All decellularized groups are significantly lower than LhCG graft in all contents, DNA, GAG and collagen with $p \leq 0.0001$, * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$, **** means $p \leq 0.0001$.

Tissue engineering products from xenogeneic or allogeneic cells are going to induce immune rejection after implantation due to the cellular components as a main source of antigen. However, the ECM of tissues is relatively tolerable due to the conservation of the components among different species. Therefore, the tissue engineering grafts could be decellularized to reduce the immune rejection which may lead to abnormal remodelling and even worse the failure of the graft. Other than the biological advantages, decellularization brings about other practical advantages which are critical to successful translation to clinical application. Since typically substantial infrastructure and logistics is a necessity of tissue engineering products, decellularization can reduce the troubles because of the living nature of the grafts. Decellularized tissue engineering products have the potential to be commercialized and industrialized to become “off-the-shelf” products. This potential not only is a logistic advantage, but also a benefit to patients

clinically, as it could be stored for relatively long period and turned into a ready-made commodity, thus expands the operation window period for patients.

To verify the biocompatibility of dLhCG graft, it was embedded in the omentum of rats for up to two weeks. The greater omentum is a highly vascularized network contributes to immune reaction, hence it is clinically relevant as an environment to test the biocompatibility of dLhCG graft. The histology of the embedded grafts reveals the migration of non-immunocytes cells into the graft without the formation of fibrous encapsulation, instead neo blood vessels and neo tissues has penetrated the grafts by the time of two weeks. (Figure 3-3) Moreover the GAG and collagen quantity in the graft has been largely preserved without degradation. The H&E staining was scored using a 5-point ordinal severity scale in a no inflammation to moderate range on lymphocytes, multinucleated giant cells, neutrophils, macrophages, mast cells and eosinophils, which indicates a high biocompatibility of the xenogeneic graft. In addition, the severity of inflammation decreases from 1 week to 2 weeks after implantation which implied that the inflammatory response was a reaction to the implantation of grafts or the surgery but not a long term immune rejection. (Table 3-1)

Overall the biocompatibility test validated the clearance of DNA after the decellularization process which avoids the induction of immune reaction thus improves the integration and bioactive molecules retention. The high biocompatibility of dLhCG realizes the utilization of xenogeneic grafts whose cells could be from different species, which resolves the problem of limited healthy cartilage for grafts, avoids donor sites morbidity and avoids two rounds of surgery in autologous implantation.

Figure 3-3.

H&E, Saf O, Masson's trichrome (MT), Alizarin red staining (AR) and immuno-histochemistry (IHC) staining with primary antibody of CD3 and CD68 for dLhCG, which is the decellularized sample, after being implanted in rat greater omentum for one and two weeks, respectively, for test of *in vivo* biocompatibility. The region enclosed in a dash-line rectangle is the position of the graft.

Table 3-1 Scoring on severity of inflammation of rat omentum embedded porcine xenogeneic dLhCG graft

	1 week	2 weeks
Lymphocytes	3	2
Multinucleated giant cells	1	0
Neutrophils	2	2
Macrophages	3	2
Mast cells	2	1
Eosinophils	2	1

3.1.3.2 Storage potential test of dLhCG grafts

To overcome the short operation window period problem and prepare the dLhCG graft as a clinically available product, the storage period or shelf life is critical too. Hence the dLhCG graft has been preserved in phosphorous buffered saline (PBS) at 4°C for up to 60 days. It is shown that quantity of all main relevant compounds including GAG and Col II is remained at similar level without significant difference according to the biochemical analysis, which is confirmed by the histology results as revealed by the similar intensity of staining especially the Safranin O staining for GAG and immunohistochemistry for Col II. Moreover, the histology results verified that the morphology of the graft is maintained with a lacunae shape like native cartilage throughout the 2 months as shown by the H&E staining (Figure 3-4). Hence it is concluded that storage in PBS at 4°C up to 2 months is not affecting the property of the graft, which proved the potential of dLhCG grafts to become an off-the-shelf product.

Fig 3-4A

Fig 3-4B

Figure 3-4.

Storage test of dLhCG up to 2 months A). histology of dLhCG stored in PBS under 4°C for 14 days, 30 days, and 60 days in comparison to fresh dLhCG respectively, by Haematoxylin and eosin (H&E) stain, Safranin-O (Saf O) stain, and immuno-histochemistry (IHC) stain with primary antibody of collagen Type II (Col II). Scale bar: 200 μm . B) Quantification of DNA residue, glycosaminoglycan (GAG) and total soluble collagen contents in all samples: dLhCG, dLhCG 14 days, 30 days and 60 days. No significant difference was observed in all content at all time points. Positive Saf O stain of GAG appears in bright red; positive IHC stain of Col II appears as emitting green fluorescence; and positive DAPI stain of cell nuclei appears as emitting blue fluorescence.

3.2 Development and characterization of LhCG+ and dLhCG+ grafts

3.2.1 Introduction

Overall incidence of osteochondral defect in the general population is estimated to be 1 to 2 million people.[213] Repair of critical-sized osteochondral defects remains a significant clinical challenge due to its limited self-repair capability where limited treatment options are available. Currently, autologous, allogeneic or xenogeneic grafts are frequently used as clinical treatment.[214] Nevertheless, the major disadvantages of such therapeutic approaches are the limited access to autologous and allogeneic grafts with a significant risk of donor site morbidity. Moreover, complications such as the risk of rejection, disease transmission and infection further restrict the use of allogeneic and xenogeneic grafts in many clinical treatments.[215] Therefore, focus has been shifted to tissue engineering as a promising alternative therapeutic approach for the treatment of orthopaedic injuries and diseases.

In tissue engineering field, there is an increasing awareness that design of osteochondral tissue engineering grafts needs to consider the *in vivo* complexity to increase the likelihood of successful osteochondral tissue repair.[216] Therefore, the utilization of multiphasic scaffolds to recapitulate the multiphasic nature of the native tissue is a developing trend in osteochondral tissue engineering. The hierarchical structure of the osteochondral tissue contains a gradient of varying tissue organization which work in concert to confer diverse structural and mechanical properties across the tissue.[217] By developing osteochondral scaffolds with tissue-specific architecture, it could induce functional regeneration of osteochondral constructs.

While many biosynthesized grafts comprise of both the cartilage and bone regions, they are not able to recreate the complex osteochondral interfacial structure which resulted in unstable conjunction and poor mechanical properties, thus limiting its advancement towards clinical translation.[218] The osteochondral transition zone has unique mechanical properties because of a high level of gradient and interfacial tissue organization between bone and cartilage.[219] An ideal tissue engineered osteochondral graft should withstand compression and friction, while interacting with different structural and biological environment at cartilage, bone and cartilage-bone interface layers.[220]

Articular cartilage contains pure hyaline cartilage which induces significant challenge on the demand of high compositional purity in tissue engineering cartilage graft.[221] As a solution, we had engineered a living hyaline cartilage graft (LhCG).[222] The prototype of LhCG was established in the previous study that exhibited similar characteristic markers as articular cartilage and preserved the tissue purity.[223] Moreover, LhCG has demonstrated its capability to repair cartilage defect and favourable integration with the host tissue in pre-clinical studies. On the other hand,

subchondral bone has relative stronger self-repair capability and larger mechanical hardness compared to cartilage.[224] Poly (lactic-co-glycolic- acid) is an FDA approved biomaterial widely used in bone tissue engineering due to its tuneable mechanical property and high biocompatibility.[225]

In this section a full-scaled osteochondral graft, LhCG+, was designed and developed combining LhCG prototype, PLGA as subchondral plug with a naturally formed interpenetration network as the gradient transition zone as shown in Figure 3-1.

3.2.2 Materials and Methods

Fabrication of PLGA Sintered microspheres scaffold (SMS): Unless stated otherwise, all chemicals used were purchased from Sigma Aldrich, Singapore and all cell-culture reagents were purchased from Invitrogen, Life Technologies. PLGA (85:15) was dissolved in dichloromethane (CH_2Cl_2) at a ratio of 1:5 (w/v). Upon complete dissolution, the mixture was added using 0.3 mm diameter needle injection at a rate of 0.01mL/s into 1% PVA solution under stirring of 500 rpm at room temperature. The resultant emulsion was kept stirring at 400 rpm overnight to allow complete evaporation of methylene chloride. The PLGA microspheres were collected by filtration, followed by washing with distilled water and vacuum dry. The microspheres were sieved to select the size of 200-300 μm .

SMS was fabricated by putting 20 mg of PLGA microspheres into silicon mould (4 mm in diameter) and heated at 70°C for 2 hours. The SMS was collected by removing the mould when returning to room temperature. After sterilization by immersing in 70% alcohol and UV overnight, the scaffolds were kept in DMEM until further usage.

Fabrication of LhCG+: Passage 1 chondrocytes and gelatine microspheres (fabricated as stated in section 3.1.2) were mixed in alginate hydrogel. 80 μL of mixture was added

on top of each SMS scaffold and 20 μ L of 102mM calcium chloride solution was dropped into each construct to facilitate the gelation process. Each construct was then cultured on agarose gel-coated tissue culture plate in chondrocyte culture (CC) medium in the humidified incubator at 37°C with 5% CO₂. Chondrocytes in the hydrogel voluntarily migrated and proliferated into the micro-cavities in SMS. After culturing for 35 days, the alginate hydrogel was removed by leaching in sodium citrate buffer for 10 mins. Henceforth, these full-scaled osteochondral constructs were named LhCG+. The total height of the entire graft is 3 mm which matches to the defect depth of the entire osteochondral defect.

Fabrication of dLhCG+: The entire LhCG+ graft was decellularized side by side using four approaches to select the optimal decellularization protocol. dLhCG+ G1 was frozen at -20°C for three hours and thawed under room temperature for four hours. The freeze-thaw cycle was repeated three times. Subsequently, the graft was soaked in 1% sodium dodecyl sulphate (SDS) solution diluted by hypotonic solution for 72 h. dLhCG+ G2 was frozen at -80°C for three hours and thawed under room temperature for four hours. The freeze-thaw cycle was repeated three times. Subsequently, the graft was soaked in hypotonic solution for 24 h followed by 1% Triton-X (1st Base) diluted in hypotonic solution for 48 h. dLhCG+ G3 was immersed in hypotonic solution for 48 h and then in 1% Triton-X in hypotonic solution for 72 h. dLhCG+ G4 was immersed in 1% SDS solution diluted by hypotonic solution for 72 h followed by 1% Triton-X in hypotonic solution for another 72 hours. Finally grafts in all groups were put into DNase I (0.5 mg/mL) and RNase A (50 μ g/mL) solution for 3 hours. Subsequently all grafts were washed by PBS for 24 h. During the decellularization process by solution methods in all groups agitation of 150 rpm was applied under 37°C. Henceforth, the dLhCG+ G2 were named dLhCG+.

Scanning Electron Microscopy: The LhCG+ graft was fixed in 4% formaldehyde and dehydrated in ethanol followed by freeze-drying. The sample was then sputter coated and observed in Field Emission Scanning Electron Microscope (FE-SEM) (JSM-7600F).

Biochemical Analysis: Samples were frozen at -20°C before freeze-drying for 24 h, followed by overnight digestion in 0.3 mg/ml papain dissolved in 0.2 mM DTT and 0.1 mM EDTA solution. DNA content was measured by the fluorometric Hoechst 33258 assay. Glycosaminoglycan (GAG) content was measured by 1,9-dimethylmethylen blue (DMMB) dye binding assay while total collagen content was quantified by using L-hydroxyproline assay from acid hydrolysed samples. Calcium was quantified by using o-cresolphthalein assay (sigma and abcam) from 0.6N chloric acid extracted samples.

