Micro- and nanotechnology detection and delivery systems for screening and optimisation of MSC chondrogenesis for cartilage regeneration

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NANYANG
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**MICRO- AND NANOTECHNOLOGY DETECTION AND
DELIVERY SYSTEMS FOR SCREENING AND
OPTIMISATION OF MSC CHONDROGENESIS FOR
CARTILAGE REGENERATION.**

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**Interdisciplinary Graduate School
Nanyang Institute of Technology in Health and Medicine**

2017

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OPTIMISATION OF MSC CHONDROGENESIS FOR
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A thesis submitted to the Nanyang Technological
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the degree of Doctor of Philosophy

2018

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ABSTRACT

Cartilage regeneration remains a clinical challenge. Majority of clinical treatments for cartilage injuries and disorders mainly help to address related complications or postpone the need for joint replacement. Mesenchymal stem cells (MSCs) are looked upon as prospective cell source for cartilage regeneration due its high proliferative and multipotency capabilities. There are several *in vitro* studies revolve around the use of different scaffold materials, physical cues, signalling molecules and growth factors to provide chondrogenic differentiation cues to MSCs. These studies often required the use of large amount of MSCs, materials and reagents for screening and validation of MSC chondrogenesis using the conventional methods. Nevertheless, with the combination of tissue engineering and micro- and nanotechnology, it will represent a more efficient system for the study of MSC chondrogenesis for cartilage regeneration. In this thesis, we focus on developing detecting system with nanosensors to screen optimal chondrogenic conditions to attain proliferative chondrocytes for cartilage regeneration. At the same time, we explored into creating a controlled-release delivery system with ultrasound-activated magnetic microbubbles in three-dimensional (3D) construct for future growth factor delivery Firstly, in chapter 2, we introduced the use of

modified polymer chains to fabricate nanosensors for real-time cell tracking. In particular, di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) was inserted in between two polycaprolactone (PCL) polymer chain through covalent bond, in which it was used to synthesis PCL-DPP-PCL NPs. We were able to monitor MSCs for 28 days and it showed no adverse effects these nanosensors have on MSC adipogenic and chondrogenic. While these nanosensors have the advantage of long-term tracking of MSC, molecular beacon (MB)-based nanosensors have additional advantage of noninvasively monitor target intracellular mRNA expression, which will be elaborated in chapter 3. We have demonstrated the potential of MB-NPs as a platform to observe the type II collagen (*Col2*) expression over a period of 28 days in MSCs embedded in 3D agarose when induced with chondrogenic medium. There was also a good correlation between the *Col2* trend observed with MB-NPs and qRT-PCR data. Chapter 4 further explored this detection platform. It was utilised to screen the optimal time point to add fibroblast growth factors (FGF2) to attain proliferative chondrocytes. High expression of *Col2* and *Ki67* were observed when FGF2 was added from days 14 to 20, with the treatment of TGF β 3 from days 0 to 13 and days 21 to 28. The fluorescence signal trend of both *Col2* and *Ki67* MBs coincided with quantitative real-time polymerase chain reaction (qRT-PCR) data. Future screening of MSC chondrogenic

conditions for cartilage regeneration can thus be realised through the use of this nanosensor platform. Lastly, we developed a controlled-release system in 3D construct with magnetic microbubble technology. We were able to coat different cargos onto the magnetic microbubbles and released in 3D construct under ultrasound stimulation. These combination techniques bring about a promising system for cartilage regeneration in the near future.

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LIST OF ABBREVIATIONS

MSC: Mesenchymal stem cell

PBS: Phosphate Buffer Solution

NP: Nanoparticle

QDs: Quantum dots

PSNP: Polystyrene nanoparticle

Cd: Cadmium

PCL: Polycaprolactone

DPP: Di(thiophene-2-yl)-diketopyrrolopyrrole)

GFP: green fluorescent protein

RNA: Ribonucleic acid

mRNA: Messenger ribonucleic acid

FRET: Forster resonance energy transfer

MB: Molecular beacon

MB-NP: Molecular beacon nanoparticle

PLGA: Poly(Lactide-co-Glycolide)

3D: Three-dimensional

2D: Two-dimensional

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

PPAR γ : Peroxisome proliferator-activated receptor gamma

Col2: Type II Collagen

ALP: Alkaline phosphatase

OCN: Osteocalcin

Col1: Type I Collagen

Col10: Type X Collagen

ST: Short term

LT: Long term

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

qPCR: Real-time polymerase chain reaction

PLL: Poly-L-lysine

PVA: Polyvinyl alcohol

TGFβ3: Transforming growth factor beta 3

FGF2: Fibroblast growth factor 2

MNP: Magnetic nanoparticle

MMB: Magnetic microbubbles

GPI: Glycosylphosphatidylinositol

PUBLICATION LIST

- 1 Liu, S., Tay, L. M., Anggara, R., Chuah, Y. J. & Kang, Y. Long-term tracking mesenchymal stem cell differentiation with photostable fluorescent nanoparticles. *ACS applied materials & interfaces* **8**, 11925-11933 (2016).
- 2 Tay, L. M. *et al.* Non-invasive Monitoring of 3D Chondrogenic Constructs using Molecular Beacon Nanosensors. *Tissue Eng Part C Methods*, doi:10.1089/ten.TEC.2016.0320 (2016).
- 3 Tay, L. M. & Xu, C. Coating microbubbles with nanoparticles for medical imaging and drug delivery. *Nanomedicine*, doi:10.2217/nnm-2016-0362 (2016).

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- 4 The effect of temporal manipulation of TGF β 3 and FGF2 on the derivation of proliferative chondrocytes from Mesenchymal Stem Cells – a study monitored by qRT-PCR and Molecular Beacon Based Nanosensors (accepted)

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Chapter 1: Introduction

1.1. Background

1.1.1 Cartilage tissue engineering

Injuries and disorders

Hyaline cartilage is a type of flexible connective tissues that is located throughout the body. Articular cartilage is a type of hyaline cartilage that can be found at the end of bones in the joints. It provides several functions such as support and reduction in friction between joints^{1, 2}. Damaged articular cartilage is often replaced with fibrocartilage scar tissues which may lead to osteoarthritis. This affects the overall biomechanical activities of the joints^{1, 3, 4}.

Another hyaline cartilage that can be found near the end of long bones is the physis, which is also known as growth plate. The unique characteristic of growth plate is that it is only present in children and adolescences undergoing puberty. Growth plate consists of mainly of resting, proliferative and hypertrophic zones with specialized chondrocytes, as shown in Figure 1.1. The directional growth and maturation of the chondrocytes progress from the resting zone brings about the longitudinal growth in long bones⁵⁻⁷.

Any defects in genes involving these processes will result in skeletal dysplasia and short stature⁷. Partial or complete growth plate arrest can take place as a result of severe growth plate injuries (typically type III, IV and V under Salter-Harris classification)^{8, 9}.

Figure 1.1. Distinct zones of chondrocytes from histology of mouse growth plate. (Reprinted from Current Biology, Vol. 11, Issue 15, Alicia White, Gillian Wallis, Endochondral ossification: A delicate balance between growth and mineralisation, Pages R589, Copyright (2001), with permission from Elsevier)

Any injury or disorder to the cartilage can lead to some degree of disability and even deformity^{5, 10}. However, unlike bone, cartilage is aneural and avascular thus it does not possess regenerative capacity^{2, 3, 10, 11}. Current treatments for cartilage injuries and disorders do not solve the root cause of the problems as they mainly address related symptoms and complications¹². Restoration of cartilage is still a clinical challenge. Even with surgical intervention such as marrow stimulation and limb lengthening, there was varying degree of success with limitations^{10, 12}.

Cell-based therapies

Over the last few decades, scientists have worked on different cell-based therapies for cartilage repair and regeneration. Chondrocyte-based therapies have been used in numerous studies for cartilage repair¹²⁻¹⁵. However, it is difficult to harvest chondrocytes as it typically requires invasive techniques, which can lead to donor site morbidity. In order to attain adequate cell number for therapy, chondrocytes harvested are normally expanded *in vitro* as they are typically low in quantity. Due to its limited proliferating capacity, chondrocytes undergo multiple expansion can result in the change of chondrocyte phenotype^{12, 16}.

On the other hand, due to its high proliferative potential and multipotency capability, mesenchymal stem cells (MSCs) are looked upon as a promising cell source for cartilage tissue engineering. MSCs are also easily obtained from numerous sources, such as bone marrow and adipose tissues^{12, 17, 18}. Under the right stimuli, these MSCs can differentiate into chondrocytes and express hyaline-like extracellular matrix^{16, 17, 19}. These stimuli include scaffold material, physical cues, signaling molecules and growth factors^{12, 19, 20}. Optimisation of MSC chondrogenesis of MSCs for cartilage regeneration commonly involves growth factors and a three-dimensional microenvironment. Previous studies have identified several growth factors (for instance: FGF2 and TGF β 3) that regulate proliferation and chondrogenesis of MSCs. Growth factor TGF β 3 is known to be essential for MSC chondrogenesis²¹⁻²³, while FGF2 has shown to promote cell proliferation and influence differentiation²⁴⁻²⁶. However, TGF β 3 alone is not sufficient for complex cartilage regeneration, such as growth plate repair. Nevertheless, the synergy effect of TGF β 3 with other growth factor(s) holds potential for complex cartilage regeneration¹². The investigation of suitable combination and optimization process using the conventional methods often requires the use of large amount of MSCs, materials and reagents. Thus, there is a need for an efficient and effective *in vitro* model to monitor

MSC chondrogenesis during this optimization process for cartilage regeneration.

1.1.2 Monitoring of MSCs using Nanosensors

The need for nanosensors

Emerging MSC-based research and therapies highlights the need for a better understanding cell fate and functions under different culture conditions and after implantation^{27, 28}. Traditionally, to understand the morphology, distribution, survival, and differentiation of MSCs, histological analysis and molecular analysis, such as quantitative reverse transcription polymerase chain reaction (qRT-PCR), are executed^{28, 29}. Yet, these techniques are laborious and destructive as it involve the invasive extraction of tissues for *in vivo* implantation and disrupt live cell samples. They are also pre-determined endpoint assay that do not provide real-time information of MSCs. Furthermore, qRT-PCR only evaluates the average population of messenger ribonucleic acid (mRNA). There is a possibility of missing out critical genetic information in small subpopulation of the cells, which includes the differences from cell-to-cell³⁰. Therefore, there is a need for a non-invasive, real-time platform for cell tracking. This provides

insightful information on the optimal treatment/transplant conditions and efficacy of stimuli at different combinations and doses^{28, 29}.

Fluorescence imaging has been commonly used in research due to its high sensitivity in low cost, ease of accessibility and spatial and temporal^{28, 31}. In order for monitoring to be possible, cells have to be first labeled with contrast agents which are typically organic dyes and fluorescent protein. However, these organic dyes often suffer from low photostability and *in vivo* metabolic degradation, thus it is only applicable for short-term imaging^{28, 31}. On the contrary, nanoparticles (NPs) are able to address these shortcomings.

NPs can be synthesized in various sizes, shapes and compositions. NPs can be designed to possess different physical properties, such as magnetic and optical properties. This allows NPs to be use with different imaging modalities²⁹. Specifically, fluorescent NPs such as quantum dots (QDs) and polymer NPs are commonly used for cell tracking. In comparison with organic dyes, nanosensors are often resistant to photobleaching with minimal impact to cell fate and functions^{28, 29}. Different targeting moieties, like antibodies, can also be conjugated on the surface of NPs to provide specific-targeting function²⁹.

Quantum dots (QDs) for cell tracking

QDs, which are highly fluorescent semiconductor NPs, have been widely used for tracking of cells in *in vitro* and *in vivo* models. Typically, QDs used in biological application consist of cadmium (Cd)-based core^{32, 33}. QDs were first applied as cell labelling probes by Chan et al.³⁴ Near infrared QDs were also utilized to pre-label MSCs before transplanting into rat infarct brain for tracking³⁵. These were possible due to their unique optical properties such as good photostability with narrow emission spectra. The fluorescence emission wavelength of QDs are also tunable with respect to its size^{28, 29, 36}. Nevertheless, signals were often lost due to cell division, diffusion and exocytosis. Poor retention in cells poses a crucial problem for long-term tracking^{37, 38}. Another concern with QDs is their cytotoxicity, which explains why they are yet available for clinical applications. The chemical composition of QDs, especially Cd, was found to induce cell death^{27, 29, 36}. Derufs et al. investigated the cytotoxicity of cadmium selenide (CdSe) QDs with a liver model consisting of primary hepatocytes. They observed a closely relation of cytotoxicity with the free Cd²⁺ ions liberated due to surface oxidation of QDs³⁹. Compared to free Cd²⁺ released on cell surface, ingested Cd²⁺ was found to result in much higher toxicity effect⁴⁰. Chen et al. also suggested that intracellular distribution of QDs played a part in the cytotoxicity effect³². CdSe/ZnS QDs observed to have inhibited

osteogenic differentiation in human bone marrow MSCs⁴¹. There have been several efforts to reduce the cytotoxicity^{42, 43}. For instance, surface modification through coating on QDs helped in reducing cytotoxicity during long-term *in vitro* cell tracking. Nevertheless, the release of Cd over time after the degradation of coating still posed a concern for *in vivo* studies³⁹.

Polymer-based nanoparticles (NPs) for cell tracking

Polymer NPs, on the other hand, are less toxic and have been widely used for drug delivery, imaging and other biological applications. An example of polymer NPs used in cell labelling is polystyrene NPs (PSNPs). PSNPs are typically synthesized using emulsion polymerisation method. They were mainly uptake by MSCs through clathrin-independent endocytosis. Through flow cytometry and confocal imaging, fluorescence signals can be observed to trace PSNPs-labelled MSCs⁴⁴. However, there are limitations to PSNPs. PSNPs are only suitable for short-term cell tracking as the fluorescent dyes in PSNPs are not protected from leaking, quenching and photobleaching⁴⁵.

Other than encapsulation method, polymer-based fluorescent NPs can also be fabricated by covalently bonding fluorescent organic dyes with polymer chains⁴³. Huang et al. developed polymeric NPs with strong fluorescence

signal and good photostability for cell imaging. They were synthesised through nanoprecipitation of a modified biocompatible and bioresorbable polycaprolactone (PCL) polymer chain with di(thiophene-2-yl)-diketopyrrolopyrrole) (DPP) to give PCL-DPP-PCL NPs. These fluorescent NPs formed were reported to be able to track HuH-7 tumour cells and MSCs for 14 days without much photobleaching. Little cytotoxicity was observed in both cell types. Nevertheless, the influence of these NPs on the function of MSCs was unknown. Long-term tracking of cells will be beneficial, especially for tissue engineering and *in vivo* studies^{43, 46}.

Molecular Beacon (MB)-based nanosensors

Nanosensors developed were mainly used in monitoring the biodistribution and migration of cells. Little was done to involve monitoring of the status and functions of cells, specifically differentiation of MSCs. To study the expression of biomarker genes, researchers utilized reporter gene assay, in which they transfected lineage specific promoter-driven fluorescent protein construct into genome of cells. The use of green fluorescent protein (GFP) that is type I collagen promoter-driven has been demonstrated by Dacic et al. and Kalajzic et al for observing of osteoblast differentiation^{47, 48}. Though labelling of cells with reporter genes is more stable, it involves genetic

modification of cells which increases the risk of mutagenesis. In addition, transfection of reporter genes is laborious and has low efficiency in stem cells^{43, 49, 50}.

An alternative monitoring of MSC differentiation is molecular beacon (MB)-based nanosensor. MB is a hair-pin shaped oligonucleotide sequence with a fluorophore and a quencher at two ends of the sequence. In the presence of target specific messenger RNA (mRNA) sequence, it will hybridize with the MB and restore the pre-quenched state of fluorophore through Forster resonance energy transfer (FRET) mechanism (Figure 1.2). Thus, MB provides the function to detect the presence of specific intracellular mRNA in living cells⁵¹.

Figure 1.2. Hybridization of molecular beacon with its complementary target (Reprinted from *Angewandte Chemie International Edition*, Vol. 48, Issue 5, Kemin Wang et al., *Molecular Engineering of DNA: Molecular*

Beacons, Pages 857, Copyright (2009), with permission from John Wiley and Sons)

For MBs to be uptaken by cells, external interventions are needed as MBs are negatively charged and too large for diffusion to take place for cell labeling. The traditional delivery methods include microinjection and electroporation. However, these techniques are tedious and of low throughput, especially for microinjection⁵¹. In addition, after MBs are internalised by the cells, they are subjected to degradation and clearance. Therefore, MBs can only be used for short-term imaging⁵².

The challenges can be addressed by encapsulating MBs in biodegradable poly(D,L-lactide-co-glycolide) (PLGA). Wiraja et al. reported that with the encapsulation of MBs in PLGA NPs (MB-NPs), they were internalised by cells mainly by phagocytosis pathway. The direct delivery of MB-NPs into cytoplasm helped MB-NPs to avoid the endogenous degradation. In addition, the steady release of MBs from the NPs over a period allows for long-term monitoring. MB-NPs also demonstrated the ability to monitor MSC osteogenic differentiation for a period of 21 days^{50, 52}.

The potential of MB-NPs as a monitoring platform for MSC differentiation can be further explored into three-dimensional (3D) cell culture construct, which resembles native environment. As tissue engineering often requires

longer repair and restoration duration, the use of MB-NPs for monitoring of MSC differentiation can be extended to more than 21 days. Furthermore, MB-NPs provide a prospective platform for non-invasive optimising and screening of culture conditions.

1.1.3 Controlled-released delivery system using magnetic microbubbles (MMB)

Microbubbles were first discovered as a good contrasting agent for ultrasound imaging back in 1968⁵³. Other than enhancing contrast in ultrasound images, microbubbles can also be used as delivery tool for therapeutic agents under ultrasound stimulation. Therefore, microbubbles hold vast potential as theranostic agents. Many studies have been done to improve the stability and functions of microbubbles⁵⁴.

The stability and functions of microbubbles depend heavily on the composition of the microbubble shell. The microbubble shell affects the clearance time in the body as well as the response of immune system to microbubbles. With a correct choice of material for the shell, it can stabilise microbubbles by reducing surface tension, thus preventing coalescence. The composition of the shell also determines mechanical properties and the

reaction of microbubble under ultrasound stimulation^{55, 56}. For instance, lipid shell has a unique characteristic of spontaneous self-assembly of phospholipids through weak van der Waals attraction. This prevents dissolution of gas core by reducing surface tension and assists the oscillation of microbubbles at resonant frequency. Therefore, it enables surface cargos to be release during inertial cavitation⁵⁶.

With the increasing interests in the potential of NPs, many researchers have incorporated NPs into microbubbles to provide them multi-functionalities, specifically multimodal contrast agents and theranostic platform⁵⁷. As many NPs are used as imaging probes, the integration of NPs and microbubbles gives rise to multimodal imaging function. Ke et al. were able to obtain hybrid image from fluorescent and ultrasound imaging by loading QDs onto microbubbles. They also demonstrated the localized release of loaded QDs through ultrasound-targeted microbubbles destruction (UTMD). This allows for fluorescent imaging of cells and tissues⁵⁸.

Microbubbles not only function as contrast agents, they have been used as delivery carriers as well. Cargos were typically embedded in the shell of microbubbles. However, this loading technique is only limited to hydrophobic cargos with limited loading capacity. These short-comings can

be resolved by NPs. Hydrophilic cargos, such as nucleic acids, can be easily encapsulated in NPs. The use of NPs not only improves loading efficiency of cargos on microbubbles, it also provides a sustainable release of cargos from the NPs^{57, 59, 60}. During inertial cavitation, the collapse of microbubbles produces shockwaves or microjets. This process enables the release of therapeutic cargos, such as drugs, and delivers across the vessel wall. However, these shockwaves or microjets can also damage the surrounding tissues. In addition, the collapse of microbubbles results in burst release of therapeutic agents, which could possibly lead to toxicity^{57, 61}.

In recent year, our group has developed a controlled-release system through stable magnetic microbubble oscillations. Microbubbles were coated with magnetic microbubbles and loaded with doxorubicin (Dox)-containing PLGA NPs. When stimulated above resonant frequency, magnetic microbubbles oscillated and released NPs in a sustainable manner. This release of NPs attained deep penetration of around 200µm, Instead of inertial cavitation, the size of microbubbles continuously shrink as they unload the cargos and iron oxide NPs from the shell. Eventually, microbubbles ceased to exist once all the cargos and iron oxide NPs detached from the shell. Gao et al. also demonstrated the delivery of Dox-PLGA particles into 3D cell-alginate hydrogel construct, with successful

penetration and cellular uptake⁶¹. This technology can be further explored into encapsulating both cells and loaded-MMBs into a 3D hydrogel construct. The loaded-MMBs in the construct under ultrasound stimulation could possibly provide more efficient delivery of cargos with closer proximity with cells. In addition, the time points and dose frequency of cargos released can be controlled by ultrasound stimulation. Thus, this technology provides a potential controlled-release delivery system which can possibly be used in regenerative therapy in the future.

1.2. Objectives and Hypothesis

The project's hypothesis is to incorporate nanotechnology and microtechnology into tissue engineering for cartilage regeneration. Specifically, the utilization of nanosensors to noninvasively monitor cellular development of MSCs during chondrogenesis progress. At the same time, nanosensors can aid in the screening process of MSC chondrogenic culture conditions. In addition, the development of a controlled-release delivery system in 3D construct using magnetic microbubbles with ultrasound stimulation can assist in cartilage regeneration.

The project is divided into four parts. First, PCL-DPP-PCL NPs have been studied to provide strong fluorescence signal and good photostability for cell tracking. However, the effects on MSC functions (i.e. cell proliferation and differentiation capabilities) have yet to be study. Therefore, the project will investigate if MSC multipotency is affected with the use of these NPs. We expects that there will be minimal effects on the functions of MSCs and the good photostability of these NPs allows long-term cell tracking. Second part of the project is to develop monitoring system using molecular beacon based nanosensors to detect expression of chondrogenic marker (type II Collagen, *Col2*) in 3D MSC construct. We speculates that these nanosensors are able to provide accurate information of the gene expression and allow for prolong monitoring of MSC differentiation in 3D construct. Three part of the project is to further expand the system into screening of culture conditions for cartilage regeneration. Specifically, we aim to obtain optimal culture condition for MSC-derived proliferating chondrocytes, which is found in growth plate, with the use of TGF β 3 and FGF2 through MB-NPs platform. Last part of the project is to propose an effective, controlled-release delivery system using magnetic microbubbles in 3D microenvironment. We aims to be able to load different cargos onto magnetic microbubbles and are able to controlled-release these cargos in 3D hydrogel to the MSCs under ultrasound stimulation.

1.3. Dissertation Overview

This thesis introduces the potential tracking and detection platform for MSC, and specifically for cartilage regeneration. Potential controlled-release delivery system using magnetic microbubbles in 3D construct will also be discussed.

Chapter 1 introduces the background and rationale for the research. The objectives and hypothesis of the thesis are also mentioned.

Chapter 2 discusses the use of modified polymer-based fluorescent NPs for long-term cell tracking. The chapter also examined the effect of these NPs on MSC's multipotency.

Chapter 3 discusses molecular beacon based nanosensors as a noninvasive platform for monitoring of intracellular mRNA expressed. The chapter also validates the use of the nanosensors for 3D MSCs chondrogenic differentiation.

Chapter 4 elaborates on the potential of the molecular beacon based nanosensors in screening the optimal culture conditions for cartilage tissue engineering.

Chapter 5 elaborates on the potential delivery system of different cargos using magnetic microbubbles in 3D agarose construct under ultrasound stimulation.

Chapter 6 concludes the whole thesis and discusses the future works for the research works.

Chapter 2: Long-term tracking mesenchymal stem cell differentiation with photostable fluorescent nanoparticles

Reprinted (adapted) with permission from (Liu, Shiyang[#], Li Min Tay[#], Raditya Anggara, Yon Jin Chuah, and Yuejun Kang. "Long-term tracking mesenchymal stem cell differentiation with photostable fluorescent nanoparticles." *ACS applied materials & interfaces* 8, no. 19 (2016): 11925-11933.). Copyright (2016) American Chemical Society.

Abstract

Mesenchymal stem cells (MSCs) have proved to be a promising and abundant cell source for tissue and organ repair in regenerative medicine. However, the cell fate, distribution and migration of these transplanted cells are still unclear due to the limited tracking methods. It is desirable to develop a biocompatible and photostable probe to label the MSCs for long-term tracking without affecting the cell proliferation and potency. Herein we apply a recently developed nanoprobe system, in which di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) is covalently linked in the middle of polycaprolactone (PCL) forming the PCL-DPP-PCL polymer complex. Although the PCL-DPP-PCL nanoparticles uptake by the MSCs did not affect the cell viability, it was interesting that they exhibited different effects on the multi-lineage potency of the MSCs in the subsequent differentiation *in vitro*. Specifically, we found that the PCL-DPP-PCL labelling was unfavourable to the MSC osteogenic differentiation, whereas the labelled MSCs exhibited the same adipogenic and chondrogenic differentiations compared to the unlabelled controls as verified by gene expressions and histological staining. Furthermore, the PCL-DPP-PCL nanoparticles remained strong fluorescence intensity even after 4 weeks of differentiation. This study indicated that PCL-DPP-PCL nanoparticles could be used for

long-term cell tracing in MSC differentiation into adipogenic and chondrogenic lineages.

2. 1 Introduction

Stem cell-based therapies, aiming to treat diseases using the self-renewal and multilineage differentiation capacities of the transplanted stem cells, have achieved great progress in the past decade⁶²⁻⁶⁵. Specifically, mesenchymal stem cells (MSCs) are much less prone to the ethical issues and the risk of developing teratoma⁶⁶. MSCs are able to differentiate into multiple types of mesoderm lineages, such as chondrocytes, osteocytes and adipocytes⁶⁶. An increasing number of MSC-based therapies, especially for tissue repair or immune disorder diseases, have been attempted since 2004⁶⁷. However, there are many major issues remaining to be resolved before successful clinical applications on real patients^{66, 68}. One of the critical challenges is the difficulty to understand the transplanted stem cells' fate, distribution and migration in the local microenvironment without good tracking methods. Compared to traditional but highly invasive histological analysis, non-invasive imaging techniques are urgently demanded to track the transplanted stem cells for monitoring the therapeutic progress. The distribution and migration of the transplanted stem cells can provide a reliable evaluation for the cell-based therapeutic effect and elucidate the optimal conditions during surgery, such as cell dosage, route of delivery and the timing of transplantation^{69, 70}. Additionally, in the applications of stem cell based tissue and organ regeneration, it is

essential to delineate the specific contributions of transplanted cells and host cells to the damaged tissues or organs⁷¹.