Histology and immunohistochemistry: The samples were frozen embedded and sectioned with a thickness of $10\mu\text{m}$ through the centre of the sample, and then stained with Hematoxylin and eosin (H&E) and Safranin-O according to standard protocols. In addition, the sections were also used for immunohistochemical staining with anti-type I (Abcam, USA) and anti-type II collagen antibodies. Subsequently the slides were incubated with goat anti-rabbit IgG-FITC (Santa Cruz, Singapore) at 37°C for 1 h. The nuclei were then counterstained by 4',6-diamidino-2-phenylindole (DAPI) and observed under fluorescence microscope.

Statistical analysis: All data in this study were shown as average $\pm$ standard deviation. One-way ANOVA statistical analysis with post hoc comparisons was used to analyse the data. $p < 0.05$ was deemed to indicate statistical significance.

3.2.3 Results and discussion

3.2.3.1 Fabrication of LhCG+ graft

Full-scaled osteochondral grafts, namely LhCG+ and dLhCG+, are fabricated as an integrated construct comprising of two units each: ECM-based scaffold as the “upper” block and degradable plastic co-polymer, PLGA-based SMS as the “lower” block. The ECM block and SMS block are physically joined together by entangling each other, forming a transition zone of interpenetrating network (IPN) at the junction - mostly on SMS side. (Figure 3-1 and 5 a, b). The ECM block and SMS block respectively plays a role of cartilage and subchondral bone substitute for full-scaled osteochondral engraftment. The employed SMS is constructed by sintering degradable polymeric microspheres, hence the bulk of this scaffold is in the form of microsphere assembly and the empty space in between the spheres forms the pores. In comparison with other forms of porous scaffolds for tissue engineering application, including those created on basis of porogen induced phase separation or electrospinning, SMS is endowed with relatively greater mass of bulk material and well-connected pores upon relatively lower porosity overall. To act as a degradable subchondral plug, once implanted into the trabecular bone environment, SMS substitute possesses greater instant strength as well as higher sustainability due to the larger amount of material, and more importantly, it favors *in situ* recellularization by facilitating cell migration due to the connected pores.

Fig. 3-5A

Fig. 3-5B

Fig. 3-5C

Fig. 3-5D

Figure 3-5.

Full-scaled osteochondral graft: LhCG+. A) Schematic illustration for fabrication of LhCG+ and macroscopic observation of the mature graft, as an integrated construct comprising of two units each: 3D cultured chondrocytes' secreted extracellular matrix (ECM)-based scaffold as the "upper" block and PLGA-based SMS as the "lower" block, in the middle of which there is a transitional zone of interpenetrating network (IPN) at the junction - mostly on SMS side. B) Scanning electron microscopy (SEM) of LhCG+: respectively differentiating the zones of ECM, IPN (SMS) and SMS bottom; and particularly highlighting the IPN zone with higher magnitudes. Scale bar: 100µm. C) In vitro histology of LhCG+ at various depths of the scaffold, i.e. at the top of the ECM based layer, at the bottom of the ECM layer right above the SMS, at the top of the SMS layer right below the ECM based layer, at the bottom of the SMS layer, by haematoxylin and eosin (H&E) staining, safranin-O (Saf O) staining, and immuno-histochemistry (IHC) staining with primary antibodies of collagen Type II (Col II) and collagen Type I (Col I), in which the zones of ECM, IPN (SMS) and SMS bottom are differentiated. Scale bar: 100µm. Positive Saf O staining of GAG appears in bright red; positive IHC staining of Col II and Col I appears as emitting green fluorescence; and positive DAPI staining of cell nuclei appears as emitting blue fluorescence. And D) Quantification of cell number, glycosaminoglycan (GAG) and total collagen contents in LhCG+, where the ECM-block and SMS-block of LhCG+ are differentiated. * means $p \leq 0.05$, ** means $P \leq 0.01$, *** means $P \leq 0.001$, **** means $P \leq 0.0001$.

The full-scaled osteochondral graft, LhCG+, was made on basis of 3D cell culture template using microcavity structure on top of SMS plug (Figure 3-1 and 3-5a). The upper ECM block of LhCG+ construct (ECM-LhCG+) is secreted by chondrocytes cultured in 3D microcavity structure. As revealed by SEM (Figure 3-5b), histology (Figure 3-5c) and biochemical analysis (Figure 3-5d), both cartilaginous composition and matrix architecture in ECM-LhCG+ block is in a hyaline cartilage phenotype as indicated by immunohistochemistry (IHC) stains of abundant Col II over lean Type I collagen (Col I) and the lacunae morphology same as native cartilage. The lower SMS block of LhCG+ construct (SMS-LhCG+) is also perfused, thus entangled, with cartilaginous component that extends from the upper ECM-LhCG+ block via the connective IPN zone over the junction (Figure 3-5a, b). Biochemical analysis indicated observably smaller cell population and significantly lower presence of cartilaginous matrices in SMS-LhCG+ block than those in ECM-LhCG+ block (Figure 3-5d), on the ground of which SEM and histology further revealed a decreasing gradient of cartilaginous constituents across the SMS-LhCG+ block from the IPN zone through the bottom of it. This decrease in cell population and matrices also go along with a decay of hyaline-like cartilaginous phenotype marked by observable up-regulation in IHC stain of Col I (Figure 3-5c). Since the SMS-LhCG+ block is designed as an open platform

accessible for *in situ* recellularization of subchondral bone cells after implantation, this result favored the purpose.

3.2.3.1 Fabrication of dLhCG+

Due to the advantages of decellularized graft, LhCG+ graft was decellularized using four different methods. Optimal decellularization of a biological tissue pursues maximal clearance of cellular antigens mainly marked by DNA contents as well as minimal loss and distortion of ECMs. Based on the dual criteria, dLhCG+ G2 excelled other dLhCG+ products considering the DNA residue and ECM component preservation. The smallest amount of DNA showed up in dLhCG+ G2, and dLhCG+ G4. Without significant reduction in DNA, the purpose of decellularization is defeated. Hence dLhCG+ G1 and dLhCG+ G3 are not satisfactory methods to decellularize LhCG+ grafts as shown by the high DNA quantity in biochemical analysis and visible staining of nucleus by DAPI. Between dLhCG+ G2 and dLhCG+ G4 group, the preservation of ECM is considered, as shown by the GAG and collagen Type II staining, the preservation of the two main component in articular cartilage is significantly higher than dLhCG+ G4 group as validated by the biochemical analysis. In addition, the finest maintenance of cartilaginous ECM architecture and micro-structure as shown by the morphology of stained sections proved that dLhCG+ G2 is the optimal decellularization methods in this set-up (Figure 3-6). From now on the dLhCG+ G2 group will be named as dLhCG+ graft and used for further test. In dLhCG+ the “upper” ECM block of dLhCG+ construct (ECM-dLhCG+) is made up of pure ECM secreted by chondrocytes, the extension of which across the junction into the “lower” SMS block of the same dLhCG+ construct (SMS-dLhCG+) is responsible for binding the two blocks together via entanglement in the IPN zone. Although the components in the SMS-dLhCG+ part are significantly lost due to the decellularization process, since the entire dLhCG+ construct is designed as

an open platform accessible for *in situ* recellularization of both chondrocytes and cells from subchondral bone, respectively, into the blocks of ECM- and SMS-dLhCG+ after implantation, the structure of the scaffold and the acellular nature facilitates the access of cells into the graft.

Fig. 3-6C

Fig. 3-6D

Fig. 3-6E

Figure 3-6.

Decellularized full-scaled osteochondral graft: dLhCG+. A) , B), C), and D) In vitro histology of dLhCG+ fabricated by decellularizing LhCG+ using four different approaches side by side, labelled as G1, G2, G3, G4, respectively, by haematoxylin and eosin (H&E) staining, safranin-O (Saf O) staining, and immuno-histochemistry (IHC) staining with primary antibodies of collagen Type II (Col II) and collagen Type I (Col I), in which the zones of ECM, IPN (SMS) and SMS bottom are differentiated. Scale bar: 100 μm . Positive Saf O staining of GAG appears in bright red; positive IHC staining of Col II and Col I appears as emitting green fluorescence; and positive DAPI staining of cell nuclei appears as emitting blue fluorescence. And E) Quantification of DNA, GAG and total collagen contents in the ECM-block of all dLhCG+ groups in comparison. The same quantifications in the SMS-block of dLhCG+ reveal too trivial outcomes to be detectable and reportable. * means $p \leq 0.05$, ** means $P \leq 0.01$, *** means $P \leq 0.001$, **** means $P \leq 0.0001$.

3.3 Conclusion

In conclusion, the decellularized ECM based man-made cartilage graft dLhCG has been successfully fabricated with improved biocompatibility and storage capacity.

Considering the benefits brought by the acellular and xeno/allo-genic nature of dLhCG grafts which broaden the source of healthy chondrocytes, avoid two rounds of surgery, elongate operation window period and facilitate clinical application, the dLhCG graft is promising to become a useful tool to treat osteochondral injury clinically.

In addition, the full-scaled osteochondral grafts d/LhCG+ have been successfully developed too. The PLGA-SMS subchondral layer was integrated with scaffold-free ECM-based cartilage layer sandwiching a transition zone to establish the cartilage-bone interface. Gelation of chondral layer on top of the porous subchondral bone layer mimics the osteochondral gradient in composition and architecture. The secretion of ECM from living chondrocytes and the migration of chondrocytes into the porous PLGA-SMS scaffold formed the layer specific osteochondral tissue zonal structure naturally. The

decellularized full-scaled osteochondral graft (dLhCG+) has been successfully fabricated as confirmed by the removal of cellular component and retention of ECM composition and its morphology. However, the decellularization process significantly reduced the ECM components' content in the subchondral mimicking layer in dLhCG+ grafts which may lead to delamination problem *in vivo*.

In the future a more advanced decellularization approach is to be developed for LhCG+ grafts, cell apoptosis induction could be considered as an alternative method to remove cells which may retain the ECM to a higher extend. Moreover, the decellularization process could be strictly optimized using orthogonal experiments to standardize the procedure and prepare dLhCG grafts for industrialization.

Chapter 4 Using dLhCG grafts to repair cartilage defect

Summary

In this study, we endeavor to repair traumatic cartilage lesions in pig knee models using novel dLhCG *allo* and *auto* grafts that is produced by 3D cultured porcine chondrocytes *in vitro* - free of non-cartilaginous constituent. Comparative trials are conducted in parallel among the developed samples to clinically available autologous chondrocyte implantation (ACI). Here, sound cartilage regeneration in 6 months after implantation has been shown, including the recoveries in form and function with correct cartilage composition, structure and phenotype. The allogeneic decellularized graft excels other counterparts, which opens this engrafting system to enjoy all superiorities and conveniences brought up by decellularization process in terms of logistic simplicity, immune biocompatibility and architectural genuineness.

4.1 Introduction

Articular cartilage, a connective tissue of diarthrodial joints, devoid of blood vessels, lymphatics and nerves, is highly specialized to adapt to a harsh biomechanical environment. The property leads to the limited capability of intrinsic healing and repair and hence the needs of articular cartilage defect repair treatment. Tissue engineering evolving from biomaterials combining scaffolds, cells and bioactive molecules is promising as a reparative rather than palliative method to treat articular cartilage defect.[164] Scaffolds are commonly fabricated using synthetic materials as templates for tissue formation or bioreactors to apply stimuli to seeded cells. However, synthetic materials bring about disadvantages involving inflammatory reactions, acidic by-

products and undesirable residues. Therefore scaffold-free grafts have been studied to circumvent the problems.[226] Moreover scaffold-free grafts preserve intact cell-ECM structure and improve functional similarity to natural tissue. Nevertheless, in the neo-tissue formation process *in vitro* the scaffold consisting of biomaterials is beneficial to the growth and development of engineered tissue as the trigger for proper cell migration by designed structures. Hence a transient scaffold could be ideal for the tissue engineering grafts where the synthetic material could be removed after graft maturation.[227]

Extracellular matrices (ECM) from a variety of tissues including lung, dermis, vasculature, liver and adipose tissue has been applied as biomimetic scaffolds for tissue regeneration taking advantages of the biochemical cues. Decellularization is a process to remove all cellular components while preserving biologically active factors, three-dimensional architectures and microstructures of the matrices. The successful application of decellularization methods broadens the source of tissue from autologous to allogeneous and xenogeneic as the cellular component, i.e. the main source of antigen causing immunoreaction that is destructive to tissue regeneration, has been largely reduced.[228] However, decellularization of cartilage, due to its dense nature, requires harsh environment which causes loss of its bioactivity and limits its function in application.

Moreover, the integration of implanted graft is a major challenge for the success of tissue engineering protocols. Effective cartilage integration takes place in the condition that matrix-free chondrocytes are induced to migrate between the native tissue and implanted graft surfaces.[229] The appropriate porosity facilitates integration of the graft with surrounding tissue and remodelling via cell migration in tissue regeneration, replacement and repair process after implantation. The compact structure of native

cartilage tissue not only makes it difficult for chondrocytes to be removed from the ECM but also for proliferated chondrocytes to grow into the matrix. The low porosity of decellularized native cartilage is a fatal drawback that leads to immunogenicity and incomplete cartilage regeneration.[192] Inefficient migration of chondrocytes into the decellularized native cartilage may cause collapse of the structure with the degradation of the matrix in long term.

Native articular cartilage tissue is unique in terms of the purity of the hyaline type cartilaginous tissue, the tight network of Type II collagen fibres and abundant highly negatively-charged proteoglycans chains as ECM. The uniqueness of cartilage on one hand fits in the scaffold-free tissue engineering paradigm on the other hand raises hurdles for decellularization of native tissue.[230] Due to the high purity of articular cartilage tissue the scaffold-free graft is in advantage which prevents the formation of fibrous tissue or capsule around the graft after implantation, on the contrary in the graft maturation process, the non-cell-adhesive supporting scaffold is critical to preserve the phenotype of articular chondrocytes and prohibit formation of fibrocartilage. Hence our lab previously developed a transient scaffold of micro cavity structure fabricated by alginate with gelatin as porogen. Utilizing the transient scaffold, a mature scaffold-free living hyaline cartilage graft (LhCG) with high purity of hyaline like cartilage tissue was produced.[231] On the other hand, although the decellularization is more frequently applied on native tissue to produce ECM as graft for regenerative medicine, articular cartilage tissue faces the difficulty of being fully decellularized and manipulated or customized. Therefore, tissue engineering decellularized hyaline like cartilaginous ECM would be a potential alternative. Hence, we have decellularized the LhCG graft to fabricate a porous ECM based scaffold-free tissue engineered graft for cartilage defect repair. (Figure 3-1)[232]

Current cartilage repair strategies are bone marrow stimulation (microfracture), osteochondral autograft transfer system (OATS) or mosaicplasty, and autologous chondrocyte implantation (ACI). Small but not large symptomatic articular cartilage lesions are commonly treated by microfracture which results in the formation of mechanically inferior fibrocartilage. OATS can create a smooth surface in the defect although; graft subsidence at the surface with postoperative weight bearing and the absence of repair within the dead space between cylindrical grafts are the limitations.[233] ACI is the surgery procedure adopting tissue engineering concept to treat cartilage defect. The first generation of ACI is the implantation of the autologous chondrocytes which is expanded *in vitro* to the defect site under the cover of the periosteal flap. The second generation uses a synthetic membrane instead of the periosteal flap as the cover.[234] The third generation uses a biodegradable material as a scaffold for chondrocytes implantation into the defect. Due to the liquid nature of the autologous chondrocyte implantation the loss of the chondrocytes severely affects the quality of the repair. In addition, overgrowth and secondary damage caused by suturing of membrane onto cartilage are frequently reported complications of ACI.[235] Nevertheless, ACI is a popular second-line treatment for cartilage injury especially in young people.[236]

In this study we aim to investigate the tissue repair capability of decellularized LhCG (dLhCG) in both autologous and allogeneous scenarios and further, in comparison to the previously developed LhCG grafts and especially in direct comparison to clinically available ACI treatment to prove the advantage of our graft to the current cartilage injury treatment. The autologous scenario was achieved by implantation of grafts cultured from chondrocytes harvested from the same pig whereas the allogeneous scenario was achieved by implantation of grafts cultured from chondrocytes harvested from a

different pig. The hypothesis of this experiment is that dLhCG repairs the cartilage defect as good as LhCG and both dLhCG and LhCG repairs cartilage defect better than ACI.

4.2 Materials and Methods

Cartilage repair capability test of LhCG and dLhCG: 12 female mix breed domestic pig (*sus scrofa domesticus*) (Innoheart Pte. Ltd., Singapore) weighing 50-60 kg were used. Analgesics (Tramadol, 2-4mg/kg, IM) and antibiotics (Synulox, 0.05ml/kg, IM) was administered to the animal once before surgery. The animal would be pre-medicated using Atropine Sulphate (50µg/kg, IM). After which, it will be anesthetized using tiletamine, zolazepam, ketamine, and xylazine (TKX cocktail) solution (0.05ml/kg, IM). Anaesthesia was maintained during the procedure using 1-3% Isoflurane. The regions of the knee were disinfected with iodine and 70% ethanol. A parapatellar cut down was made to expose the knee joint, and the patella was laterally dislocated. In the first stage of surgery 8 pieces of 3 mm diameter cartilage chips were harvested from patella groove at each knee joint using biopsy punch. The cartilage chips were stored in phosphate buffered saline (PBS) solution under cooling by ice pack and transported to the lab which would be processed on within 2 hours. The primary chondrocytes were harvested from the fresh cartilage chips and LhCG and dLhCG grafts were fabricated as reported in section 3.1.2 and the ACI cell suspension were prepared. Two months after the first stage of surgery, a second stage of surgery was performed in which one beyond critical-size full-thickness cartilage defects were made with minimal damage to the subchondral bone on each condyle of the knee (8 mm in diameter). Hence totally 48 lesions were created, among which 10 were left untreated as negative controls, 10 were implanted with Allo dLhCGs; 10 were implanted with Auto dLhCGs; 9 were implanted with Auto LhCGs and 9 were treated by ACI.

ACI cell suspension preparation and implantation: When the chondrocytes were 80-90% confluent in flask, they were cryopreserved in CC medium in liquid nitrogen. Two weeks before the second round of surgery, the cells were thawed and plated into new flasks to further proliferate to reach the required cell concentration in ACI cell suspension which is 12 million cells/ml. In the operation procedure, a patch of membrane made by decellularized and devitalized porcine skin has been sewn over the cartilage defect. 80 μ L of such cell suspension which matched to the defect volume were injected underneath the membrane.

Implanted tissue harvesting: Six months after second stage surgery, the pigs were sacrificed by intravenous injection of pentobarbital (150 mg/kg). The femur distal end including the implant, its corresponding subchondral bone and the surrounding host tissue were collected.

Biomechanical Test: Native articular cartilage in the same weight-bearing region from the hind legs of the same pig were used as positive controls in the biomechanical testing. For the analysis of biomechanical properties, the Young's modulus of the harvested samples were measured using biomechanical equipment (Instron, USA). A constant, compressive crosshead moving at the speed of 1mm/min was applied on the LhCG, dLhCG, and ACI repaired tissue, and auto-regenerated tissue until the end-of-test criterion was reached (80% of the specimen height). A stress-strain curve was then obtained. The Young's modulus of the tested tissue was calculated by linearization of the raw data.

Histology and immunohistochemistry: All samples were paraffin embedded and sectioned with a thickness of 10 μ m through the centre of the sample, and then stained with Hematoxylin and Eosin (H&E), Safranin-O, Masson's Trichrome and Picrosirius

red according to standard protocols respectively. In addition, the tissue sections were also used for immunohistochemistry with anti-type I (Abcam, USA), anti-type II and anti-type X collagen and anti-CD3 and anti-CD68 antibodies. The slides were treated according to the protocol of Ultra Vision Quanto Detection System HRP DAB kit (Thermo Scientific, Singapore) and observed under bright field. The *in vivo* regenerated cartilage was scored using the classical scoring system – Histology histopathology grading system (HHGS) and Wakitani Scoring system. (Supplementary Table 4-1 and 4-2) The criteria considered by this histological scoring system include structure, cell morphology, safranin-O staining and tide mark integrity. Samples were scored independently by three evaluators who were blind to experimental data. The scores of the three observers were averaged to obtain a representative score.

Biochemical Analysis: Samples were frozen at -20°C before freeze-drying for 24 h, followed by overnight digestion by 0.3 mg/ml papain dissolved in 0.2 mM DTT and 0.1 mM EDTA. DNA content was measured by the fluorometric Hoechst 33258 assay. GAG content was measured by DMMB dye binding assay while total collagen content was quantified by L-hydroxyproline assay from acid hydrolysed samples.

Microcomputed Tomography (μCT): The femoral ends of tissue from pigs sacrificed after 6 months were fixed in 10% neutral buffered formalin for at least 5 days prior to microcomputed tomography (μCT) analysis. The qualitative and quantitative analysis of neo-tissue regeneration within defect site in all the five groups was examined using high resolution μCT (inspeXio SMX-90CT imaging system, Shimadzu, Japan) scanning.

The bone sample was mounted into the sample holder with femur axis perpendicular to scanning plane. Scanning was carried out at an X-ray source voltage of 90 kV and beam current of 110 μ A. The X-ray projections were captured at rotation interval of 1.2° around 360° of the sample with voxel of 0.1 mm/pixel. X-ray projections were reconstructed using inspeXion main software. Defect site was visualized through all three planes coronary, sagittal and transverse of defect with adjacent native tissue. For quantification of bone volume to tissue volume (BV/TV) in regenerated neo-tissue, a cylindrical volume of interest (VOI) of 8 mm diameter corresponding to the defect region was selected in the dataset.

Microarray analysis: The Agilent SurePrint G3 Custom GE 8x60K 1 color (Agilent, USA) was used as microarray platform to examine the global gene expression of cells in tissues harvested from all groups, native cartilage, subchondroal bone and synovium. 100 ng of total RNA was labeled with Low Input Quick Amp Labeling Kit and purified by Qiagen RNeasy Kit (Qiagen, Singapore). 600 ng of cyanine 3-CTP labeled cRNA was hybridized onto Agilent Sure Print G3 Pig GE 8x60K Microarray for 17 h at 65°C 10 rpm in Agilent hybridization oven. After wash the hybridized microarray slide was scanned on Agilent High Resolution Microarray Scanner. Processing and analysis of the microarray data were performed with the Agilent Feature Extraction software. The data were normalized by percentile shift to 75th percentile per sample and transformation to median of all samples per gene.

Statistical analysis: All data in this study were shown as average $\pm$ standard deviation. One-way ANOVA statistical analysis with post hoc comparisons was used to analyse the data. $p < 0.05$ was deemed to indicate statistical significance.

4.3 Results and Discussion

The purpose of development of tissue engineering graft is to repair cartilage defect that is beyond the critical size. The critical size reported in domestic pig (*sus scrofa*) was 6.3 mm in diameter. As such one defect was created at each femoral condyle of 8 mm in diameter. The animals have been allocated into *Allo* and *Auto* groups where the *Allo* group meaning *Allo* dLhCG group receives samples developed from chondrocytes harvested from other pigs whereas *Auto* groups including *Auto* dLhCG group, LhCG group and ACI group receive samples developed from chondrocytes harvested from the same pig. The negative control was untreated defect that were left empty without any implantation treatment after defect creation, while the positive control was the healthy native cartilage near the defect region. The ACI group was applied according to the guidance given by Carticel[®], an FDA approved ACI product. This design embodies the hypothesis by three progressive sub-hypothesis: firstly the LhCG and dLhCG grafts repair cartilage defect effectively and is superior in function recovery of damaged cartilage to ACI treatment; secondly the dLhCG grafts repair the cartilage defect as efficient as LhCG grafts which is with living cells, in other words, the decellularization process is not diminishing the cartilage repair efficacy of LhCG grafts; and thirdly the *Allo* dLhCG grafts although fabricated from allogeneic chondrocytes do not induce immune rejection and repair cartilage defect as efficient as *Auto* dLhCG grafts.