Fluorescence imaging has been widely applied in various biological fields as a non-invasive technique because of its unique advantages, such as high sensitivity, rapid acquisition, reduce radiation, low cost and easy access⁷⁰⁻⁷⁵. The most popular labelling markers for fluorescence imaging are organic dyes, such as DAPI (4',6-diamidino-2-phenylindole) and Alexa Fluor family fluorophores. However, most organic dyes can only be used for short-term imaging because of photobleaching effect⁷¹. Another emerging type of fluorescent labels are quantum dots (QD), which emit strong fluorescence due to quantum confinement effect and the excitation and emission spectra are highly tunable based on their unique sizes^{71, 76}. However, the typical semiconductor and heavy metal-based quantum dots could release metal ions particularly under UV illumination, which are highly toxic to the cells both *in vitro* and *in vivo*⁷⁷⁻⁷⁹. Cadmium selenide (CdSe)/zinc sulfide (ZnS) QDs were developed to label human MSCs during osteogenic differentiation by liposome-mediated transportation⁸⁰. It was found that although the labelled MSCs showed normal proliferation, the gene expressions of specific osteogenic markers including osteopontin, osteocalcin and alkaline phosphatase were significantly inhibited in the presence of QDs⁸⁰. In another prior study, MSCs labelled with commercial

quantum dots (QTracker 800, Thermo Fisher Scientific) were delivered to nude rats with segmental bone defects to track the cell distributions during the bone healing process after transplantation⁷⁷. Although clear fluorescence could be observed *in vivo* from the treated rats, the QD-labelling reduced the function of transplanted cell and thereby the bone healing responses. To address this major issue, QD probes need to be modified with biomolecules or biocompatible polymers to reduce their cytotoxicity before applications. For example, MSCs were labelled CGGGRGD (Cys-Gly-Gly-Gly-Arg-Gly-Asp) was conjugated to CdSe/ZnS QDs for induced differentiations into adipogenic, chondrogenic, and osteogenic lineages⁷¹. These bioconjugated QDs were shown to be able to maintain the viability as well as the multipotency of the labelled human MSCs (hMSCs). In addition to quantum dots, other nanoprobes, such as phosphonate-functionalised polystyrene nanoparticles loaded N-(2,6-diisopropylphenyl)-perylene-3,4-dicarboximide dye were also used for long-term tracing of hMSC differentiation⁸¹.

Previously, Huang and co-workers have prepared a synthetic polymer platform based on modified polymer chains. Di(thiophene-2-yl)-diketopyrrolopyrrole (DPP) was inserted in between biocompatible and bioresorbable polycaprolactone (PCL) polymers through covalent interaction, which is abbreviated as PCL-DPP-PCL⁸². These nanoparticles

exhibited bright fluorescence and strong photostability for cancer and stem cell imaging. However, the potential influence of this polymeric nanoprobe on MSC potency and differentiation remains unclear. Herein we aim to study the long-term tracing of MSCs labelled with PCL-DPP-PCL and to investigate the potential effect of these nanoparticles on the MSC multipotency, especially the differentiation capability. Specifically, we labelled hMSCs with PCL-DPP-PCL nanoparticles and then induced these labelled hMSCs into different cell lineages including adipocytes, chondrocytes, and osteocytes respectively, which were characterized by gene expression and histological staining. Additionally, the residue fluorescence within the differentiated labelled cells was quantified with flow cytometry and imaged with confocal laser scanning microscopy.

2. 2 Materials and Method

Fabrication of PCL-DPP-PCL NPs

The detailed procedure to prepare and characterize the PCL-DPP-PCL nanoparticles (NPs) can be found in the previous report⁸². The prepared NPs were dispersed in sterile 1X PBS before use.

Cell culture

Human bone marrow derived mesenchymal stem cells (hMSCs) of consented patients in the National University Hospital (Singapore) were isolated using Institution Review Board (IRB) approved protocol ⁸³. Expansion medium for cell expansion consists of 10% Fetal Bovine Serum (FBS) (Life Technologies, Singapore) and 100 units/ml penicillin-streptomycin (Life Technologies, Singapore) in low glucose Dulbeco's Modified Eagle Medium (DMEM) supplemented with sodium pyruvate and GlutaMax™ (Life Technologies, Singapore). Cells were incubated in 37°C, at 5% CO₂. After 48 h, unattached MSC were removed. While adherent cells remained in culture flasks for further expansion. The hMSCs in passages 3 and 4 were used for this study.

Cellular labelling by nanoparticles

The hMSCs were seeded in 6-well plates in expansion medium. After cell attachment overnight, the original medium was replaced by 0.2 mg/mL PCL-DPP-PCL NPs in medium. Then the cells were grown for 3 and 7 days before the excess NPs were rinsed with 1X PBS.

For confocal laser scanning microscopy, the cells were fixed by formalin and then permeabilized by 0.1% Triton-X. Then the samples were blocked by 1% BSA and stained with Alexa Fluor® 633 phalloidin for F-actin and DAPI for nucleus. The 3-color laser source (405 nm, 561 nm and 633 nm) in a confocal microscope (Carl Zeiss, LSM 710) provided excitation for the fluorophores in DAPI (410-549 nm), PCL-DPP-PCL (566-622 nm) and Alexa Fluor® 633 phalloidin (638-747 nm).

For quantitative analysis by flow cytometry, the cells were trypsinized by 1×TrypLE™ Express Enzyme (Life Technologies, Singapore) and then centrifuged. 1X PBS solution was added into the cell pellet to prepare monodispersed cell suspension (500 µl) for flow cytometry (LSR II, BD). The PE channel (excitation: 561 nm; emission: 567-597 nm) was used to detect the fluorescence signals from PCL-DPP-PCL NPs. Regular cells without NP treatment were used as negative control. Approximately 1×10^4 cell events were recorded and the data were analysed by FlowJo software.

For quantitative analysis by flow cytometry, the cells were trypsinized by 1×TrypLE™ Express Enzyme (Life Technologies, Singapore) and then centrifuged. 1X PBS solution was added into the cell pellet to prepare monodispersed cell suspension (500 µl) for flow cytometry (LSR II, BD). The PE channel (excitation: 561 nm; emission: 567-597 nm) was used to detect the fluorescence signals from PCL-DPP-PCL NPs. Regular cells without NP treatment were used as negative control. Approximately 1×10^4 cell events were recorded and the data were analysed by FlowJo software.

Cell proliferation

The influence of PCL-DPP-PCL NPs on hMSC proliferation was evaluated using PrestoBlue assay. Firstly, 2000 cells/well labelled and unlabelled hMSCs were seeded on a 24-well plate and cultured for 14 days at 37°C and 5% CO₂. The medium was changed every 2-3 days. The absorbance was measured at day 3, 7, 10, and 14. Percentage (%) reduction was calculated based on the formula provided by the manufacturer.

Cell differentiation

The hMSCs of passage 3 or 4 were cultured in three T75 flasks. When cells became confluent after 3-4 days, PCL-DPP-PCL NPs (0.2 mg/mL) were added to the culture medium. Regular cells without NP treatment were used as control. After further incubation for 3 and 7 days, the excess NPs were removed by PBS washing for thrice before the cells were trypsinized by 1×TrypLE™ Express Enzyme. The cell suspension in DMEM medium from each group was placed on 4-well plates for osteogenic and adipogenic differentiations respectively, while other cells were seeded in sterile conical tubes for chondrogenic differentiation. The DMEM medium was replaced by differentiation induction medium after one day.

MSC differentiations

6.0×10^4 and 6.0×10^3 hMSCs were seeded on 1.9 cm² Nunc™ 4-well plates (Thermo Fisher Scientific, Singapore) in expansion medium for adipogenic and osteogenic differentiations respectively. Cells were first incubated overnight for effective attachment. Then the expansion medium was replaced by differentiation medium on the following day for induction of adipogenic or osteogenic differentiations. The adipogenic medium comprised high glucose DMEM (without L-glutamine) with 10% FBS, 10

µg/ml insulin (Life Technologies, Singapore), 1 mM sodium pyruvate (Life Technologies, Singapore), 100 units/ml penicillin-streptomycin, 0.1 µM dexamethasone (Sigma-Aldrich, Singapore), 200 µM indomethacin (Sigma-Aldrich, Singapore) and 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, Singapore). The osteogenic medium included low glucose DMEM, 10% FBS, 1× Glutamax™ (Life Technologies, Singapore), 100 units/ml penicillin-streptomycin, 50 µg/ml ascorbic acid (Sigma-Aldrich, Singapore), 10 mM β-glycerophosphate (Sigma-Aldrich, Singapore), 1 mM sodium pyruvate, and 0.1 µM dexamethasone. The cells were cultured in humidified conditions at 37°C, 5% CO₂ with the differentiation medium refreshed every 2-3 days. After adipogenic and osteogenic differentiation, the cells were washed with PBS twice before trypsinized by 1×TrypLE™ Express Enzyme. Then the cells were mixed with DMEM medium and spun down. The cell pellets were resuspended in 500 µl PBS for flow cytometry.

Induction of hMSC chondrogenic differentiation was performed in a 3-dimensional culture condition. 2.0×10^5 hMSCs were added to a 15 ml conical tube and then centrifuged to form a cell pellet, which was cultured in expansion medium to allow pellet stabilization. The expansion medium was replaced by chondrogenic medium on the second day. The chondrogenic medium consisted of high glucose DMEM, 1× Glutamax™, 50 µg/ml ascorbic acid, 100 units/ml penicillin-streptomycin, 1 mM sodium pyruvate,

0.1 μ M dexamethasone, 4 mM proline (Life Technologies, Singapore) and ITS (BioLab, Singapore). 10 ng/ml transforming growth factor β 3 (TGF β 3) (R&D Systems, Singapore) was freshly supplemented to the chondrogenic medium during the medium replacement every 2-3 days. Pellets after chondrogenic differentiation were firstly homogenized in collagenase type I for 2 h at 37°C with 5% CO₂ and then spun down. Then 1 $\times$ TrypLE™ Express Enzyme was added to the pellet for several minutes and cells were centrifuged after adding DMEM medium. Finally, the cells were resuspended in PBS for flow cytometry.

Histological analysis

The adipogenic and osteogenic differentiation samples were collected at day 21, while the chondrogenic pellets were harvested at day 28. The samples were washed with PBS before fixation with 10% formalin (Sigma-Aldrich, Singapore) for overnight. The fixed samples were rinsed with PBS prior to histological staining.

The adipogenic lineage was stained with Oil Red O solution to identify intracellular oil droplet accumulation that can be found in mature adipocytes⁸⁴. After 15 min incubation, the samples were rinsed with 70% ethanol and then distilled water to remove the excess staining solution

before being counterstained with hematoxylin (Sigma-Aldrich, Singapore) for 30 sec and rinsed with distilled water. For osteogenic lineage, cells were stained with Alizarin Red S solution (Sigma-Aldrich, Singapore) for 5 min, followed by gentle agitation with distilled water to remaining solution. The cells subjected to osteogenic differentiation presented brick-red colour due to calcium deposition. The chondrogenic pellets were embedded in FSC 22 Mounting Media (Leica, Singapore) and left at -20°C for about 2 h. 5 μm thick tissue sections of were sectioned using a Leica CM1900 cryostat (Leica Microsystems, Singapore) and were collected using Mezel-Glaser Superfrost Plus slides (Thermo Fisher Scientific, Singapore).

Immunohistochemistry staining was done to analyse collagen type II expression by using UltraVision™ Quanto Detection System HRP DAB kit (Thermo Fisher Scientific, Singapore). The samples on the slides were first incubated with digestive enzyme containing 0.5% pepsin (Sigma-Aldrich, Singapore) at 37°C in humid condition for 20 min. Staining was then done following the protocol of the kit. Mouse monoclonal anti-Col II antibody (Merck Millipore, Singapore) with a dilution factor of 1:500 was used and samples were incubated at 4°C overnight. The chondrogenic sections were counterstained with hematoxylin (Sigma Aldrich, Singapore). The coverslips were dehydrated after staining.

Gene expression

Adipogenic and osteogenic samples were harvested at day 14, while chondrogenic pellets were only collected at day 21. Prior to RNA isolation, chondrogenic pellets were incubated in 0.25% collagenase type I for 2 h at 37°C water bath. The RNA was isolated using RNeasy mini kit (Qiagen, Singapore) and quantified with NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific, Singapore). RNA was reverse transcribed into cDNA with 5× iScript Reverse Transcription Supermix (Biorad Laboratories, Singapore). Real-time Polymerase Chain Reactions (RT-PCRs) were implemented with SYBR Green PCR Master Mix kit (Life Technologies, Singapore), together with StepOnePlus Real-Time PCR System (Life Technologies, Singapore) to quantify the specific mRNA expression in the samples. The steps for RT-PCRs were as followed: denaturation at 95°C (10 min), 40-cycle amplification of 95°C denaturation (15 sec) and 60°C extension (1 min). Primers used to amplify cDNA of adipogenic cDNA include *GAPDH*, PPAR-G and Leptin. Meanwhile, *GAPDH*, Col I, OCN and ALP primers were used for osteogenic cDNA, and *GAPDH*, Col I and Col II primers were used for chondrogenic cDNA. The primer sequences used were shown in Table 2.1. All data were normalized according to the housekeeping gene *GAPDH*, and the mRNA of target genes were relative

to their respective level at day 0 right before induction of differentiation.

Gene expression level was determined using the equation $2^{-\Delta\Delta C_t}$.

Table 2.1. Primer sequences for specific biomarkers used in RT-PCR.