The function of articular cartilage is to provide a smooth, lubricated surface to allow smooth gliding movements between bones with low frictional coefficient and to bear weight loads as a shock absorber. The same composition and structure of the regenerated tissue to the native tissue is the key criteria in determining the quality of repair which leads to the ideal recovery of function of the cartilage. As revealed by the histology results clinically available ACI and all grafts developed in our lab were able

to achieve cartilage regeneration that completely fills up the defect in contrary to the partially filled defect that was left empty without any implantation of graft as a negative control. (Figure 4-1a-h) However the ACI group has shown an irregular surface with pannus formation on the top surface of the cartilage which could be due to the involvement of a membrane made by porcine skin Col I and Type III collagen (Col III) to cover the chondrocytes suspension in the liquid phase to prevent leakage. The membrane although is a must to preserve chondrocytes brings impurities to the regenerated tissue as shown by the negative staining in Col II and positive staining in Col I (Figure 4-1 d, e) which consequently results in the inferior mechanical property. (Table 4-1) Hence it is concluded that LhCG and dLhCG groups repair the defect more efficiently than currently clinically available ACI treatment.

Table 4-1 Mechanical test of *in vivo* regenerated cartilage tissue from *Allo* dLhCG, *Auto* dLhCG, LhCG, ACI engraftment and auto regenerated tissue in comparison to native cartilage

Sample	Compressive modulus
Native	0.46±0.02
Untreated defect	0.29±0.33
ACI	0.29±0.27
<i>Allo</i> dLhCG	0.39±0.16
<i>Auto</i> dLhCG	0.30±0.27
<i>Auto</i> LhCG	0.32±0.17

Fig 4-1A

Allo dLhCG

Auto dLhCG

Auto LhCG

Untreated defect

ACI

2mm

Fig 4-1B

Allo dLhCG

Auto dLhCG

Auto LhCG

Untreated defect

ACI

2mm

Fig 4-1C

Allo dLhCG

Auto dLhCG

Auto LhCG

Untreated defect

ACI

2mm

Fig 4-1D

Allo dLhCG

Auto dLhCG

Auto LhCG

Untreated defect

ACI

2mm

Fig. 4-11

Fig 4-1J

Figure 4-1.

Histology of cartilage repair by engraftment of *Auto* LhCG, *Auto* dLhCG and *Allo* dLhCG in comparison to ACI at time point of 6 months after engraftment. A) Hematoxylin and eosin (H&E) staining, B) Masson's trichrome (MT) staining, C) Safranin-O (Saf O) staining, and immuno-histochemistry (IHC) staining with D) primary antibody of Type II collagen (Col II), E) Type I collagen (Col I), F) Type X collagen (Col X), G) CD3 and H) CD68. The negative control, namely the untreated defects that are left void of grafts, is marked as "Untreated defect". Two arrows are used to indicate the location and width of the experimental defects originally made. Scale bar: 2 mm. I) Picrosirius staining under polarized light and bright field, which reveals ultra-structural histology of articular cartilage zones of the Samples. The Samples include LhCG, *Auto* dLhCG and *Allo* dLhCG, clinically available ACI treatment, the negative control "Untreated defect" and the positive control – intact, surrounding native cartilage of the host – marked as "Native". Scale bar: 200 µm. Positive MT staining of collagens appears blue; positive IHC staining by polymerization of 3',3'-diaminobenzidine(DAB) catalyzed by horse radish peroxidase (HRP) conjugated on secondary antibody appears in golden brown. Picrosirius stains relatively raw matrix fibers in brown or dark yellow and relatively delicate fibers in green or light yellow under polarized light. J) The histological scores based on both Histological Histochemical Grading System (HHGS) and Wakitani scoring system. A low score (min. 0; max. 14) defines a native-like cartilage tissue with full thickness defect filling and good integration with adjacent host cartilage. (*) indicates statistical significance ($p < 0.05$); ** indicates $p \leq 0.01$, *** indicates $p \leq 0.001$.

GAG and Col II are the two composites that endows the cartilage with the superior

mechanical property. GAG plays important role in reservation of water molecules whose incompressibility contributes to the compression strength of articular cartilage. However, it is observable that almost all decellularization process decreases the GAG quantity due to the relatively smaller molecular weight and size (Figure 3-2). Interestingly after six months *in vivo* remodelling process, the regenerated tissue has been shown to be more intensively stained by Safranin O in dLhCGs and ACI groups compared to LhCG groups. Although decellularization has caused reduction in GAG quantity in dLhCGs compared to LhCGs, the porosity has been increased too. The higher porosity facilitates the migration of cells into the dLhCGs not mentioning the liquid nature of ACI in comparison to LhCG hence results in better *in vivo* remodelling and larger quantity of GAGs secreted by migrated chondrocytes. On the other hand, the purity of collagen in dLhCGs and LhCG were high as shown by the strong staining of Col II but no positive staining of Col I which was opposite to ACI group as revealed by the brownish staining of Col I especially at the centre of the regenerated tissue. Other than the impurities brought by the covering membrane, the upregulation of secretion of Col I in ACI could be due to the di-differentiation of chondrocytes causing the generation of fibrocartilage which decreases the mechanical strength of neo-tissue. Although dense ECM affects the migration of chondrocytes into the grafts, it maintains the morphology of chondrocytes resided inside to ensure the purity of the regenerated cartilage phenotype. Both LhCG and dLhCGs are ECM rich hydrogels whose bioactivity prevents di-differentiation of chondrocytes. Overall, as confirmed by the higher but not significantly different total scoring of dLhCG groups than LhCG groups (Figure 4-1g), dLhCG grafts repairs the cartilage defect as good as LhCG grafts because the dLhCG grafts benefit from the dual advantages of easy

migration of chondrocytes hence better *in vivo* remodelling and better maintenance of cell morphology hence high purity of cartilage phenotype.

Fig 4-2A

Fig 4-2B

Fig 4-2C

Figure. 4-2

Biochemical analysis of cartilage repair of LhCG, Auto dLhCG and Allo dLhCG in comparison to ACI at time point of 6 months. The negative control, namely the untreated defects that are left void of grafts, is marked as “Untreated defect”; the positive control, namely the intact surrounding native tissues of the host, is marked as “Native”. The analyses include DNA assay, glycosaminoglycan (GAG) assay, and total soluble collagen assay. The quantity of each composition are normalized by A) wet weight, B) dry weight and C) volume of the defect. * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$, **** means $p \leq 0.0001$

Native cartilage owes a zonal structure of superficial zone, middle zone and deep zone from top to bottom which was validated by the picrosirius staining to show different

orientation and alignment of collagen fibres and different cell arrangement in each zone.(Figure 4-1f) The *Allo* dLhCG group is the closest to the native cartilage by showing three zones of structure consisting of the superficial zone with collagen packed and parallel to the surface of cartilage, the middle zone with collagen oblique and chondrocytes dispersed and the deep zone of collagen perpendicular to the surface of cartilage tissue and chondrocytes in columnar orientation. Combined with the biochemical composition that is most like native cartilage (Figure 4-2a-c) the *Allo* dLhCG grafts achieved the largest compression strength as shown by compressive modulus that reaches 85% of native cartilage. (Table 4-1) In addition, the subchondral bone status in all groups has been verified to be with minimal to no damage by micro-CT. (Figure 4-3)

Figure 4-3.
Representative μ CT images of coronal, sagittal and transverse sections of regenerated tissue with surrounding native tissue A) without any treatment or treated by clinically available ACI and B) treated by tissue engineering grafts *Allo* dLhCG, *Auto* dLhCG and *Auto* LhCG at 6 months

In summary, The LhCG and dLhCGs grafts repaired the cartilage defect with more like native articular cartilage tissue than clinically available ACI treatment as shown by the total score in both Histology histopathology grading system (HHGS) and Wakitani scoring system comprehensively considering structure, cell morphology and integration and the mechanical property as the main function of articular cartilage. Especially *Allo* dLhCG group achieved the same level of repair efficacy to *Auto* LhCG group. The success of allogeneic decellularized graft broadens the cell source for graft fabrication; reduces two rounds of surgery in ACI and autologous grafts to one round and avoids the donor site morbidity problem; furthermore, elongates the window period of operation for patients and overcome the hurdle for clinical application caused by living cells.

Fig 4-4

Figure 4-4.

Gene expression profiling of cells in all samples. DNA microarray analysis was performed on RNA extracted from cells in *Allo* dLhCG, *Auto* dLhCG, *Auto* LhCG, ACI and auto regenerated tissues, which were compared to cells in native surrounding tissues, namely native cartilage, native subchondral bone and native synovium. Expression level were sorted to observe patterns on a list of 15 marker genes. Bars represent the fold change in gene expression i.e. up-regulation (upwards) or down-regulation (downwards).

The positive results triggered our interest to study the mechanism behind the repair process. As a decellularized graft *Auto* and *Allo* dLhCG grafts are supposed to be not carrying any living cells however as shown by the DNA content (Figure 4-2) and various staining in histology (Figure 4-1) after 6 months *in vivo* remodelling process, the regenerated neo cartilage-like tissue owns living cells inside. The elucidation of the cell type is critical to understand the repair process and hence a microarray was conducted using the RNA extracted from all the samples and the surrounding native cartilage, native subchondral bone and native synovium tissues. It was observed when all the related marker genes are presented as gene expression profile across all samples that *Allo* and *Auto* dLhCG samples both showed similar pattern as native cartilage as shown by the same trend of up or down regulation of genes. The genes were arranged in an order following the fold of change of expression level in microarray results of

native cartilage, as such the genes to the left were upregulated whereas genes on the right were down regulated. (Figure 4-5) The same trend of expression profile was observed in both *Allo* and *Auto* dLhCG grafts. Although the fold of changes was not the same exactly, the trend was the same to native cartilage. On the contrary the gene expression pattern of native subchondral bone and synovium tissue was the opposite where the genes on the left were down regulated and genes on the right were upregulated generally. Among all the genes, the key chondrocyte markers including Col II (COL2A1), aggrecan (ACAN), cartilage oligomeric matrix protein (COMP) and collagen type XI (COL11A1) were all upregulated which indicates the secretion of various characteristic proteins in cartilage. The expression level that was higher than native cartilage is very possibly due to the response to the injury environment to compensate for the loss of cartilage tissue after the creation of the defect. Moreover, the expression profile of *Auto* LhCG is a mixture of the chondrocytes that resided in the living graft and the cells migrated from the surrounding native tissue. Despite the difference between the LhCG grafts and native cartilage, both dLhCG grafts and LhCG grafts performed significantly better than ACI treatment whose COL2A1 gene expression has been down regulated and COL10A1 gene has been upregulated compared to native cartilage which coincided with the frequently reported complication of hypertrophic phenotype after implantation that leads to failure of treatment ultimately. In addition, it was observed that majority of the bony and synovial markers including alkaline phosphatase (ALPL), bone sialoprotein (IBSP) and fibroblast growth factor 2 (FGF2) in the *Allo* and *Auto* dLhCG grafts were down regulated in comparison to native bone. The comparison of expression profile of cells in *Allo* and *Auto* dLhCG grafts to cells in native cartilage, subchondral bone and synovium tissue revealed that the cells resided in the decellularized graft 6 months

after implantation were chondrocytes that migrated from the surrounding cartilage tissue. Cartilage has been reported with limited self-regeneration capability though; the graft has successfully stimulated the proliferation of chondrocytes and regeneration of neo cartilage tissue.