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
<i>GAPDH</i>	ACAAC TTTGGTATCGTGG AAGG	GCCATCACGCCACAGTTT C
PPAR-G	TACTGTCGGTTTCAGAAA TGCC	GTCAGCGGACTCTGGATT CAG
Leptin	TGCCTTCCAGAAACGTGA TCC	CTCTGTGGAGTAGCCTGA AGC
OCN	CGCCTGGGTCTCTTCACT AC	CTCACACTCCTCGCCCTA TT
ALP	ACCACCACGAGAGTGAA CCA	CGTTGTCTGAGTACCAGT CCC
Col I	CAGAACGGCCTCAGGTA CCA	CAGATCACGTCATCGCAC AAC
Col II	CCAGATGACCTTCCTACG CC	TTCAGGGCAGTGTACGTG AAC

Confocal laser scanning microscopy

The adipogenic and osteogenic lineages (day 21) were washed twice by PBS, fixed by formalin and then permeabilized by 0.1% Triton-X. Then the samples were blocked by 1% BSA and stained with Alexa Fluor® 633 phalloidin for F-actin and DAPI for nucleus. We used three laser channels (405 nm, 561 nm and 633 nm) in a confocal microscope (Carl Zeiss, LSM 710) to excite the fluorophores of Alexa Fluor® 633 phalloidin, DAPI and PCL-DPP-PCL NPs. Their fluorescence emission signals were detected in three individual channels (410-549 nm, 638-747 nm and 566-622 nm respectively).

For chondrogenic lineage, the cell pellets were rinsed with PBS twice before formalin fixation. The fluorescence of the labelled samples were observed by confocal microscope (excitation: 561 nm; emission: 566-622 nm). We applied z-stack function to image different layers of the pellet. Additionally, the cryosection slides of pellets were also imaged with confocal microscopy.

Cytotoxicity assay

The cytotoxicity of PCL-DPP-PCL NPs on hMSCs during cell labeling was

tested by PrestoBlue assay. Firstly, 6000 cells/well unlabeled hMSCs were seeded on a 96-well plate. After cell attachment, the original medium was changed with 0.2 mg/mL PCL-DPP-PCL NPs in medium. The cells were incubated with the NPs for 3 and 7 days respectively before the absorbance (measurement wavelength: 570 nm; reference wavelength: 600 nm) were quantified by a micro-plate reader (Infinite M200 Pro, Tecan Asia, Singapore). Cell viability was calculated based on the manufacturer's instructions. The unlabeled hMSCs were used as control with relative cell viability set as 100%.

Fabrication of porous scaffold

Porous scaffold was fabricated using salt leaching and solvent casting techniques. Poly(L-Lactide-co-Epsilon-Caprolactone) (70:30) (Purasorb®, Singapore), also known as PLCL, was dissolved in 10% w/v chloroform (Fischer Scientific, analytical grade, Singapore). NaCl (Sigma-Aldrich) with particle size of 150-200 μm was mixed in the PLCL solvent, which was then casted at a constant rate to form approximately 200 μm thick scaffold and dried. The salt component was then leached by washing with water forming a porous scaffold.

The PLCL scaffolds were pretreated with 3 mg/ml chitosan (Sigma-Aldrich, Singapore) in 4 mM HCl using aminolysis method [22] before they were soaked in 50% ethanol and dried. Subsequently, these scaffolds were immersed in 10% (w/v) 1,6-hexanediamine (Alder BioPharmaceutical, Singapore) in isopropanol (Fischer Scientific, Singapore) and then 1% glutaraldehyde (Sigma Aldrich, Singapore) after washing with 1× phosphate buffered saline (PBS) (Life Technologies, Singapore). Finally, the scaffolds were incubated in 3 mg/ml chitosan (Sigma Aldrich, Singapore) in 4 mM HCl at 4°C overnight. Chitosan-modified PLCL scaffolds (2 mm diameter) were sterilized using 70% ethanol for 2 h and washed. Approximately 5×10^4 hMSCs were seeded on each scaffold and cultured.

Statistical analysis

The data were analysed following Student's t-test to determine the statistical significance among experimental groups with $n \geq 3$ using One-Way ANOVA and Tukey HSD test. The difference between data was regarded statistically significant when $p\text{-value} < 0.05$.

2.3 Results and Discussion

For abbreviation, the PCL-DPP-PCL NPs are simply referred to as NPs henceforth. The brief workflow of this study is presented in Figure 2.1. Firstly, the original hMSCs were incubated with NPs for 3 days (ST: short term) and 7 days (LT: long term) with blank cells as control. Then, these labelled cells were induced into adipogenesis, chondrogenesis and osteogenesis. To study the influence of NPs on hMSC proliferation and multipotency, we divided the MSCs into 3 major experimental groups, i.e., unlabelled control, short-term staining for 3 days (NP-ST) and long-term staining for 7 days (NP-LT).

Figure 2.1. Schematic illustration of long-term tracking human mesenchymal stem cells during adipogenic, chondrogenic and osteogenic differentiations using PCL-DPP-PCL nanoparticles.

2. 3.1 Cell labelling with NPs

We firstly investigated the cellular uptake of NPs. The confocal fluorescence microscopy in both individual channels and ortho-views (Figure 2.2A and Figure 2.3) indicated that NPs were internalized into the cytoplasm of hMSCs, which was consistent to the finding in the previous

work⁸². Additionally, prolonged incubation time from 3 days to 7 days for cell staining enhanced the cellular intake of NPs with increased fluorescence intensity (Figure 2.2A), which was further verified by flow cytometry showing the peak intensity shift (Figure 2.2B).

Figure 2.2. (A) Cellular uptake of NPs (yellow) in hMSCs imaged by confocal laser scanning microscopy with counterstaining of nuclei with DAPI (blue) and F-actin were stained by Alexa Fluor® 633 phalloidin (red). Scale bar: 100 μ m. (B) Quantitative analysis of cellular uptake of NPs by flow cytometry. (C) Cell proliferation measured by PrestoBlue assays. Data presented as mean $\pm$ SD. *p-value <0.05, **p-value < 0.01. n = 3.

Figure 2.3. Corresponding ortho-views by taking different z stacks for the confocal laser scanning microscope images of NP-ST (A) and NP-LT (B) in Figure 2.2A. Cell nuclei were also stained with DAPI (blue) and F-actin with Alexa Fluor® 633 phalloidin (red), and the fluorescence of NPs were coloured as yellow.

The staining process did not show any considerable effect on the cell viability compared with the unlabelled control (Figure 2.4), indicating that the NPs had minimum cytotoxicity to the hMSCs. For long-term tracking of cell differentiation, the NP labelling is desired to have minimum effect on cell proliferation to maintain the multipotency of hMSCs. The PrestoBlue assay measured the reduction ability of live cells up to 2 weeks (Figure 2.2C), which revealed that the short-term labelled group (NP-ST) and the long-term labelled group (NP-LT) did not have any significant inhibition effect on the hMSC proliferation as compared to the unlabelled control.

Figure 2.4. Relative cell viability of hMSCs during NP-labelling (concentration: 0.2 mg/mL) measured by PrestoBlue assay. The mean viability of unlabelled control was set as 100%. Data presented as means $\pm$ SD (n = 3).

2. 3.2 Multilineage differentiations of labelled hMSCs

Figure 2.5 showed the long-term effect of NP labelling on hMSC-derived adipogenic differentiation after 3 weeks. The confocal microscopy indicated that differentiated adipocytes retained strong fluorescence due to NP staining (yellow, Figure 2.5A) for both NP-ST and NP-LT groups. The observation was further quantified by flow cytometry analysis, which also showed relatively stronger fluorescence of NP-LT than NP-ST (Figure 2.5B). Compared to the NP-labelled hMSCs before adipogenic differentiation (Fig. 2.2A-B), the cellular fluorescence intensity did not exhibit significant decrease after 3 weeks indicating the strong photostability of the labelling NPs.

A

B

C

D

Figure 2.5. MSC differentiation in adipogenic lineage. (A) Confocal fluorescence images of the differentiated adipocytes (day 21). Scale bar: 100 μm . (B) The retained fluorescence due to NP staining in the differentiated adipocytes (day 21) measured by flow cytometry. (C) Leptin and PPAR- γ expressed by cells were (day 14) quantified by RT-PCR assay. The results were normalized to the data on day 1 (start of differentiation). Data presented as mean $\pm$ SD. *p-value < 0.05, **p-value < 0.01. n = 4 for leptin and n = 3 for PPAR- γ . (D) Oil Red staining for oil droplets in the differentiated adipocytes (day 21). Scale bar: 250 μm .

The variation of cell morphology and fluorescence of NP labelling at intermediate stages (day 6, 13 and 20) in the adipogenic differentiation were shown in Figure 2.6. Figure 2.5D displayed the accumulation of intracellular oil lipids in the differentiated adipocytes by histological staining, providing the qualitative evidence of successful adipogenesis in the NP-labelled cells as compared with the unlabelled control. To evaluate the adipogenic differentiation quantitatively, we measured the gene expression of two important adipose biomarkers including leptin and peroxisome proliferator-activated receptor γ (PPAR- γ), which regulate fatty acid storage and glucose metabolism. The RT-PCR assay (Figure 2.5C) revealed that

the time of NP staining before induction of adipogenic differentiation influenced the adipose gene expression. Specifically, the NP-ST group exhibited a similar level of leptin and an upregulation of PPAR- γ as compared to the unlabelled control. In contrast, the gene expressions of leptin and PPAR- γ in the NP-LT group were both downregulated significantly. These results implied that prolonged NP staining and higher concentrations of internalized NPs could interfere with the hMSC-derived adipogenic differentiation, for which the underlying mechanism of cellular transduction was still unclear and required further investigation. Nevertheless, we have demonstrated that short-term NP staining for 3 days (NP-ST) could provide strong fluorescence labelling without compromise of adipogenesis for long-term tracking of hMSC differentiation. Therefore, we applied short-term NP staining in the following chondrogenic and osteogenic differentiations.

Figure 2.6. Microscopic images of the NP-labelled hMSCs during adipogenic differentiation. The images of NP-ST and NP-LT groups were obtained by merging the bright field contrast images and the corresponding fluorescent images. Scale bar: 250 μm .

The chondrogenic lineage was analysed after 3-4 weeks of induced differentiation as shown in Figure 2.7. We observed strong fluorescence within the formed cartilage tissue pellet labelled with NPs (Figure 2.7B) in addition to its cryosection (Figure 2.7A). We further homogenized the tissue

pellet, digested the matrix proteins and obtained the suspension of chondrocytes for flow cytometry, which showed that the individual hMSC-derived chondrocytes retained strong fluorescence of the NP labels even after 4 weeks of chondrogenesis (Figure 2.7C). Three important chondrogenic markers, including type II collagen (*Col2*), aggrecan (AGG) and type I collagen (*Col1*), were analysed using RT-PCR assay for their gene expressions in the chondrogenic pellets at day 21. The gene expressions of *Col1* and AGG in the labelled chondrogenic pellets were similar to those in the unlabelled control (Figure 2.7D). As the most abundant collagen in native cartilage tissues⁸⁵, *Col2* exhibited high level of expression in both labelled and unlabelled groups (Figure 2.7E). Although the *Col2* expression in the labelled pellets exhibited relatively lower fold change compared to the unlabelled control, the data did not show significant difference statistically. Moreover, both groups exhibited a high *Col2/Col1* ratio above 2,000 fold difference (Figure 2.7F), which was a significant and characteristic feature of hyaline-like cartilage tissue formation^{85, 86}. Immunohistochemistry staining further demonstrated the similar density of *Col2* in labelled and control tissue pellets after 4 weeks of chondrogenesis (Figure 2.7G). Therefore, the NP-labelling was also proved to be amicable for long-term tracking of chondrogenic differentiation of hMSCs.

Figure 2.7. MSC differentiation in chondrogenic lineage. (A) The cryosections of the hMSC-derived tissue pellets (day 28). Scale bar: 100 μ m. (B) Fluorescence images of the original pellets (day 28) obtained from confocal laser microscope. Scale bar: 200 μ m. (C) Flow cytometry

measurement of the trypsinized chondrocytes (day 28). (D–F) The mRNA expression of *Col1*, *Col2* and aggrecan (day 21) quantified by RT-PCR assay. Data were normalized according to the results at day 1 (start of differentiation). Data presented as mean $\pm$ SD. n = 4 for *Col1*, n = 3 for AGG and *Col2*. (G) Immunohistochemistry staining of *Col2* in the chondrogenic pellets (day 28). Scale bar: 250 μ m.

The last differentiated group was the osteogenic lineage. Confocal fluorescence microscopy after 3 weeks of osteogenic differentiation (day 21) indicated strong fluorescence in the hMSC-derived osteocytes (Figure 2.8A). However, the NP-labelled osteocytes exhibited much lower intensity of Alizarin Red staining as compared to the unlabelled control (Figure 2.8B), suggesting that the presence of NPs suppressed the calcium deposition considerably during osteogenesis. We also quantified the gene expressions of three important osteogenic markers, including *Col1*, osteocalcin (OCN) and alkaline phosphatase (ALP), using RT-PCR assay after induction of differentiation for 14 days. The RT-PCR data revealed significant downregulation of all three osteogenic gene markers in the NP-labelled group (Figure 2.8C). These results implied that the NP labelling, even by short-term staining of 3 days, had inhibited the osteogenic differentiation of hMSCs. It was previously reported that TiO₂ nanoparticles exhibited adverse effects on the osteogenic differentiation of MSCs by

influencing ALP, OCN and osteopontin (OPN) gene expressions in a size-dependent and dose-dependent manner. MSC viability and proliferation capabilities were also affected⁸⁷. In the present study, on the other hand, we showed that the synthetic polymer-based NPs with staining concentration of 0.2 mg/ μ L did not affect the cell viability and proliferation, while they compromised the osteogenic differentiation of MSCs.