4.4 Conclusion

In conclusion, the hyaline cartilage grafts developed in our lab including *Allo* dLhCG, *Auto* dLhCG, LhCG grafts repaired cartilage defect successfully and more importantly superior to currently clinically available ACI treatment in terms of mechanical property, histology, biochemical composition, and residing cells' gene expression profile. Moreover, the decellularized grafts no matter allogeneic or autologous achieved the same level of repair in comparison to living cellular LhCG grafts as shown by the mechanical property, histology and biochemical composition.

Considering the benefits brought by the acellular and allogeneic nature of *Allo* dLhCG grafts which broaden the source of healthy chondrocytes, avoid two rounds of surgery, elongate operation window period and facilitate clinical application, the *Allo* dLhCG graft is promising to become a useful tool to treat cartilage injury clinically.

In the future, to prepare dLhCG grafts as a commercialized and industrialized product, the decellularization methods should be further optimized and standardized using orthogonal experiments.

Chapter 5 LhCG+ and dLhCG+ graft to repair osteochondral defect

Summary

In this tissue engineering study, we endeavor to repair traumatic osteochondral lesions in rabbit knee models using full-scaled living and decellularized xenografts (d/LhCG+) that is produced by 3D cultured porcine chondrocytes *in vitro* on top of sintered poly (lactic-co-glycolic acid) microspheres scaffold. Comparative trials are conducted in parallel among samples with or without graft decellularization. Here, sound osteochondral regeneration of both cartilage and subchondral bones from engraftment within 100 days after implantation has been shown in living grafts, including the recoveries in form and function with correct osteochondral composition, structure and phenotype that is superior to decellularized grafts. Both living and decellularized grafts showed accelerated osteochondral regeneration effect in comparison to defects left untreated.

5.1 Introduction

Volumetric loss in cartilage and subchondral bone due to injury or degenerative diseases are commonly treated by osteochondral grafts. Autologous, allogeneic or xenogeneic grafts are currently the most frequently used grafts; however, the application of these grafts is limited by source. Donor site morbidity is a long-standing risk of autologous grafts, especially for cartilage tissue which has a very limited self-repair capability.[237] Whereas the risk of rejection, disease transmission and infection, the inability to be customized and the inherent heterogeneity is the restriction for allogeneic and xenogeneic grafts. Therefore, tissue engineering field becomes a promising alternative

method to find solutions for orthopaedic injuries and diseases. The complex osteochondral structure and the limited self-repair capacity of articular cartilage has always been a challenging task in osteochondral defect repair.[238] An ideal graft to repair a large osteochondral defect should be able to provide mechanical stability during the early postoperative stage while induce tissue repair and regeneration. To repair osteochondral defect composed of the bone, cartilage and the bone-cartilage interface, grafts ranging from single phase to bilayer / multi-phase osteochondral grafts have been adopted.

Both the articular cartilage and the underlying subchondral bone are affected by an osteochondral injury.[239] The osteochondral zone has unique mechanical properties because of a high level of gradient and interfacial tissue organization between bone and cartilage. In the developmental process, bone is formed from cartilage via endochondral ossification process and after maturation the two tissues are complementing each other through intricate mechanisms. From the lubricating and resistive cartilage to hard and mineralized subchondral bone below it, morphological, physical and mechanical features are changing gradually to allow a smooth transition.[240] The healing abilities of cartilage and bone are different too. Cartilage layer of the defect lacks the self-repair capability due to the low metabolic rate, whereas the subchondral bone layer has a stronger self-repair capability. To enhance graft integration and further establish long-term functionality, the regeneration of the osteochondral interface is critical.[241] An ideal tissue engineered osteochondral graft should withstand compression and friction, while interacting with different structural and biological environment at cartilage, bone and cartilage-bone interface layers. To develop an interface mimicking the native tissue, biocompatible and biodegradable scaffolds with highly porous microstructures is

desired to facilitate cell attachment, proliferation and stimulation of neo tissue infiltration.

Clinical application of a tissue engineering product containing living cells encounters significant logistic hassles; more importantly, concerns and demands of host-graft immune biocompatibility may further confine it to autologous cell sourcing only, which makes additional constraints in operation by twice surgeries, strict window period, and limited source.[242] Decellularization is a promising method to avoid the problems involved by cellular components in grafts. After decellularization tissue engineering extracellular matrix (ECM) with tissue specific architecture are capable to induce regeneration and differentiation without supplementation of biologic additives. This advantage has made them an attractive alternative from both cost and regulatory standpoints.[243] Although decellularization process has been successfully applied on many tissue types, the dense ECM nature of cartilage tissue causes a unique challenge due to diffusion limitations. The tissue needs to be disrupted mechanically to aid decellularization, but this step destroys the mechanical properties of the matrix. Additionally, the dense ECM nature of cartilage also impedes cell migration into the matrix, hindering tissue regeneration.[190] Hence, there is a need for an acellular matrix with low immunogenicity that resembles native cartilage in terms of its biochemical make-up.

In this study, we endeavor to repair traumatic osteochondral lesions via a biphasic tissue engineering graft with a naturally formed interpenetration zone from cartilage phase to bone phase. The anisotropic graft is characterized for the different architecture and composition at the cartilage phase, bone phase and cartilage-bone interface.[244] The decellularization potential on the graft has been tested by histology and biochemical analysis. The osteochondral lesion repair capability has been tested in a rabbit

osteocondral defect model by examining the mechanical property and biological property of the regenerated tissue. The cell and tissue morphogenesis and mineralization were examined by histology, biochemical analysis and microtomography.[245]

5.2 Materials and Methods

Osteochondral defect repair capability of d/LhCG+: 15 male New Zealand White Rabbits (3 – 4 months old, supplied by Innoheart Pte. Ltd., Singapore) weighing 3 to 4 kg were used. Rabbits were given an intramuscular injection of mixture of Ketamine (10-15mg/kg) and Domitor (0.1- 0.2mg/kg) for anesthesia. Subsequently, the regions of the knee were shaved and disinfected with iodine and 70% ethanol. A parapatellar cut down was made to expose the knee joint, and the patella was laterally dislocated. Two osteochondral defects were made on condyles of the knee (3 mm in diameter, 3 mm in depth). The graft was press-fit into the defect and the cut was closed by silk suture.

Biochemical Analysis: Samples were frozen at -20°C before freeze-drying for 24 h, followed by overnight digestion in 0.3 mg/ml papain dissolved in 0.2 mM DTT and 0.1 mM EDTA. DNA content was measured by the fluorometric Hoechst 33258 assay. GAG content was measured by DMMB dye binding assay. Total collagen content was quantified by using L-hydroxyproline assay from acid hydrolysed samples. Calcium was quantified by using o-cresolphthalein assay (sigma and abcam) from 0.6N hydrochloric acid extracted samples.

Histology and immunohistochemistry: The harvested samples from osteochondral defect repair experiment were paraffin embedded. The samples were sectioned with a thickness of $10\mu\text{m}$ through the centre of the sample, and then stained with hematoxylin and eosin (H&E), Safranin-O, Masson's Trichrome and Picrosirius red according to standard protocols. In addition, the sections were also used for immunohistochemical staining

with anti-type I (Abcam) and anti-type II collagen antibodies. The slides were treated according to the protocol of Ultra Vision Quanto Detection System HRP DAB kit (Thermo Scientific, Singapore) and counterstained by hematoxylin and observed under bright field.

Biomechanical Test: Native osteochondral tissue in the same weight-bearing region from the hind legs of the same rabbit were used as positive controls in the biomechanical testing. For the analysis of biomechanical properties, the Young's moduli of the harvested samples were measured using biomechanical equipment (Instron, USA). A constant compressive crosshead moving at the speed of 1mm/min was applied on the LhCG+ repaired tissue, dLhCG+ repaired tissue and auto-regenerated tissue until the end-of-test criterion was reached (80% of the specimen height). A stress-strain curve was then obtained. The Young's modulus of the tested tissue was calculated by linearization of the elastic region of the curve.

Microcomputed Tomography: The femoral ends of tissue from rabbits sacrificed after 50 days and 100 days were fixed in 10% neutral buffered formalin for at least 5 days prior to microcomputed tomography (μ CT) analysis. The qualitative and quantitative analysis of neo-tissue regeneration within defect site in LhCG+ group was examined using high resolution μ CT (inspeXio SMX-90CT imaging system, Shimadzu, Japan) scanning. The bone sample was mounted onto the sample holder with femur axis perpendicular to scanning plane. Scanning was carried out at an X-ray source voltage of 90 kV and beam current of 110 μ A. The X-ray projections were captured at rotation interval of 1.2° around 360° of the sample with voxel of 0.1 mm/pixel. X-ray projections were reconstructed using inspeXion main software. Defect site was visualized through all three planes coronary, sagittal and transverse of defect with adjacent native tissue.

For quantification of BV/TV in regenerated neo-tissue, a cylindrical VOI of 3 mm diameter corresponding to the defect region was selected in the dataset.

Statistical analysis: All data in this study were shown as average $\pm$ standard deviation. One-way ANOVA statistical analysis with post hoc comparisons was used to analyse the data. $p < 0.05$ was deemed to indicate statistical significance.

5.3 Results and discussion

Osteochondral repair was executed by *in situ* engraftment of full-scaled osteochondral grafts, LhCG+ and dLhCG+, respectively. To fit the osteochondral defect, the thickness of ECM-d/LhCG+ blocks and SMS-d/LhCG+ blocks roughly matches the depth of defect in cartilage and subchondral bone layer, respectively. (Figure 3-1b and Figure 3-5a). This design serves to testify the repairing efficacy of these full-scaled osteochondral grafts, namely LhCG+ and dLhCG+, in terms of osteochondral regeneration *in situ*.

Table 5-1 Mechanical test of regenerated osteochondral tissue by LhCG+ and

Samples	Young's Modulus (MPa)	
	Day 50	Day100
Untreated	0.85 ± 0.75	0.16 ± 0.22
dLhCG+	1.46 ± 1.93	3.37 ± 2.18
LhCG+	0.48 ± 0.58	7.80 ± 5.55
Native	10.99 ± 3.26	

dLhCG+ grafts

Figure. 5-1

Histology of osteochondral repair by engraftment of full-scaled osteochondral implants: LhCG+ and dLhCG+. Testing time points are on Day 50 and Day 100, respectively. A) Macroscopic observation of the defect at the two testing time points, Scale bar: 1mm. B) Haematoxylin and eosin (H&E) stain, C) Masson's trichrome (MT) stain, D) Safranin-O (Saf O) stain, and immuno-histochemistry (IHC) stain with E) primary antibody of collagen type II (Col II) and F) collagen type I (Col I). The negative control, namely the untreated defects that are left void of grafts, is marked as "untreated". Two arrows are used to indicate the location and width of the experimental defects originally made. Scale bar: 500µm. G) Polarizing microscopy of picrosirius staining, which reveals ultra-structural histology of both articular cartilage zone (Cart) and subchondral bone zone (Sub-B) of the Samples. The Samples include LhCG+, dLhCG+, the negative control "Untreated" and the positive control – intact, surrounding native Cart and Sub-B of the host – marked as "Native". Positive Saf O staining of GAG appears in bright red; positive MT staining of collagens appears blue; positive IHC staining uses 3,3' - diaminobenzidine (DAB) which precipitates in the presence of horseradish peroxidase (HRP) conjugated protein appears in brown. Picrosirius stains relative raw matrix fibers in red or dark yellow, stains relatively delicate fibrils in green or light yellow.

Fig5-2A

Fig5-2B

Figure 5-2.

Biochemical analysis of osteochondral repair by engraftment of full-scaled osteochondral implants: LhCG+ and dLhCG+. The negative control, namely the untreated defects that are left void of grafts, is marked as “untreated”; the positive control, namely the intact, surrounding native tissues of the host, is marked as “native”. The samples harvested from the articular cartilage zone are marked as “Cart” and the samples harvested from the subchondral bone zone are marked as “Sub-B”. The analyses include DNA assay, glycosaminoglycan (GAG) assay, collagen assay and calcium assay. Testing time points are on Day 50 and Day 100, respectively. The samples are normalized by A) wet weight and B) dry weight. * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$

As revealed by various histological stains (Figure 5-1), from Day 50 to Day 100, the performance of osteochondral repair by LhCG+ significantly excels that of dLhCG+; while dLhCG+ significantly excels that of the untreated defect, which remains completely negative all the time. For the full-scaled osteochondral graft LhCG+ graft, by the time of Day 50, the cartilage layer has been recovered to partial thickness of the native cartilage. In addition, the subchondral layers consisting of PLGA based SMS block has been partially degraded with the infiltration of neo-tissue. In the remaining part of the SMS block the porosity is like surrounding native trabecular bone. By the time of Day 100 the LhCG+ group eventually achieves similarly positive regeneration of both hyaline-like articular cartilage and subchondral bones considering the dimensional, compositional, phenotypic and architectural restoration, which also

appears comparable to surrounding native tissues of the host. Noticeably, as the engineered subchondral bone plugs, the PLGA based SMS-block has vanished in form; the successful remodeling of subchondral bones is implied. The regenerative performance of decellularized full-scaled osteochondral graft dLhCG+ appears significantly less positive than that of LhCG+ at both time points, especially on Day 100 when no comparable regeneration is accomplished either in articular cartilage or in subchondral bones. The tendency revealed by biochemical quantification (Figure 5-2) and mechanical test (Table 5-1) appears in line with the histology. Overall, osteochondral graft is successful in osteochondral regeneration with similar tissue architecture, composition and ultrastructure and hence the similar mechanical property, which is 70% of native osteochondral tissue. However, the decellularization influences the repair efficacy of the osteochondral graft. This is probably due to the delamination caused by the weakened IPN entanglement after decellularization. Hence a more advanced decellularization method could be tested out to prepare the graft to become clinically available.

Fig5-3

Figure 5-3.

Representative μ CT images of coronary, sagittal and transverse sections of regenerated tissue with surrounding native tissue treated by full-scaled osteochondral implants: LhCG+ at Day 50 and Day100. Scale bar: 3 mm.

Table 5-3 Bone volume to total volume ratio of regenerated subchondral bone in LhCG+ group

Samples	BV/TV (%)	
	Day 50	Day100
LhCG+	0.72 $\pm$ 0.03	0.91 $\pm$ 0.05
Native	0.99 $\pm$ 0.02	0.98 $\pm$ 0.02

As revealed by the mechanical, histology and biochemical analysis, the LhCG+ group showed a promising tissue repair capability. As an osteochondral graft the specific healing capacity should be examined at different zones. Although only chondrocytes were resided in the graft, the subchondral bone region is healed too according to the tests. Hence the subchondral bone tissue morphogenesis and mineralized matrix development at the defect site was examined qualitatively (Figure 5-3) and further bone specific parameter were quantified in LhCG+ group at Day 50 and Day 100 (Table 5-2). The defects were reconstructed into images through coronary, sagittal and transverse planes using VGStudio. The LhCG+ group exhibited closure of defect with no adverse degeneration of tissue based on the increased intensity of image as time passed by. Well directed regeneration in the subchondral bone region progressing towards the closure of defect was observed in the LhCG+ groups as shown by the higher density at the

surrounding region than the centre. This may be due to the porous structure of PLGA-SMS scaffold, which facilitates bone growth towards the defect with higher porosity and relatively high mechanical strength to bear the load in the regeneration procedure. The reconstructed micro CT images have been volume rendered and the cylindrical defect volumes of interest (VOI) has been analysed to identify the extend in mineralization in regenerated neo-tissue (Table 5-2). The value BV/TV indicates the fraction of a given volume of interest (VOI, i.e. the Total Volume TV) that is occupied by mineralized bone (Bone Volume). The BV/TV of the reparative tissue was able to reach 70% of the native tissue after 50 days and progressed on to 93% after 100 days. The quantitative assessment of BV/TV is in consistent to the qualitative image intensity and showed increase after 100 days compared to 50 days in LhCG+ group and ultimately reached a level that is like native subchondral bone. The substantial mineralization of new tissue in LhCG+ group could be due to the matching degradation profile of PLGA scaffold with the regeneration profile of the injured subchondral bone. Overall the μ CT affirmed the ossification and bone tissue regeneration process in the subchondral bone region in response to the engraftment of LhCG+ grafts.

5.4 Conclusion

The biomimetic gradient LhCG+ scaffold exhibited superior tissue coverage at the defect macroscopically. Furthermore, the histology studies showed the regeneration of subchondral bone and cartilage tissue as evidenced by same phenotype neo-tissue as the native healthy cartilage. In addition, confined subchondral bone formation with BV/TV same as the native tissue was seen in animals engrafted with gradient LhCG+ graft. More importantly, the biomechanical compression test demonstrated higher Young's modulus in LhCG+ group, which implies strong load-bearing capacity of regenerated neo-tissue at 70% of native tissue. Nevertheless, in the untreated defect no neo-tissue

was regenerated in the cartilage layer and the tissue degenerate gradually. Hence, the engineered osteochondral mimetic LhCG+ graft with cartilage layer, subchondral bone layer and gradient interface showed accelerated osteochondral tissue regeneration. However, the dLhCG+ grafts did not achieve satisfactory level of tissue repair effect, which could be due to the reduction of ECM in the transition zone. The reduction of ECM affects the strength of connection between the cartilage and the subchondral bone layer which may lead to the delamination of the two layers and hence unsatisfactory repair efficacy. Therefore, in the future a decellularization method that can retain the ECM to a higher extent and achieve the same level of cellular component removal is desired and should be researched on.

Chapter 6 dLhCG and LhCG graft to repair osteochondral defect

Summary

In this tissue engineering study, we endeavor to repair traumatic osteochondral lesions in rabbit knee models using a novel decellularized man-made hyaline-like cartilage xenograft that is produced by 3D cultured porcine chondrocytes in vitro - free of non-cartilaginous constituent. Comparative trials are conducted in parallel among samples with or without graft decellularization. Here, sound osteochondral regeneration of both cartilage and subchondral bones from cartilage engraftment within 100 days after implantation has been shown, including the recoveries in form and function with correct osteochondral composition, structure and phenotype. The decellularized grafts and living grafts achieved the same level of repair efficacy, hence the decellularized graft excels the counterpart due to superiorities and conveniences brought up by decellularization process in terms of logistic simplicity, immune biocompatibility and architectural genuineness.

6.1 Introduction

Osteochondral lesions contain lesions in both cartilage and subchondral bone tissue. To repair osteochondral lesions, the key challenge lies in regeneration of articular cartilage, as subchondral bones are capable of self-regeneration given a shield by fine cartilage on top; however, the self-regenerating ability of articular cartilage is significantly compromised due to its avascular and nerveless nature. Therefore, a successful treatment of traumatic osteochondral lesions relies on successful cartilaginous repair.[246]

In this study, we endeavor to repair traumatic osteochondral lesions via tissue engineering strategy. It is hypothesized that, given a finely regenerated articular cartilage shielding on top, the restoration of subchondral bones, and even overall osteochondral complex, can be managed afterwards via self-remodeling *in situ*. Therefore, successful cartilage tissue engineering is of the key point of this study.[234] Cartilage tissue engineering aims and performs to restore cartilage function by developing cartilage substitutes using appropriate cells and biomaterials. As an elegant connective tissue in musculoskeletal family, cartilage is endowed with compositional purity and structural delicacy, which builds up the bases of cartilage's biomechanical properties and functionalities. In cartilage, parenchyma and stroma are combined; vessels and neurons are void.[247] This feature induces significant challenge on the demand of high compositional purity in engineered cartilage graft.[248] Particularly in articular cartilage graft, two levels of purities are demanded: free of non-cartilaginous constituents and exclusiveness of hyaline cartilage. Besides the challenge on high purities, there are another two challenges in cartilage tissue engineering: genuine architecture of the graft to match the structural delicacy of native cartilage; and, integration of the graft with the host counterpart after implantation - given the intrinsic obstruction of connection and diffusion between ECM-dense tissues.[249]

Collectively, the general hypothesis and corresponding design of this study lie in pursuit of osteochondral restoration achieved via *in situ* tissue remodeling by providing articular cartilage repair via engraftment of tissue-engineered cartilage substitutes followed by self-regeneration of subchondral bone. To this end, dLhCG, as a novel decellularized man-made hyaline-like cartilage xenograft was developed from porcine chondrocytes and implanted to repair traumatic osteochondral lesions in rabbit knee models. This study focused on testing the osteochondral repair efficacy of acellular dLhCG— that is,

to test the efficacy of decellularized graft for osteochondral repair which targets to repair cartilage layer without deliberate repair on subchondral bone layer.

6.2 Materials and Methods

Histology and immunohistochemistry: The samples were sectioned with a thickness of 10 µm through the center of the graft, and then stained with Hematoxylin and Eosin (H&E), Safranin-O, Masson's Trichrome and Picrosirius red where applicable according to standard protocols. In addition, the tissue sections were also used for immunohistochemistry with anti-type I (Abcam, USA) and anti-type II collagen antibodies. The slides were treated according to the protocol of Ultra Vision Quanto Detection System HRP DAB kit (Thermo Scientific, Singapore) and counterstained by Hematoxylin and observed under bright field.

Biochemical Analysis: Samples were frozen at -20 °C before freeze-drying for 24 h, followed by overnight digestion by 0.3 mg/ml papain dissolved in 0.2 mM DTT and 0.1 mM EDTA solution. DNA content was measured by the fluorometric Hoechst 33258 assay. GAG content was measured by DMMB dye binding assay while total soluble collagen content was quantified by using L-hydroxyproline assay from acid hydrolyzed samples. Calcium content was quantified by the chromogenic complex formed between calcium ions and o-cresolphthalein.

Osteochondral defect repair capability of dLhCG and LhCG: 24 male New Zealand White Rabbits (3 – 4 months old, supplied by Innoheart Pte. Ltd., Singapore) weighting 3 to 4 kg were used. Rabbits were given an intramuscular injection Ketamine (10-15mg/kg) and Domitor (0.1- 0.2mg/kg) followed for anesthesia. Subsequently, the regions of the knee were shaved and disinfected with iodine and 70% ethanol. A parapatellar cut down was made to expose the knee joint, and the patella was laterally

dislocated. Two critical-size osteochondral defects were made on patellar groove of the knee (3 mm in diameter, 3 mm in depth). The grafts were press-fit in and the opening was closed by silk suture.

Biomechanical Test: Young's modulus of each harvested sample was measured using biomechanical equipment (Instron, USA) in an unconfined set-up where the samples were kept in-situ at the patella groove. A constant, compressive crosshead moving at the speed of 1mm/min was applied on the LhCG and dLhCG repaired tissue, and auto-regenerated tissue inside the defect that were untreated until the end-of-test criteria were reached (80% of the specimen height or 100 N compression force whichever earlier). A stress-strain curve was then obtained. The Young's modulus of the tested tissue was calculated by linearization of the elastic region of the curve. Native articular cartilage in the same weight-bearing region from the hind legs of the same rabbit was used as positive control in biomechanical test.

Microcomputed Tomography (μ CT): The femoral ends of tissue from rabbits sacrificed 50 days and 100 days after implantation were fixed in 10% neutral buffered formalin for at least 5 days prior to μ CT analysis. The qualitative and quantitative analysis of neo-tissue regeneration within defect site in all groups was examined using high resolution μ CT (inspeXio SMX-90CT imaging system, Shimadzu, Japan) scanning. The bone sample was mounted into the sample holder with femur axis perpendicular to scanning plane. Scanning was carried out at an X-ray source voltage of 90 kV and beam current of 110 μ A. The X-ray projections were captured at rotation interval of 1.2° around 360° of the sample with voxel of 0.1 mm/pixel. X-ray projections were reconstructed using VGStudio software. Defect site was visualized through all three planes coronary, sagittal and transverse of defect with adjacent native tissue. For

quantification of BV/TV in regenerated neo-tissue, a cylindrical VOI of 3 mm diameter corresponding to the defect region was selected in the dataset.