Figure 2.8. MSC differentiation in osteogenic lineage. (A) Confocal fluorescence images of the differentiated osteocytes (day 21). Scale bar: 100 μm . (B) Calcium deposition were stained with Alizarin Red after 21 days of osteogenic differentiation. Scale bar: 250 μm . (C) The mRNA expression of Col1, OCN and ALP (day 14) quantified by RT-PCR assay. Data were normalized according to the results at day 1 (start of differentiation). Data presented as mean $\pm$ SD. n = 4. *p-value <0.05, **p-value < 0.01.

2. 3.3 MSC labelling in 3D scaffold

All above results were based on MSC labelling for conventional 2-dimensional (2D) cell cultures or pellet culture. In the recent advances of tissue engineering, 3-dimensional (3D) scaffolds have become more popular due to many advantages in better mimicking the native cellular environment, which consists of a 3D network with a complex extracellular environment and a highly porous nanotopography⁸⁸. Cells cultured in 3D scaffolds tend to behave more like their in-vivo counterparts, which may work as a better model for tissue engineering and other applications such as drug discovery and cellular signalling⁸⁹. The special mechanical properties of these 3D scaffolds also provide supports to the cells and

newly formed tissues, which is important for weight-bearing tissues such as cartilage and bone^{83, 90}. To prove the concept, we seeded the hMSCs on a porous scaffold composited from Poly(L-Lactide-co-Epsilon-Caprolactone) (PLCL) (Figure 2.9) and cultured the cells for 14 days.

Figure 2.9. Scanning electron microscope images for PLCL porous scaffold under different magnifications: (A) 100×, (B) 250× and (C) 500×.

The 2D and 3D images in Figure 2.10 obtained by confocal fluorescence microscopy demonstrated the distribution of NP-labelled MSCs scattered in the scaffold. The cell nuclei stained with DAPI was indicated as blue dots in the magnified inset of Figure 2.10. It was however difficult to distinguish the nuclei from the strong background fluorescence under excitation of 405 nm due to the auto fluorescence of PLCL scaffold⁹¹. Meanwhile, the bright fluorescent NPs clearly indicated the distribution of labelled hMSCs in the scaffold under excitation of 561 nm. This simple test showed that these NPs could also be used as an excellent contrast agent for cell tracking in

the 3D scaffold environment, providing important information on the location, distribution and migration of the labelled cells.

Figure 2.10. Confocal fluorescence images of hMSCs seeded in a 3D scaffold (day 14). 3D images were reconstructed using ZEN 2012 software by taking different z-stack images. Scale bar: 100 μm .

2. 4 Conclusion

In summary, PCL-DPP-PCL nanoparticles are able to label hMSCs by internalization without affecting the cell viability and proliferation behaviours. It was found that PCL-DPP-PCL NPs labelling for 3 days at concentration of 0.2 mg/ μL did not show any negative effect on MSC adipogenic and chondrogenic differentiations, while the osteogenic

differentiation was compromised compared to the unlabelled control. Furthermore, the labelling NPs can maintain strong fluorescence even after 4 weeks, indicating these NPs can work as efficient fluorescent probes for long-term tracing during adipogenic and chondrogenic differentiations. By replacing DPP with the near infrared dye in this system, this strategy could be extended for long-term stem cell tracking applications *in vivo*.

Chapter 3: Non-invasive monitoring of 3D chondrogenic constructs using molecular beacon nanosensors

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Abstract

Chondrogenic differentiation of human mesenchymal stem cells (MSCs) in three-dimensional hydrogel holds promise as a method for repairing injured articular cartilage. Given MSC plasticity (its potential to mature into alternative lineages), non-destructive monitoring is critical for the optimization of chondrogenic differentiation conditions and the evaluation of the final product. However, conventional validation/assessments of the differentiation process (i.e., quantitative reverse transcription polymerase chain reaction [qRT-PCR] and histology) are end-point assays requiring disruption of the sample. This report introduces molecular beacon (MB)-based nanosensors to achieve non-invasive monitoring of chondrogenic differentiation. These nanosensors consist of biodegradable poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs) encapsulating MBs to detect Type II Collagen (*Col2*) and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNAs that serve as lineage-specific and housekeeping biomarkers, respectively. The steady release of MBs from MB-NPs allows long-term monitoring of MSCs undergoing chondrogenic differentiation over a period of 28 days. Dual-coloured MB loading ensures accurate assessment of *Col2* mRNA expression level, where potential heterogeneity in nanosensor uptake and retention by MSCs are taken into account. When normalized nanosensor signal was compared against qRT-PCR result, a

tight correlation was observed ($R^2 = 0.9301$). Finally, nanosensor usage was compatible with MSC potency with minimal influence on chondrogenic, adipogenic, and osteogenic differentiation.

3.1 Introduction

Cartilage tissue engineering is a technique of great promise to regenerate damaged articular cartilage. This concept aims to regenerate or reconstruct the cartilage by implanting constructs consisting of chondrogenic cell seeded scaffolds, with or without the delivery of bioinductive-factors^{92, 93}. Currently, a few cell-based strategies have been clinically investigated, for examples the implantation of autologous chondrocytes in fibrin glue and implantation of minced cartilage in polymers⁹³.

Autologous chondrocytes are usually preferred for constructing the cartilage tissue to prevent immune response. Still, this requires an invasive and painful cartilage biopsy which might induce donor morbidity. In addition, chondrocytes obtained from the patients are often inadequate in number and culture expansion is thus needed⁹³. Chondrocytes have slow proliferation rates and are prone to de-differentiate during culture expansion, which leads to the down-regulation of cartilage-specific genes^{94, 95}. This has led to more interest in the use of mesenchymal stem cells (MSCs) as it is able to undergo chondrogenesis under the influence of the cell microenvironment and growth factors⁹⁶. Typically, MSCs are cultured within a three-dimensional (3D) scaffold (e.g. hydrogel) which resembles the native cartilage microenvironment. It allows the deposition of

neocartilage matrices and maintains native chondrocytes rounded morphology^{97, 98}. Biological factors such as the transforming growth factor super family (e.g.TGFβ3) are employed to induce chondrogenic differentiation of MSCs and the secretion of extracellular matrix⁹⁹.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a popular assay to examine the expression of chondrogenic lineage markers (mainly Collagen 2, *Col2*) while histological and immunohistochemical validation are performed to assess the deposition of cartilaginous extracellular matrix proteins. These analyses however, are end-point assays that involve disruption of samples, and do not provide real-time monitoring of chondrogenic differentiation^{97, 100-102}. One alternative strategy involves the incorporation of reporter genes, in which a fluorescent reporter gene is designed to generate a signal when the gene of interest is activated¹⁰³. However, risks of insertional mutagenesis following transfection render cells transformed using this approach unsuitable for cell therapy¹⁰⁴. Nanotechnology, on the other hand, holds potential to provide a safe, non-destructive and highly efficient method for continuous monitoring of MSCs chondrogenic differentiation *in vitro*. Many studies have used nanoparticles (NPs) as cell labelling tools or contrasting agents in both *in vitro* and *in vivo* environments due to their unique optical, magnetic and/or acoustic attributes^{28, 36}. NPs can come in different sizes, shapes, building

blocks (e.g. polymer, iron, silica), and surface conjugation^{27, 29}. The flexibility and properties of NPs allows non-invasive, yet real-time cell tracking and monitoring over a wider observation period³⁶. These nanoparticles can be visualized in various imaging modalities like optical imaging and magnetic resonance imaging (MRI)^{27, 36}. However, majority of the current NPs for cell labelling only provide the insight of temporal-spatial dynamics of cells, not much was done in observing cellular differentiation²⁹.

Recently, our group has developed a smart nanosensor platform for non-invasive and continuous monitoring of cell differentiation for a period of at least 28 days¹⁰⁵. This smart nanosensor is fabricated by encapsulating molecular beacons (MB) in biodegradable poly(lactic-co-glycolic acid) (PLGA) nanoparticles (MB-NPs). After cellular internalization, PLGA degrades gradually and releases the MBs into the cytoplasm continuously⁵². In the presence of their target intracellular mRNA, it hybridised with released MBs. MBs regain fluorescence from their pre-quenched state to provide semi-quantitative mRNA expression levels. The sustained release manner of the PLGA nanoparticles overcomes the previously reported short monitoring time of MB because of rapid cellular clearance^{106, 107}. Hence, long-term yet precise monitoring of mRNA expression can be executed, which has allowed us to observe the osteogenic differentiation of human MSCs over a period of three weeks⁵⁰.

In this study, we explore the potential of this nanosensor platform for long-term, non-invasive monitoring of chondrogenic differentiation of MSCs in 3D hydrogel. Specifically, MBs of different fluorophores targeting the mRNAs of articular cartilage specific Type II collagen (*Col2*) and housekeeping reference gene, glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), were encapsulated in PLGA NPs to act as smart nanosensors. Dual MB loading with different fluorophores (FITC & Cy3 for *Col2* & *GAPDH*-MB, respectively) ensures accurate assessment of *Col2* mRNA expressed has into account of potential heterogeneity in nanosensor uptake and retention among the MSCs population. MSCs were labelled with these nanosensors before being encapsulated in 3D agarose hydrogels and induced chondrogenic differentiation for 28 days (Figure 3.1). Intracellular mRNA expression levels were then determined through imaging and quantification of the cell fluorescence intensity.

Figure 3.1. Schematic of the molecular beacon containing poly(lactic-co-glycolic acid) nanoparticles for longitudinal tracking of MSC chondrogenesis in 3D hydrogel matrix. MSC, mesenchymal stem cell.

3. 2   Experimental: Materials and methods

Chemicals and reagents were all purchased from Sigma-Aldrich and used without further purification except for those mentioned. Dulbecco's modified eagle medium (DMEM) with high glucose (4.5 g/L) and L-glutamine, DMEM with low glucose (1 g/L), fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/mL), collagen I (rat tail), TrypLE™ express enzyme, insulin, Glutamax™, sodium pyruvate, proline, TRIzol® reagent, SYBR Green PCR Master Mix kit were from Life technologies (USA), and DNase I were obtained from Invitrogen™. ITS premix universal culture supplement was obtained from Corning®. Transforming growth factor β 3 (TGF β 3) was purchased from R&D System (Minneapolis, MN). SensiFast™ cDNA synthesis kit was from Bioline (UK) while RNeasy Mini Kit was from Qiagen. EGM™ growth media was obtained from Lonza. UltraVision™ Quanto Detection System HRP DAB kit was purchased from Thermo Fisher Scientific (USA). Mouse monoclonal anti-Co/2 antibody was obtained from Merck Millipore.

Synthesis and characterization of MB-NPs: *GAPDH* and *Col2* MB sequences (Table 3.1) were adapted and modified from previous studies^{108, 109}, and verified using basic local alignment search tools (BLAST). Nanosensors were fabricated using double emulsion technique according to the published protocol.^{50, 52} Briefly, 100 µl of MBs in nuclease-free water (0.5 nmol *GAPDH* MB and *Col2* MB each) were added to the mixture of 10 mg PLGA in 0.5 ml chloroform and 1% span 80 (v/v) at 30,000 rpm for 30 seconds (Scilogex D160). Double emulsion was then formed by adding the first emulsion into 5ml of 3% polyvinyl alcohol (PVA) solution to be homogenized again at the same speed and duration. Remaining chloroform in the solution was allowed to evaporate and nanosensors were rinsed thrice with nuclease-free water to eliminate excess PVA solution. Nanosensors were then lyophilised and stored at -80°C.

To calculate the MB encapsulation efficiency, the supernatant left during MB-NP synthesis were collected and quantified the amount of free MBs. Specifically, DNase I (40 U/ml) were added to the supernatant containing free MBs for 15 min. The fluorescence intensity was then measured by fluorescence microplate reader. By extrapolating from the MB calibration curve, the concentration of MBs in the supernatant was obtained. The

values were normalised with the initial MB quantity to give the encapsulation efficiency of MBs during the synthesis.

Field emission scanning electron microscopy (JEOL JSM-6007F) at 5 kV was performed to examine the morphology of MB-NPs. Zeta potential and hydrodynamic diameter were measured by Zetasizer nano Z (Malvern).

MB release profiles from MB-NPs: 2 mg of MB-NPs was incubated in 37°C PBS for 30 days. Supernatants were collected at day 0, 0.5, 1, 2, 3, 4, 6, 9, 12, 15, 21, 30 and treated with DNase I (40 U/ml), followed by fluorescence readings using fluorescence microplate reader. The percentages of *GAPDH* MB and *Col2* MB released from nanosensors were quantified after extrapolating the MB concentration from the MB calibration curve.

MBs hybridization study: To examine the specificity of the MB, *GAPDH* and/or *Col2* complementary oligonucleotide sequences (Table 3.1) were mixed with 0.5 μ M of the corresponding MBs. Samples were incubated in the 96-well plate at 37°C for 10-15 min. Fluorescence intensity was quantified by fluorescence microplate reader with the excitation/emission

wavelength of 485/517 nm for FITC and 550/570 nm for Cy3. All the groups were normalized with DNase1-treated MBs (as positive control; 40 U/ml) and PBS treated samples (as negative control).

Table 3.1. The sequences of molecular beacons and molecular beacon targets used

<i>MBs</i>	<i>Sequences (5'-3')</i>
<i>GAPDH MB</i>	[Cy3]-CGACG-GAGTCCTTCCACGATACCA-CGTCG-[BHQ2]
<i>Col2 MB</i>	[FITC]-CCCGAT-CCTGCAGCACCTGTCTCACC-ATCGGG-[BHQ1]
<i>GAPDH target</i>	TGGTATCGTGGAAGGACTC
<i>Col2 target</i>	GGTGAGACAGGTGCTGCAGG

Cell Culture: Human MSCs were harvested from consented patients' bone marrow through National University Hospital (Singapore) with the guidance of the Institution Review Board (IRB) approved protocol.¹¹⁰ Isolated MSCs were cultured in expansion medium consisting of sodium pyruvate and GlutaMax™ supplemented low glucose DMEM with the addition of 1% penicillin-streptomycin (P/S) and 10% FBS. Culture medium was replaced every 2-3 days till flasks reached approximately 80% confluence.

Human umbilical vein endothelial cells (HUVEC) (Lonza) was expanded in collagen I (50 µg/ml in 20mM acetic acid, rat tail) (Gibco) coated culture flask. EGM™ growth media (Lonza) was used and medium was changed every 2-3 days till flasks reached 80% confluency.

Cell labelling with MB-NPs: The condition of MB-NPs cell labelling was according to our previous publication.¹¹¹ 50,000 MSCs were seeded on each well in a 6-well plate to reach 80% confluency. 4 mg nanosensors were first mixed with 50 µl of poly-L-lysine (PLL) solution (Mw 70,000-150,000, 0.01%w/v) for 30 min. PLL-coated nanosensors were rinsed with PBS, thereafter centrifuged. It was then dispersed in expansion medium to reach 1mg/ml. MSCs were cultured with the nanosensor solution overnight before rinsing with PBS thrice. The cells were then treated with TrypLE™ treatment for further study.

To quantify the labelling efficiency with flow cytometry (LSR Fortessa™ X-20, BD Biosciences), cells were then re-suspended in 800 µl solution (50% PBS and 50% culture medium). FACSDiva software (BD Bioscience) and FlowJo (TreeStar) were used to analyse the data.

Adipogenic and osteogenic differentiation of MSCs in 2D cell culture

plate: MSCs were seeded on 12-well plate (1.2×10^5 and 1.2×10^4 cells per well for adipogenic and osteogenic differentiation respectively). Adipogenic or osteogenic medium was introduced to the cells the following day. Adipogenic medium consisted of high glucose DMEM, $0.1 \mu\text{M}$ dexamethasone, 10% FBS, 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) 100 Units/ml P/S, 1 mM sodium pyruvate, $10 \mu\text{g/ml}$ insulin, and $200 \mu\text{M}$ indomethacin. Osteogenic medium comprised low glucose DMEM with 10 mM β -glycerophosphate, Glutamax™, 100 Units/ml P/S, 1 mM sodium pyruvate, $0.1 \mu\text{M}$ dexamethasone, $50 \mu\text{g/ml}$ ascorbic acid and 10% FBS. Medium was changed every 2-3 days.

Chondrogenic differentiation in 3D agarose hydrogel: $25 \mu\text{l}$ of 5×10^6 cells/ml MSCs were mixed with $25 \mu\text{l}$ 3% agarose solution (Agarose Type VII, Sigma Aldrich) to attain 2.5×10^6 cells/ml in 1.5% final concentration of cell-agarose hydrogel. Triplicate of $50 \mu\text{l}$ cell-agarose droplets were dropped onto the base of 12-well plate using pipette. The drops were left to solidify for 5 min before being placed into the normal culture medium. Medium was substituted with chondrogenic medium one day later. Chondrogenic medium consisted of high glucose DMEM, 100 Units/ml P/S,

Glutamax™, 1 mM sodium pyruvate, 0.1µM dexamethasone, 4mM proline, 50 µg/ml ascorbic acid and ITS (BioLab) and 10 ng/ml TGFβ3. Chondrogenic constructs were cultured for 28 days and medium was changed every 2-3 days. Constructs were also cultured in expansion medium as the control group.

Confocal imaging: Chondrogenic constructs were imaged using confocal laser scanning microscopy (LSM710, Carl Zeiss) to observe fluorescence emitted by *GAPDH* MB (Cy3:550/570 nm) and *Col2* MB (FITC:485/517 nm). Different slices of 3D constructs were captured using z-stack function. Images were processed using LSM Image Browser (Zeiss) and fluorescence was quantified using ImageJ software.

To image MB-NPs internalization in MSC samples, MB-NPs carrying only *GAPDH* MB- (Cy3; 570 nm) were labelled to MSCs prior to encapsulation in 3D agarose hydrogel. After 2 weeks of culture, samples were collected for counter-staining. 5 µM Calcein AM (membrane staining; 515 nm) and 2 drops/ml of Hoescht 33342 (nuclear staining; 461nm) were incubated with samples for 15 min before it was rinsed thrice with PBS. Samples were then fixed using formalin and washed with PBS before imaging.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis: 3 weeks after chondrogenic induction, 3D constructs were homogenized with TRIzol® Reagent, followed by chloroform. After thorough mixing, the mixture was spin-down at 12,000 g for 15 min at 4°C. RNA-containing aqueous phase was collected. Total RNA was then purified from with solution with RNeasy Mini Kit and quantified using NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). Reverse transcription using SensiFast™ cDNA Synthesis Kit was done followed by real-time Polymerase Chain Reaction (qPCR) with SYBR Green PCR Master Mix kit using StepOnePlus Real-Time PCR System (Life Technologies). Samples underwent denaturation for 10 min at 95°C and 40 cycles of amplification (denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute per cycle). The sequences of the primers utilised are listed in Table 3.2. Data obtained for target mRNA expression as normalized against endogenous *GAPDH* gene, employing the comparative C_T method formula.

Table 3.2. Primer sequences utilised in quantitative reverse transcription polymerase chain reaction

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
<i>GAPDH</i>	ACAAC TTTGGTATCGTGG AAGG	GCCATCACGCCACAGTT TC
<i>Col2</i>	CCAGATGACCTTCCTACG CC	TTCAGGGCAGTGTACGT GAAC
<i>Leptin</i>	TGCCTTCCAGAAACGTGA TCC	CTCTGTGGAGTAGCCTG AAGC
<i>PPARγ</i>	TACTGTCGGTTTCAGAAA TGCC	GTCAGCGGACTCTGGAT TCAG

Immunohistological and histological analysis: Samples are treated with 10% formalin and rinsed with PBS. Chondrogenically differentiated 3D constructs were fixed after 4 weeks of culturing. Thereafter, the samples were embedded in FSC 22 Mounting Media (Leica) and freeze at -20°C for at least 2 hours. Cryosectioning was done using Leica CM1900 cryostat (Leica Microsystem). 7 μ m section slices were collected with Mezel-Glaser Superfrost Plus slides (Thermo Fisher Scientific). Immunohistochemical staining was performed using UltraVision™ Quanto Detection System HRP DAB kit and mouse monoclonal anti-*Col2* antibody to validate *Col2* deposition.

Adipogenically and osteogenically differentiated samples were fixed after 3 weeks of culture. Adipogenic and osteogenic samples were then subjected to Oil Red O staining and Alizarin red staining respectively. Lipid droplets in adipogenic differentiated samples were stained using Oil Red O solution for 30 seconds. Mineralization of osteogenic differentiated samples was stained using Alizarin Red S solution for 5 min.

Statistical analysis: Statistical difference among experimental groups was evaluated using one-way analysis of variance (ANOVA) and Tukey HSD test with $n \geq 3$. Difference was considered significant when $*p < 0.05$ and $**p < 0.01$.

3.3 Results

3.3.1. Characterization of nanosensor

MB-NPs targeting both *Col2* and *GAPDH* mRNA were synthesized according to our previous work⁵² and their size was controlled around 1 μm (Figure 3.2A). Each MB-NP carried approximately 140 MB strands. They had an almost-neutral surface charge (-0.06 mV), but became positively-charged (~ 16.6 mV) after surface modification with PLL⁵⁰. Figure 3.2B illustrated good encapsulation efficiency of MBs in MB-NPs.

Figure 3.2. Characterization of MB-NPs. (A) Hydrodynamic diameter of MB-NPs; inset: SEM image of MB-NPs; (B) MB encapsulation efficiency in MB-NPs. MB, molecular beacon; NP, nanoparticle.

We first examined the release of MBs from MB-NPs in PBS for 30 days period, which is the time period in which MSC chondrogenesis typically occurs. As shown in Figure 3.3A, there were two stages of release. In the first stage, ~50% of encapsulated MBs were released from the particles in the first 4 days. Subsequently, steady release continued over the next 26 days with ~30% of encapsulated MBs liberated into the supernatant.

At day 30, the supernatant was allowed to hybridize with oligonucleotide targets complementary to the MB (Table 3.1). The restoration of pre-quenched MB fluorescence (~5-fold for *Col2* and ~4.5-fold for *GAPDH* MB) (Figure 3.3B) signifies the retention of MB sensor functionality throughout the 28 days experimental period.

Figure 3.3. Characterization of MB-NPs: (A) Release profile of *GAPDH* and *Col2* MBs from MB-NPs; (B) Hybridization study of MBs released from MB-NPs at day 30 with its oligonucleotide target sequences (normalized

against DNase I-treated samples). MB, molecular beacon; NP, nanoparticle.

When normalized fluorescence intensity of supernatant at day 30 (Figure 3.3B) was compared against that of fresh MB samples, we noted a slight increase in fluorescence intensity which suggests that some MBs lose its original hairpin structure upon NP fabrication and 4-week hydrolysis (Figure 3.4C & D). Nevertheless, this should not significantly alter the monitoring procedures, as overall MB functionality is still retained.

Figure 3.4. Characterization of MB-NPs. (A) hybridization study of fresh MBs with its target oligonucleotide sequences (normalized against DNase I-treated samples); (B) fluorescence signal comparison of fresh MB and MB released from MB-NPs at day 30. (* represents $P < 0.05$). MB, molecular beacon; NP, nanoparticle.

3.3.2. Labelling MSCs with nanosensors

To verify the final intracellular localization of the MB-NPs, we labelled MSCs with *GAPDH* MB-NPs (Cy3; 570nm) and counter-stained with calcein AM and Hoescht33342. We observed the red signal emitted by *GAPDH* MBs was in the cytoplasm of the cell, surrounded by the green signal from the cell membrane (Figure 3.5C).

To evaluate labelling efficiency of MB-NPs, flow cytometry analysis was performed on *GAPDH* MB-NP-labelled MSCs. As shown in Figure 3.5D, strong fluorescence could be observed from most of the cells (~91%) as a result of the liberated *GAPDH* MB (~154-fold of unlabelled cells). This echoes the stable, strong expression of *GAPDH* mRNA in MSCs¹¹².

Figure 3.5. Characterization of MB-NPs: (A) Orthogonal confocal image of MSCs after labelling. *GAPDH* MB (Cy3; 570 nm) was located outside the nucleus (Hoechst 33342; 461 nm), but within the cell membrane (calcein AM; 515 nm). (B) Flow cytometry analysis of MSCs showing highly efficient MB-NP internalization. MB, molecular beacon; NP, nanoparticle.

3.3.3. Tracking the chondrogenic differentiation process of MSCs with MB-NPs

Next, we confirmed MB-NP capability to distinguish cells with varying *Col2* expression. In particular, we examined three groups in 3D construct (i.e. undifferentiated MSCs (UD), chondrogenic-differentiated cells (TGF β 3), and human umbilical vein endothelial cells (HUVEC)) at day 28. As shown in Figure 3.6A, TGF β 3 group exhibited the strongest green fluorescence signal while UD group showed a weaker signal. There was little or nearly no green fluorescence observed for the HUVEC even at day 28. All three groups had stable and strong red fluorescent signal from *GAPDH* MBs. By normalizing *Col2* MB signal against the stable *GAPDH* MB signal, *Col2* expression was quantified (Figure 3.6B). Signal from TGF β 3-induced MSCs was ~2 times of that in un-induced MSCs, while *Col2* signal in HUVEC was undetectable. These *Col2* signal expressions were supported with qRT-

PCR results showing a greater fold expression of *Col2* in TGFβ3 group compared to UD group (Figure 3.6C).

Figure 3.6. The identification of chondrogenically-differentiated MSCs with MB-NPs. (A) Confocal images of undifferentiated MSCs (UD), chondrogenically-differentiated MSCs (TGFb3), and HUVEC cells on day 28. Scale bar: 100 mm. (B) Quantification and normalization of *Col2* MB signal against *GAPDH* MB signal in (A). (C) Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of undifferentiated MSCs (UD) and chondrogenically differentiated MSCs (TGFb3) at day 21. Samples were normalized with *GAPDH* expression using the comparative CT formula $2^{-\Delta\Delta CT}$ (** represents $p < 0.01$). HUVEC, human umbilical vein endothelial cell.

We further evaluated the non-invasive monitoring properties of the nanosensor over the 28-day period of chondrogenic differentiation (Figure 3.7A). It was clear that the green signal from Col MB increased steadily from day 7 to day 28 while the signal from *GAPDH* MB remained consistent and stable. Crucially, we noted how both green and red signals were confined within the cells (Figure 3.7A & 3.6B), indicating that observed fluorescent signals indeed came from hybridization of MBs with its target mRNA, and not from non-specific MB dis-integration. When the normalized *Col2* fluorescence signal (Figure 3.7C) was compared with the qRT-PCR result over the four time points (Figure 3.7D), a relatively good correlation ($R^2=0.9301$) was observed.