Microarray analysis: The Agilent SurePrint G3 Custom GE 8x60K 1 color (Agilent, USA) was used as microarray platform to examine the global gene expression of cells in dLhCG, LhCG and auto-regenerated tissue, native cartilage and native subchondral bone. 100 ng of total RNA was labeled with Low Input Quick Amp Labeling Kit and purified by Qiagen RNeasy Kit (Qiagen, Singapore). 600 ng of cyanine 3-CTP labeled cRNA was hybridized onto Agilent Sure Print G3 Rabbit GE 8x60K Microarray for 17 h at 65°C 10 rpm in Agilent hybridization oven. After wash the hybridized microarray slide was scanned on Agilent High Resolution Microarray Scanner. Processing and analysis of the microarray data were performed with the Agilent Feature Extraction software. The data were normalized by percentile shift to 75th percentile per sample and transformation to median of all samples per gene.

Statistical analysis: All data in this study were shown as average $\pm$ standard deviation. One-way ANOVA statistical analysis with post hoc comparisons was used to analyze the data. $p < 0.05$ was deemed to indicate statistical significance.

6.3 Results and discussion

Osteochondral repair was executed by *in situ* engraftment of LhCG and dLhCG, respectively, in animal models in parallel. Traumatic osteochondral defects of diameter 3 mm penetrating the articular cartilage layer into the subchondral bone were created on the patellar groove of rabbit knees in the hind legs. Surgical implantation of LhCG and dLhCG were conducted respectively as testing samples; untreated defects that were left void of grafts were employed as negative controls. The surrounding native

osteocondral tissues that were kept intact during the entire experiment were taken as positive control. Two time-points were taken for characterization and analysis on the 50th day (Day 50) and 100th day (Day 100) after the implantation surgery, respectively. The key design for this animal experimentation lies in the use of solely cartilage-based grafts to cover the full thickness of osteochondral defects. (Figure 3-1a.) This design embodies the hypothesis that, given a finely regenerated articular cartilage shielding on top, the restoration of subchondral bones and further the entire osteochondral complex, can be managed afterwards via self-remodeling *in situ*.

Fig 6-1A

Fig 6-1B

Fig 6-1C

Fig 6-1D

Fig 6-1E

Fig 6-1F

Fig 6-1G

Figure 6-1.

Histology of osteochondral repair by engraftment of cartilage implants, decellularized living hyaline cartilage graft (dLhCG) and LhCG, at testing time points of Day 50 and Day 100, respectively. A) Photographies of regenerated tissue 50 days and 100 days after implantation of dLhCG and LhCG graft and untreated defect that were left empty. Scale bar: 3 mm B). Haematoxylin and eosin (H&E) stain, C) Masson's trichrome (MT) stain, D) Safranin-O (Saf O) stain, and immuno-histochemistry (IHC) stain with E) primary antibody of collagen Type II (Col II) and F) collagen Type I (Col I). The negative control, namely the untreated defects that are left void of grafts, is marked as "Untreated". Two arrows are used to indicate the location and width of the experimental defects originally made. Scale bar: 500µm. G) Polarizing microscopy of picrosirius stain, which reveals ultra-structural histology of both articular cartilage zone (Cart) and subchondral bone zone (Sub-B) of the Samples. The Samples include LhCG, dLhCG, the negative control "Untreated" and the positive control – intact, surrounding native Cart and Sub-B of the host – marked as "Native". Scale bar: 500µm. Positive Saf O stain of GAG appears in bright red; positive MT stain of collagens appears blue; positive IHC stain using 3,3' Diaminobenzidine (DAB) which precipitate in the presence of horseradish peroxidase (HRP) conjugated Col II and Col I appears in brown. Picrosirius stains relative raw matrix fibers in brown or dark yellow, stains relatively delicate fibrils in green or light yellow, and stains the superficial edge of articular cartilage with a bright red line.

Fig 6-2A

Fig 6-2B

Figure 6-2.

Biochemical analysis of osteochondral repair by engraftment of cartilage implants: decellularized living hyaline cartilage graft (dLhCG) and LhCG. The negative control, namely the untreated defects that are left void of grafts, is marked as “Untreated”; the positive control, namely the intact surrounding native tissues of the host, is marked as “Native”. The samples harvested from the articular cartilage zone are marked as “Cart” and the samples harvested from the subchondral bone zone are marked as “Sub-B”. The analyses include DNA assay, glycosaminoglycan (GAG) assay, and total soluble collagen assay. The Day 50 and Day 100 samples are normalized by A) wet weight and B) dry weight. * means $p \leq 0.05$, ** means $p \leq 0.01$, *** means $p \leq 0.001$, **** means $p \leq 0.0001$

As far as the efficacy of tissue regeneration is concerned, histological evaluation and functional test are of the most importance and recognized as the gold standard. Hematoxylin and eosin (H&E) staining reveals general composition and morphology of biological tissue specimens. From Day 50 samples of LhCG and dLhCG, it is observed that the osteochondral defects are fully filled by cartilage-like neo-tissues, thus the articular surface is recovered from the traumatic dent; newly generated bony matrix appears in the region further underneath; and the neo-tissues tends to integrate with surrounding native tissues. (Figure 6-1a, b). This observation and preliminary determination are corroborated with positive Masson's Trichrome (MT) staining specifically revealing collagen in blue color (Figure 6-1c) and positive Safranin-O (Saf O) staining specifically revealing GAG in red (Figure 6-1d). Further IHC stains indicate that, besides proteoglycans, the neo-matrices consist of abundant Col II and trace amount of Col I (Figure 6-1e, f). Contrastively, Day 50 untreated sample remains voids of tissue regeneration in most of the defective area but only some random scar-like tissue deposition at the bottom; there is also free of tissue repair further underneath in the region that is affected in the subchondral bone. These histological findings are generally in line with the biochemical quantifications (Figure 6-2a, b). Importantly, on top of the histological indication, biochemical DNA assay further confirms the occurrence of *in situ* recellularization by proliferative cell migration from surrounding host tissues into the originally acellular dLhCG implant.

Table 6-1 Mechanical test of *in vivo* regenerated osteochondral tissue by LhCG and dLhCG grafts

Samples*	Young's Modulus (MPa)	
	Day 50	Day100
Untreated	4.81 ± 4.85	2.59 ± 4.08
dLhCG	3.00 ± 2.19	7.58 ± 2.86
LhCG	3.54 ± 1.92	7.70 ± 2.25
Native	7.23 ± 1.39	7.48 ± 2.79

The major mechanical property of osteochondral tissues is practically represented by compression strength, which is determined by matrix density, composition, phenotype and architecture. On the ground of biochemical quantification and histological analysis about the matrix constituents and morphology, picosirius staining further reveals ultrastructure of the regenerated tissues via polarizing microscopy (Figure 6-1g); putting them together, the correlation between material basis and mechanical performance can be understood. In picosirius images of native osteochondral tissues, relative raw matrix fibers that are stained in brown or dark yellow are presented in subchondral bone area; relatively delicate fibrils that are stained in green or light yellow are finely aligned generally perpendicularly to the osteochondral tidemark in articular cartilage, on top of which the superficial edge is lined out in red color. Relatively more similar ultrastructure is revealed by picosirius imaging from the Day 50 samples of dLhCG than LhCG in terms of finer matrix alignment, which is responsible for better mechanical strength of the former than the latter at this time point (Table 6-1), despite the constituent and histological similarities between them. The apparently high mechanical strength of the scar-like tissues deposited at the bottom of defect in Day 50 untreated sample is of an outcome from condensed aggregation of raw fibers, which poses to be an anomaly. Collectively, progressive tissue regeneration towards osteochondral repair has been witnessed in Day 50 samples of LhCG and dLhCG. Up to this time point, engineered tissue regeneration remains solely in cartilaginous nature throughout the full entity of

the graft. Nevertheless, as the wound has been well covered from physical exposure, the condition for further osteochondral restoration towards a full recovery is satisfied.

After another 50 days' development *in vivo*, histological stains reveal that, in Day 100 samples of LhCG and dLhCG, the osteochondral defects are completely healed. The traumatic dents have vanished. Both articular cartilage and subchondral bone neo-tissues are generated in their respective zones and integrated together along the tidemark. They are also integrated with their respective surrounding native tissues of the host with no visible boundary. (Figure 6-1a-f). Successful regeneration of hyaline-like articular cartilage is proven by the positive staining of GAG and Col II as well as the negative staining of Col I; and, efficacious regeneration of subchondral bone is proven by the positive staining of Col I as well as relatively negative staining of GAG and Col II and the pink color of H&E staining. Relatively better restoration of subchondral bones is observed in dLhCG samples than that in LhCG samples. In Day 100 dLhCG samples, the regenerated subchondral bones together with affected trabecular bones underneath have accomplished their remodeling, becoming morphologically consistent with their native counterparts around; but in Day 100 LhCG samples, despite successful integration with surrounding tissues, the desired morphological consistency has not been achieved yet. Further investigations on ultrastructure of Day 100 samples of LhCG and dLhCG were conducted by picrosirius imaging. (Figure 6-1g). It reveals fine arrangements of matrices including those in regenerated articular cartilage and subchondral bone from both samples. In comparison with native counterparts, again regenerated subchondral bone tissue in dLhCG samples is more like native subchondral bone tissue, nevertheless regenerated articular cartilage tissue in LhCG samples is more like native cartilage tissue as shown by the finer alignment of cartilaginous fibrils in both superficial and deep zones.

The same composition, architecture and ultrastructure of both regenerated tissue from LhCG and dLhCG samples explains the same compression strength as the native osteochondral tissue (Table 6-1). The Young's Modulus in LhCG group is higher than native cartilage which could be due to the strong ossification process as revealed by histology of a more intensive Masson's Trichrome staining at the regenerated subchondral bone region (Figure 6-1c) and higher calcium level than native cartilage as shown in biochemical analysis (Figure 6-2). The phenomena could be an evidence of ongoing remodeling process due to the superposition of self-regeneration of subchondral bone and endochondral ossification induced by cartilage implant which could ultimately be remodeled to tissue same as surrounding native subchondral bone. Contrastively, Day 100 untreated sample still lacks repair. The entire osteochondral defect is mostly filled up by the same type of scar-like tissues and the mechanical strength even decayed from that of Day 50. Besides the mechanical profile, histological findings are also generally in line with the biochemical quantifications (Figure 6-2a, b).

Figure 6-3.
Representative μ CT images of coronary, sagittal and transverse sections of regenerated tissue with surrounding tissue treated by dLhCG and LhCG implants in comparison to untreated defect that were left void without any implantation of graft marked as “Untreated” at A). Day 50 and B). Day100. Scale bar: 2 mm.