Figure 3.7. Tracking the chondrogenic differentiation process of MSCs with MB-NPs: (A) Confocal images of chondrogenically differentiating MSCs in 3D agarose hydrogel from day 7 to day 28. Scale bar: 100 μ m. (B) 3D reconstruction of chondrogenically differentiating MSCs in 3D agarose hydrogel at day 28. (C) Quantitative signal profile of *Col2* MB signal in chondrogenically differentiated MSCs over a period of 28 days, normalized against its corresponding *GAPDH* MB signal. (D) Correlation plot between normalized fluorescence intensity (*Col2/GAPDH*) of chondrogenically

differentiating MSCs and normalized $2^{-\Delta CT}$ expression of *Col2* mRNA relative to *GAPDH* mRNA at different time points.

3.3.4. Stem cell status of the MB-NP labelled MSCs

Finally, we examined whether the tri-lineage differentiation potential of MSCs had been compromised by the MB-NP labelling through immunochemical and histological staining. Nanosensor-labelled and unlabelled MSCs were allowed to undergo three common differentiation lineages; chondrogenesis, adipogenesis and osteogenesis. Upon collection, they were fixed and stained for their respective markers. As revealed in Figure 3.8, there was minimal difference between unlabelled and MB-NP labelled groups.

Figure 3.8. Immunohistochemical and histological staining of chondrogenic, adipogenic, and osteogenic differentiated MSCs with and without the MB-NP labelling.

In addition, qRT-PCR for adipogenic differentiated MSCs with and without MB-NPs labelling was performed for further validation. Insignificant fold difference was found following MB-NPs labelling, on both adipogenic markers ($P>0.2$ for *Leptin*, $P>0.1$ for Peroxisome proliferator-activated receptor gamma, *PPAR γ*) (Figure 3.9). This confirmed that the nanosensors did not significantly affect the MSC's multi-potency.

Figure 3.9. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of adipogenic differentiated MSCs samples at day 14 with and without MB-NPs labelling. Samples were normalized with *GAPDH* expression using the comparative C_T method formula $2^{-\Delta\Delta C_T}$. (ns represents non-significant). MSC, mesenchymal stem cell.

3. 4 Discussion

The combination of human MSCs, 3D scaffold and bioinductive-factors has been a typical tissue engineering approach used to address articular cartilage injuries^{113, 114}. In order to validate successful chondrogenesis, analyses such as qRT-PCR and histology are commonly executed. These necessitate the disruption of samples and thus, temporal expression of chondrogenic biomarkers (e.g. *Col2* expression) in chondrogenic-induced MSCs cannot be fully comprehended. In order to monitor the differentiation process in real-time, researchers then employed reporter gene assays to detect changes in biomarker gene expression. These reporter genes are regulated by tissue-specific promoters and introduced in living cells through various means of transfection¹¹⁵. In general, it can be divided into non-viral and viral-based delivery. In one example, non-viral liposome-based transfection was applied by Feichtinger et al to introduce osteocalcin-2 reporter gene for osteogenic differentiation monitoring¹¹⁶. Meanwhile, lentiviral transfection was employed by Bagó et al to deliver luciferase-fluorescent chimeric reporters to track both vascularization and bone differentiation of MSCs¹¹⁷. Nonetheless, there are limitations associated with gene reporter assays. For instances, certain degree of toxicity to cells

has been reported for liposomal transfection reagent¹¹⁸, while the use of viral vectors risks include insertional mutagenesis¹⁰⁴.

In view of this need, the current study demonstrates the potential of MB-NPs for non-invasive, real-time monitoring of chondrogenic differentiation of MSCs in 3D agarose hydrogel. The co-encapsulation of 2 MBs (Col2-MBs and GAPDH-MBs) in PLGA NPs provides us with the fluorometric assessment, which is important to facilitate accurate depiction of cellular mRNA expression, given heterogeneity in nanosensor uptake and retention.

Initially, we demonstrated the retention of MB specificity and structural integrity following their encapsulation in the particles and their 30 days exposure to external micro-environment. When MSCs were labeled with these MB-NPs, MB signals were identified from the cytoplasm of the cell. Based on our previous observation⁵², MSC labelling with 1µm MB-NPs have occurred chiefly through phagocytosis, thus resulting in direct delivery of MB-NPs to the cytoplasm region. In this way, pre-mature degradation of MBs from the acidic condition of the endo/lysosomal pathway can be avoided. This then translates to better cellular retention, by minimizing NPs dilution due to exocytosis¹⁰⁷.

MB-NP's functionality was verified on three cell types expressing different *Col2* expression level (undifferentiated MSCs, chondrogenic-induced MSCs and HUVECs). Normalised *Col2* MB signal demonstrated the functionality of the nanosensor, with chondrogenically-differentiated MSCs showing the strongest signal while HUVECs have negligible signal. Moreover, when normalized signal was compared with qRT-PCR results, it was exciting to observe a relatively good correlation throughout the differentiation period of 28 days ($R^2=0.9301$). From the in-solution hybridization study, we noted the presence of noise signal from released MBs at the final time point (Figure 3.3B). Slight non-specific MB activation (especially towards the later time point), have likely compromised this correlation value. Nevertheless, this correlation suggests that our proposed MB-NPs can indeed reflect MSCs *Col2* expression, in a non-invasive manner while they are undergoing 3D differentiation within the agarose hydrogel. In addition, our data (Figure 3.6 and 3.7) illustrated that multipotency of MSCs was not compromised with the labelling of MB-NPs. This is further supported by our previous study on MSCs labelled with PLGA nanosensors. When subjected to tri-lineage differentiation (chondrogenesis, adipogenesis and osteogenesis), no visible compromise was observed through their respective histochemical staining. Insignificant alteration of osteogenic and chondrogenic gene markers of

labelled and unlabelled samples further corroborates cyto-compatibility of PLGA nanosensors¹⁰⁵.

Due to its release profile which occurs over a 28-day period, the accuracy of the nanosensor expression would eventually drop beyond this point, as there would not be further supply of functional MBs to saturate the cytoplasm. Nevertheless, it is noteworthy that our 28 days monitoring period is significantly longer compared to previous studies that utilize MBs for non-invasive biomarkers tracking in 3D conditions. Previously, Illieva M. and Dufva M. transfected Sox2 and Oct4 MBs to study neural stem cells differentiation as 3D neurospheres¹¹⁹. However, their observation period was quite limited to about a week due to dilution of intracellular MB concentration. With regards to this, multiple times of MB loadings may be required to maintain sufficient amount of MBs, as implemented previously by Desai et al for their osteogenic differentiation tracking¹⁰⁸. In the future, beside prolonging MB release period, MB-NP specificity can be further improved by employing FRET MBs which work in tandem to reveal target mRNA expression¹⁰⁶. In addition, backbone-modified MBs can also be utilized to further reduce false signal generation resulting from MB degradation especially in longer periods of culturing¹²⁰.

We envision that this nanosensor platform holds potential as a screening platform to optimize induction conditions for cartilage tissue engineering. Signal expression profile of cells or cell aggregates can be utilised as a functional readout to identify the best combination of growth factors in a longitudinal and high throughput manner. Additionally, given the versatility of our MB-NPs, multiple gene markers at different chondrogenic stages can be simultaneously tracked (e.g. *Col10* and *Col2*), to identify factors that best replicate growth plate generation *in vitro*.

3.5 Conclusion

This paper presents the use of a nanosensor platform based on co-encapsulation of MBs in biodegradable PLGA NPs for real-time monitoring of MSC chondrogenic differentiation in 3D agarose hydrogels. Unlike the typical differentiation assessment techniques which involve disruption of samples at multiple time points, the sustained release of Col-MBs and *GAPDH*-MBs from MB-NPs allows facile, non-invasive yet accurate longitudinal assessment of intracellular *Col2* mRNA expression over a period of 28 days. Throughout this period, increasing normalized *Col2* signal reflected successful chondrogenic differentiation on induced MSCs, relative to undifferentiated ones. Crucially, the signal profile was well correlated against readings from standard qRT-PCR analysis. Moreover, nanosensor labeling is biocompatible, without compromising MSCs differentiation capability. With a better understanding of the temporal chondrogenic gene expression trend using the current platform, one can screen and identify the best combination of growth factors for *in vitro* generation of growth plates.

Chapter 4: The effect of temporal manipulation of TGF β 3 and FGF2 on the derivation of proliferative chondrocytes from Mensenchymal Stem Cells – a study monitored by qRT-PCR and Molecular Beacon Based Nanosensors

(Accepted)

Abstract

Proliferative chondrocytes are critical to realize regeneration of damaged epiphyseal growth plate. However, acquiring autologous replacement cells involves highly invasive procedures and often ends up with limited cell quantity. Mesenchymal stem cells (MSCs) are a potential source of chondrogenic cells for the treatment of cartilage disorders and injuries. This article aims at optimizing the differentiation conditions of MSCs into proliferative chondrocytes, by focusing on the influence of growth factors (i.e. fibroblast growth factor 2 (FGF2) and transforming growth factor beta 3 (TGFβ3)) and the treatment duration. The differentiation process in 3D hydrogel is non-invasively monitored by using a nanosensor containing two molecular beacons (MBs) that target two critical biomarkers for proliferative chondrocytes (i.e. collagen type-II mRNA and Ki67 mRNA). The observation from the nanosensors matched quantitative real-time polymerase chain reaction (qRT-PCR) results well and further confirmed through immunostaining. Thus, the nanosensors provide a non-invasive mean to screen and monitor culture samples.

Introduction

Epiphyseal growth plate is a complex yet vulnerable cartilage tissue that is responsible for the growth of long bones in children¹²¹. It comprises of mainly three distinct zones: resting, proliferative and hypertrophic zones¹²². Chondrocytes in the proliferative zone are actively proliferating and play a critical role in providing a sustainable chondrocyte population for spatial growth along the long axis of the bone¹²². Typically, these proliferative chondrocytes will exit the proliferation cycle and mature into hypertrophic chondrocytes and subsequently progress into ossification^{121, 122}. Any injury or disorder affecting the function of chondrocytes in any zones would result growth disruption such as angular deformity or even growth arrest^{121, 123}. Due to its avascular nature, physeal cartilage has limited access to progenitor cells for regeneration. Yet, current clinical treatments, such as excision of the bony bridge and followed by insertion of interposition materials, only manage the complications related to growth plate injuries, instead of the actual restoration of growth plate function^{123, 124}.

Mesenchymal stem cells (MSCs) are a potential cell source for cartilage regeneration due to its extensive proliferation and multi-lineages differentiation capability¹²⁴⁻¹²⁶. Chondrogenic differentiation of MSCs can be achieved under the influence of culture conditions, for instance defined set

of growth factors^{126, 127}. The challenge remains as to understand the factors contributing to the MSC differentiation process towards the different zonal chondrocytes. Researchers had attempted to study the effects of growth factors on chondrogenesis using *in vitro* model by varying different type of growth factors, concentration and duration^{123, 126}. The common growth factors used in chondrogenesis studies include transforming growth factor- β (TGF- β) and fibroblast growth factor (FGF)¹²⁶.

Typically, quantitative reverse transcription polymerase chain reaction (qRT-PCR), biochemical and immunohistochemical analysis of cartilaginous matrix formation are used to identify the presence of critical biomarkers in the process. However, these are end-point assays and highly labor-intensive for large number of samples^{102, 128}. In this regard, non-invasive gene expression monitoring as enabled by molecular beacon based nanosensors (MB-NPs) provides a promising alternative to streamline and enhance efficiency of the screening process. Previously, we formulated molecular beacon (MB)-based nanosensors (MB-NPs) that allows longitudinal monitoring of intracellular mRNA expression for 28 days¹²⁹. Following successful internalisation of these nanosensors by cells, encapsulated MBs can be gradually released into the cytoplasm upon hydrolysis of poly-lactic-co-glycolic acid (PLGA) nanoparticles (NPs). The presence of its target mRNA, when hybridises to the specific MB, allows the

restoration of MB's fluorescent dye from its pre-quenched state⁵². Thus far, these nanosensors had enabled us to monitor both MSC osteogenic differentiation in two-dimensional (2D) and chondrogenic differentiation in three-dimensional (3D) culture conditions^{130, 131}.

In this work, we investigate the optimal MSC culture condition to generate proliferative chondrocytes by focusing on the temporal manipulation of growth factors. Specifically, combinations of TGF β 3, widely-used MSC chondrogenic inducing factor, and FGF2, proliferation promoting factor, were examined under various treatment periods^{132, 133}. In particular, culture conditions whereby TGF β 3 was introduced except for when FGF2 was added was compared with samples treated throughout with only TGF β 3 (which typically results in hypertrophic chondrocytes instead). To assess the differentiation of MSC into proliferative chondrocytes, chondrogenic marker, collagen type II (Col2), and proliferative marker, Ki67, were analysed through conventional validation methods¹³⁴. Meanwhile, tracking with dual MB nanosensors was introduced as an efficient alternative to facilitate *in vitro* screening of MSC chondrogenesis in 3D construct. Dual MB nanosensors employed in the study comprised of Col2-specific and Ki67-specific MBs, which were tagged with FITC and Cy3 fluorophores respectively. MSCs were incubated with these dual MB nanosensors before embedded in 3D agarose hydrogels for chondrogenic induction. The

fluorescence signals emitted from hybridised MBs were routinely imaged and quantified for analysis. Figure 4.1 presents the experimental schematic for screening of culture conditions to obtain MSC-derived proliferative chondrocytes through qRT-PCR and nanosensors.