Table 6-2 Bone volume to total volume ratio of regenerated subchondral bone in LhCG+ group

Samples	BV/TV	
	Day 50	Day100
Untreated	0.197±0.03	0.280±0.06
dLhCG	0.597±0.05	0.618±0.07
LhCG	0.675±0.06	0.769±0.05
Native	0.997±0.01	0.985±0.02

To further examine the property of regenerated subchondral bone tissue, a micro-CT was conducted in LhCG and dLhCG groups in comparison to untreated defect. By the time of Day 50, an observable progressive healing in LhCG and dLhCG has shown up especially in comparison to untreated defect at the sagittal and coronal planes. By the

time of Day 100, complete healing in the subchondral bone layer was observed in dLhCG group at the transvers plane. (Figure 6-3) Although the intensity of CT image is still weaker at the regenerated tissue region compared to native subchondral bone around, the coverage of the defect site is complete without any void space. LhCG grafts showed a strong ossification as evident by the higher intensity at the defect site especially revealed by images in the coronal and sagittal planes. Although the intensity seems to be less than the surrounding subchondral bone as viewed from the transverse plane, the repair effect is significantly better than untreated defect which has shown with no bony regeneration at the defect site. The observation is validated by the quantification of bone volume to tissue volume ratio (BV/TV) (Table 6-2) and the calcium quantification at subchondral bone zone in biochemical analysis (Figure 6-2a, b). Collectively, all these histological, biochemical, biomechanical and imaging data suggest a successful full-thickness osteochondral repair by engraftment of LhCG and dLhCG, respectively, as man-made pure cartilage grafts without any subchondral bone mimetic part. Especially as a decellularized graft, dLhCG possesses a general superiority.

In comparison to the LhCG+ and dLhCG+ graft developed and evaluated in Chapter 3 and 5, it is observable that dLhCG and LhCG graft significantly exceeded the corresponding counterparts with the subchondral plug, namely dLhCG+ and LhCG+. For the LhCG and LhCG+ graft, it is observable that the repair efficacy at the cartilage layer by 100 days are similar yet the remodeling at subchondral bone is not complete as shown by the void pocket in the histology. Moreover, by the time of 50 days, the cartilage has already been fully covered in the LhCG but only partially filled in the LhCG group. Therefore, it implies that with the fine coverage of cartilage on top, the subchondral is able to regenerate without deliberate repair due to the relatively strong self-regenerative capability. In addition, the subchondral bone in the LhCG+ graft would be further

remodeled to fully repair both cartilage and subchondral bone. When dLhCG graft is compared to dLhCG+ graft, the difference in the repair efficacy is more obvious as both cartilage and subchondral is not showing full recovery in dLhCG+ graft as opposite to dLhCG graft. It further consolidated the previous preliminary conclusion that without a fine recovery on cartilage, the subchondral bone is not able to completely regenerate. Therefore, it is hypothesized that, given a finely regenerated articular cartilage shielding on top, the restoration of subchondral bones, and even overall osteochondral complex, can be managed afterwards via self-remodeling *in situ*.

Nonetheless, it worth mentioning that the experiment condition is not completely parallel since the LhCG and dLhCG experiment is conducted at the patella groove but the LhCG+ and dLhCG+ experiment is conducted at the condyle. To examine the hypothesis, the experiment needs to be conducted in parallel at the same site. On the other hand, the result may be because of the decellularization process applied. Since LhCG+ and LhCG actually had similar tissue repair efficacy, although at slightly different pace, the significant inferior tissue repair efficacy of dLhCG+ graft could be due to the loss of the tissue engineered cartilage layer after decellularization. More advanced decellularization method could be applied such as induction of apoptosis of cells to avoid unnecessary damage on the ECM. Otherwise, the graft could be additionally cross-linked before decellularization. Nevertheless, the real tissue repair efficacy should be evaluated *in vivo*.

6.4 Conclusion

In conclusion, we achieved sound osteochondral restorations via engraftment of pure cartilaginous implants, LhCG and dLhCG, which do not contain engineered subchondral bone plugs. Considering practical usage, the decellularized cartilage graft,

namely dLhCG, further excels other counterparts with its overall superiorities and conveniences particularly brought up by acellular property in terms of logistics simplicity, immune biocompatibility and architecture genuineness.

Although an ideal osteochondral recovery was achieved using engraftment of monophasic pure cartilaginous grafts, the detailed mechanism of the recovery process is to be revealed. The hypothesis that repair of cartilage layer is sufficient to finally achieved the full osteochondral defect recovery is better supported when the detailed mechanism is verified. The mechanism could be examined by designing more time points in the remodeling process after implantation *in vivo*. The histology, gene expression profiling and protein level profiling such as western blot and immunohistochemistry are to be conducted to display the process of the remodeling and hence examine the validness of the hypothesis.

Chapter 7 Conclusion and future work

7.1 Conclusion

In this thesis, tissue engineering osteochondral grafts were developed and evaluated for two main purposes: cartilage defect repair and osteochondral defect repair. Two design philosophies were adopted to develop the osteochondral grafts, namely hyaline cartilage like and full-scaled design. Simultaneously cellular and acellular version of the grafts were fabricated by applying suitable decellularization approaches on to the tissue engineering grafts. Therefore, ECM based tissue engineering osteochondral grafts were generated which owes the advantages of high biocompatibility and logistic simplicity.

To achieve the first purpose, decellularized living hyaline cartilage graft (dLhCG) was used to repair cartilage defect which has been established to overcome hurdles brought about by living cells in grafts to enter clinical application including two rounds of surgery, limited cell source, donor site morbidity, short window period and regulation concern. The *in vitro* and *in vivo* evaluation has proved the potential of the graft being clinically applied as a cartilage injury treatment graft and commercialized as an “off-the-shelf” product. In both studies, the dLhCG grafts have provided ideal microenvironment to facilitate cell infiltration which is beneficial to *in vivo* remodelling in the regeneration process. Firstly, dLhCG grafts have been characterized as porous scaffold that owes the genuine composition and architecture of native cartilage tissue. Subsequently dLhCG grafts has been validated as a highly biocompatible scaffold as revealed by the low inflammatory response and high cell migration induction capability when embedded in omentum of rats. Secondly, the cartilage defect repair efficacy of dLhCG grafts have been confirmed by the same mechanical property, histology, composition and gene expression profile of the regenerated tissue after engraftment of

dLhCG grafts to native cartilage as shown by the *in vivo in situ* implantation into condyles of pigs.

To achieve the second purpose, PLGA augmented LhCG grafts (LhCG+) and the decellularized version dLhCG+ were developed following full-scaled design philosophy to repair osteochondral defects. In addition, the LhCG and dLhCG grafts were used to repair osteochondral defect too. The theory behind this design is the difference in self-repair capability – the strong self-repair capability of subchondral bone and weak self-repair capability of cartilage. It turned out that the LhCG+ grafts and both LhCG and dLhCG grafts were capable to repair osteochondral grafts as revealed by the similar mechanical property, morphology and composition of regenerated tissue to native osteochondral tissue in both cartilage layer and subchondral bone layer. Moreover, although the experiment was not conducted side by side, the mechanical property which is the main function of osteochondral tissue was shown to be more like as native cartilage in dLhCG grafts stimulated regenerated tissue. Therefore, the hypothesis that the key in repair of osteochondral tissue lies in the successful repair of cartilage tissue is supported. In addition, the superiority brought about by the acellular nature of dLhCG grafts makes the grafts promising as a useful tool in orthopaedic surgery.

Collectively, the above findings demonstrated the potential of the developed tissue engineering grafts especially dLhCG grafts as a clinically available product for osteochondral defect repair. Moreover, the hypothesis that the key in repair of osteochondral defect lies in the successful regeneration of cartilage layer is preliminarily validated further when the repair efficacy of dLhCG and dLhCG+ grafts were analysed comprehensively. Supported by the superior performance of dLhCG grafts in all aspects including mechanical, histological, biochemical and imaging properties. dLhCG grafts the monophasic hyaline-like cartilage graft without any mimicking part of subchondral

bone excels the dLhCG+ and LhCG+ grafts that is with the augmented subchondral plug, implies cartilage repair as the key of osteochondral defect repair.

7.2 Future work

Although the hypothesis that successful repair of cartilage tissue is critical for osteochondral tissue regeneration is preliminarily verified. The mechanism behind requires a more systematic study including gene expression profiling, protein profiling and cell type profiling at more time points in the regeneration process to reveal the details of the procedures.

On the other hand, the unsatisfactory repair efficacy of dLhCG+ graft might not falsify the design philosophy of full-scaled grafts, instead, it could be the problem of the graft. Delamination is a big challenge in developing full-scaled grafts, which leads to the non-ideal regeneration of mismatching neo tissue type. Hence better “dLhCG+” could be developed in the future with strong connection in the transition zone from cartilage layer to subchondral bone layer.

The dLhCG graft is successful in repair of both cartilage and osteochondral defects which makes the graft promising to become a clinically available graft and off-the shelf product. Hence to satisfy the industrialization requirement, a strict orthogonal experiment is to be conducted to optimize the best decellularization condition for LhCG graft.

Moreover, dLhCG graft as an ECM based graft whose regeneration stimulating effect has been shown in the osteochondral repair experiment could be used and an *ex vivo* niche to study the mechanism and process happened in the endochondral ossification process and utilized to repair large bone defect. More generally, other ECM based scaffolds fabricated using the same procedure, namely, 3D cell culture in a transient

scaffold followed by decellularization after maturation of graft could be used to develop grafts for other types of tissues and used a platform to study various mechanism of interactions between various bioactive molecules or vesicles with ECM.

List of publications

Journal articles

Nie, X., & Wang, D. (2016). Adhesion of a Living Hyaline Cartilage Graft as an Industrial

Product for Cartilage Repair. *International Journal of Advanced Research in Chemical Science*, 3(6), 27–31.

Nie, X., & Wang, D.-A. (2018b). Decellularized orthopaedic tissue-engineered grafts: biomaterial scaffolds synthesised by therapeutic cells. *Biomaterials Science*.

Book chapter

Nie X. L., Wang, D. A.*, “Bioactive hydrogels and their applications in regenerative medicine”, In: "*Polymeric Biomaterials for Tissue Regeneration: from Surface/Interface Design to 3D Constructs*", Changyou Gao., Ed.; Springer, Inc.: Heidelberg, Germany; 2016; p57-74. DOI 10.1007/978-981-10-2293-7_3 3.

Manuscript in progress

Nie X.L., Wang D.A. A full-scaled tissue engineering graft for osteochondral defect repair (In preparation)

Nie X.L., Wang D.A. A manmade decellularized hyaline cartilage graft for cartilage defect repair (In preparation)

Nie X.L., Wang D.A. Tissue engineering cartilage grafts for osteochondral defect repair (In preparation)

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Supplementary Table 3-1 5-point ordinal severity scale

Grade	severity
0	no inflammation
1	minimal
2	mild
3	moderate
4	severe

Supplementary Table 4-1 Histology histopathology grading system

Histology histopathology grading system	
	Grade
I. Structure	
a. Normal	0
b. Surface irregularities	1
c. Pannus and surface irregularities	2
d. Clefts to transitional zone	3
e. Clefts to radial zone	4
f. Clefts to calcified zone	5
g. Complete disorganisation	6
II. Cells	
a. Normal	0
b. Diffuse hypercellularity	1
c. Cloning	2
d. Hypocellularity	3
III. Safranin-O staining	
a. Normal	0

b. Slight reduction	1
c. Moderate reduction	2
d. Severe reduction	3
e. No dye noted	4
IV. Tidemark integrity	
a. Intact	0
b. Crossed by blood vessels	1

Supplementary Table 4-2 Wakitani Scoring System

Cell morphology		
	Hyaline cartilage	0
	Mostly hyaline cartilage	1
	Mostly fibrocartilage	2
	Mostly non-cartilage	3
	Non-cartilage	4
Matrix staining intensity (metachromasia)		
	Normal (compared with host adjacent cartilage)	0
	Slightly reduced	1
	Markedly reduced	2
	No metachromatic stain	3
Surface regularity (total smooth area compared with entire area of cartilage defect)		
	Smooth (>3/4)	0
	Moderate (>1/2-3/4)	1
	Irregular (1/4-1/2)	2
	Severely irregular (<1/4)	3
Thickness of cartilage (compared with that of surrounding cartilage)		
	>2/3	0
	1/3-2/3	1
	<1/3	2
Integration of donor with host adjacent cartilage		
	Both edges integrated	0
	One end integrated	1
	Neither edge integrated	2