Figure 4.1: Schematic of the screening process to achieve MSC-derived proliferative chondrocytes in three-dimensional (3D) agarose. Validation assays involved the conventional quantitative reverse transcription polymerase chain reaction (qRT-PCR) and molecular beacon nanosensors (MB-NPs) tracking

4.1. Experimental: Materials and Methods

All chemicals and reagents were acquired from Sigma-Aldrich except specifically mentioned. High glucose (4.5 g/L) Dulbecco's modified eagle medium (DMEM) and L-glutamine was purchased from Lonza. SYBR Green PCR Master Mix kit, TRIzol® reagent, fetal bovine serum (FBS), Glutamax™, sodium pyruvate, 0.25% Trypsin-EDTA, proline, insulin and penicillin-streptomycin (10,000 U/mL) were purchased from Life technologies (USA), while DNase I were obtained from Promega. SensiFast™ cDNA synthesis kit was from Bioline (UK) and RNeasy Mini Kit was from Qiagen. Transforming growth factor β 3 (TGF β 3) was purchased from R&D System (Minneapolis, MN), while fibroblast growth factor 2 (FGF2) was bought from ProSpec. ITS premix universal culture supplement was from Corning®. UltraVision™ Quanto Detection System HRP DAB kit was bought from Thermo Fisher Scientific (USA). Mouse monoclonal anti-Col2 antibody was obtained from Merck Millipore. Mouse monoclonal anti-Ki67 antibody was purchased from Dako.

Synthesis and characterization of MB-NPs: Col2 MB sequences (targeted towards Col2 mRNA) was designed according to our previous study¹⁰⁸. Ki67 MB was designed based on NCBI Reference Sequence:

NM_002417.3, in which it targeted targeting base number 225 to 244 of Ki67 mRNA. Both MB sequences were validated through basic local alignment search tools (BLAST) and were shown in Table 1. Nanosensors containing both MBs were then fabricated through double emulsion technique, in accordance to protocol published previously¹³⁰. Briefly, 200 μ l nuclease-free water containing 1 nmol GAPDH MB and 1 nmol Col2 MB was added to a mixture of 20 mg PLGA (50:50) in 1 ml chloroform with 1% span 80 (v/v) and homogenized at 30k rpm for 30 seconds (Scilogex D160). Double emulsion was formed by adding the above emulsion into 10ml of 3% polyvinyl alcohol (PVA) solution for another round of homogenization (30k rpm, 30 seconds). The chloroform in the mixture was evaporated under magnetic stirring (500rpm). Then, nanosensors were rinsed thrice with nuclease-free water prior to freeze-drying process. Finally, nanosensors were kept at -80°C until usage.

Table 4.1. Sequences of MBs and target oligonucleotide sequences used

MBs	Sequences (5'-3')
Ki67 MB	[Cy3]- <u>CAACG</u> -CGTCGACCCCGCTCCTTTTG- <u>CGTTG</u> -[BHQ2]
Col2 MB	[FITC]- <u>CCCGAT</u> -CCTGCAGCACCTGTCTCACC- <u>ATCGGG</u> -[BHQ1]

Ki67 target	CAAAAGGAGCGGGGTCGACG
Col2 target	GGTGAGACAGGTGCTGCAGG

MBs hybridization study: To examine MB probes' specificity, Ki67, Col2, or both complementary target sequences (Table 4.1) were mixed with 0.5 μ M MB solution. Samples were then allowed to hybridize for 15 min at 37°C. Fluorescence intensity was subsequently measured with H4 synergy microplate reader for both FITC (485/517 nm) and Cy3 (550/570 nm). Intensity of all the groups were normalized against that of DNase1-treated MBs (40 U/ml) and untreated samples (negative control).

Cell Culture: Human bone marrow MSCs were harvested from consented patients from the National University Hospital (Singapore) with reference to the approved protocol of the Institution Review Board (IRB)¹³⁵. MSCs were expanded in culture medium consisting of 10% FBS, L-glutamine supplemented high glucose DMEM and 1% penicillin-streptomycin (P/S) after isolation. MSCs were cultured at 37°C, 5% CO₂ condition. Medium was changed every 2-3 days.

Cell labelling with MB-NPs: To enhance cellular internalization, 4 mg nanosensors were coated with 50 μ l of poly-L-lysine (PLL) solution (Mw 70,000-150,000, 0.01%w/v) for 20 min and thereafter, excess PLL solution was removed through centrifugation. MSCs were incubated in 1 mg/ml PLL-coated nanosensors in culture medium overnight before rinsing with PBS thrice. With cell growth at approximately 80% confluency, PLL-coated nanosensors were added to the MSCs overnight before rinsing with PBS thrice. The cells were subsequently treated with trypsin for further experiments.

Cell-agarose hydrogel 3D construct

MSCs were mixed with agarose solution (Agarose Type VII, Sigma Aldrich) to attain 20 μ l of cell-agarose hydrogel. The final cell density of the cell-agarose hydrogel is 5×10^6 cells/ml with 0.75% (w/v) agarose concentration. These MSC-agarose droplets were dropped onto the base of flat-bottom 96-well plate using pipette. Culture medium was added after the droplets were solidified and incubated at 37°C, 5% CO₂ condition.

Chondrogenic differentiation in 3D agarose hydrogel: Medium was replaced with chondrogenic medium the following day after the formation of cell-agarose construct. The formulation of chondrogenic medium includes high glucose DMEM, 100 Units/ml P/S, Glutamax™, 1 mM sodium pyruvate, 0.1µM dexamethasone, 4mM proline, 50 µg/ml ascorbic acid and ITS (BioLab). There were three tested groups (T, TF7 and TF14). 10 ng/ml TGFβ3 was supplemented to the chondrogenic medium throughout the study for experiment group “T”. For the group of TF7, TGFβ3 was also added from day 0 to 6 and day 14 to 28 while 10 ng/ml FGF2 was added between day 7 and 13. For the group of TF14, TGF-β3 was added from day 0 to 13 and day 21 to 28 while 10 ng/ml FGF2 was added between day 14 and 20. All constructs were cultured for 28 days under 37°C. Chondrogenic medium was changed every 2-3 days.

Adipogenic differentiation of MSCs in 3D agarose hydrogel: Adipogenic medium comprised of high glucose DMEM with 10% FBS, 100 Units/ml P/S, 1 mM sodium pyruvate, 0.1 µM dexamethasone, 10 µg/ml insulin, 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) and 200 µM indomethacin. There were four tested groups (A, T, T7DA7 and T14DA14). For group A, the adipogenic medium was introduced to the cell-agarose 3D construct for the

whole experimental period (i.e 28 days). For group T, the chondrogenic medium supplemented with 10 ng/ml TGF- β 3 was used throughout the study. For T7DA7, the chondrogenic medium supplemented with 10 ng/ml TGF- β 3 was used for 7 days before the adipogenic medium was added. For T14DA14, the chondrogenic medium supplemented with 10 ng/ml TGF- β 3 was used for 14 days before the adipogenic medium was added till day 28. Medium was replaced every 2-3 days.

Confocal imaging: Fluorescence signals emitted by Ki67 MB (Cy3: 550/570 nm) and Col2 MB (FITC: 485/517 nm) from labelled cell-agarose constructs were imaged using confocal laser scanning microscopy (LSM710, Carl Zeiss). Images were processed using LSM Image Browser (Zeiss) and signals were quantified with ImageJ software.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis: 3D cell-agarose constructs were homogenized with TRIzol® Reagent for 5 min before the addition of chloroform. The mixture was incubated for 3 min, followed by centrifugation at 12,000 g for 15 min at 4°C. RNA at the aqueous phase was extracted. Isolation and purification of RNA was done using the RNeasy Mini Kit. Total RNA was measured with

NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). Subsequently, reverse transcription was executed using SensiFast™ cDNA Synthesis Kit. Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) was performed on StepOnePlus Real-Time PCR System (Life Technologies) using SYBR Green PCR Master Mix kit with the condition as follows: 10 min of denaturation at 95°C, followed by 40 cycles of amplification with denaturation at 95°C for 15 seconds and extension at 60°C for 1 minute per cycle. The primer sequences utilized are listed in Table 4.2. Data obtained was quantified with respect to endogenous glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, applying the comparative C_T method formula $2^{-\Delta\Delta C_T}$.

Table 4.2. Primer sequences used in qRT-PCR

Gene	Forward primer sequence (5' – 3')	Reverse primer sequence (5' – 3')
GAPDH	ACAACTTTGGTATCGTGG AAGG	GCCATCACGCCACAGTTT C
Col2	CCAGATGACCTTCCTACG CC	TTCAGGGCAGTGTACGTG AAC
Ki67	CTTTGGGTGCGACTTGAC G	GTCGACCCCGCTCCTTTT
PPAR γ	TACTGTCGGTTTCAGAAA TGCC	GTCAGCGGACTCTGGATT CAG

Immunohistological and histological analysis: Samples are fixed with 10% formalin overnight followed by washing with PBS. Fixed samples were embedded in FSC 22 Mounting Media (Leica) and froze at -20°C for overnight. Samples were cryosectioned to 7 μ m slices with Leica CM1900 cryostat (Leica Microsystem) which were then collected with Mezel-Glaser Superfrost Plus slides (Thermo Fisher Scientific).

To assess Col2 deposition, immunohistochemical staining was performed using UltraVision™ Quanto Detection System HRP DAB kit, together with

mouse monoclonal anti-Col2 antibody. For Ki67 protein staining, immunostaining was executed. Section slides for Ki67 staining sectioned tissues were pre-treated with 0.1% Triton-X-100. Slides were, then incubated with mouse monoclonal anti-Ki67 antibody for 2 hours, and thereafter goat anti-mouse IgG (H+L) Antibody antibody (Rhodamine conjugate) for another 2 hours after PBS washing. Slides were washed with PBS 3 times before counter staining stained with DAPI.

Statistical analysis: One-way analysis of variance (ANOVA) and Tukey HSD test were carried out to evaluate the statistical difference among experimental groups with $n \geq 3$. Experimental groups were considered significant different when p-value < 0.05 (*) and < 0.01 (**).

4.2. Results

4.3.1. Optimal time point for FGF2 addition for proliferative chondrogenesis

MSC has been used for chondrogenesis studies with a typical differentiation period of about 3-4 weeks prior to end-point assessment. Chondrocytes derived from MSC have a tendency to progress to the hypertrophic stage^{121, 125}. In contrast, proliferative chondrocytes are of early-stage differentiation. Therefore, it is critical to optimize induction culture conditions. For this paper, FGF2 was used to induce the proliferative capacity in chondrocytes (i.e. MSCs undergoing differentiation by TGF β 3-supplemented chondrogenic medium was treated with FGF2).

In particular, we investigated the time point to add FGF2 to obtain MSC-derived proliferative chondrocytes. Figure 4.2A illustrated the timeline for the addition of different medium. At day 7 (TF7) and day 14 (TF14), FGF2 was added to chondrogenic medium for 7 days before reverting back to TGF β 3-supplemented chondrogenic medium. Then, Col2 mRNA expression was studied to assess the chondrogenic commitment of MSCs, while evaluation of Ki67 mRNA expression provides us with proliferative capability of the samples. TF14 exhibited significant Col2 and Ki67

expressions, as shown in Figures 4.2B and 4.2C. Though Col2 fold expression here was lower than T (1.33-fold lower), it was significantly higher than TF7 (2.62-fold higher). Importantly, Ki67 expression of TF14 samples was upregulated unlike in both T and TF7 groups (1.76-fold as compared to 0.85 and 0.9-fold, respectively).

Figure 4.2. Screening of conditions for proliferative chondrocytes with TGFb3 and FGF2. (A) Illustration of experiment conditions. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) validation for (B) Col2 and (C) Ki67 markers. Samples were normalized with GAPDH expression using the comparative CT formula $2^{-\Delta\Delta CT}$. (ns: non-significant, *p<0.05, **p<0.01).

Moreover, such qRT-PCR results were substantiated with Col2 immunohistochemical staining (Figure 4.3A) and Ki67 immunostaining (Figure 4.3B). Stained samples were then quantified as shown in Figure 4.3C for Col2 proteins and Figure 4.3D for Ki67 protein. Notably, TF14 samples exhibited comparable Col2 protein level to that treated with only TGF β 3 (T), but with significantly greater level of Ki67 protein. Meanwhile, TF7 samples did not show upregulated Ki67 protein, and instead exhibited weaker Col2 protein. These results suggest that TF14 culture condition (with FGF2 applied on day 14 to day 21) is more suitable for derivation of proliferative chondrocytes, rather than T or TF7.

Figure 4.3. (A) Immunohistochemical staining for Col2 protein. (B) Immunostaining of Ki67 (Rhodamine) and DAPI. Quantitative staining intensity of (C) Col2 and (D) Ki67 proteins (normalized with DAPI staining).

4.3.2. Validation of successful early chondrocytes

With the upregulation of both markers (KI67 as well as Col2 mRNA) when MSCs were subjected to TF14 conditions, we next investigated the extent by which it has committed towards the chondrogenic lineage prior to FGF2 addition. To evaluate the duration of chondrogenic induction needed for MSCs to undergo early chondrogenesis, MSCs were treated with TGF β 3-supplemented medium for a period of 7 days (T7A) or 14 days (T14A) before replacing with adipogenic medium as depicted in Figure 4.4A. MSCs treated with just chondrogenic (T) or adipogenic (A) medium were used as controls. The presence of chondrocyte marker, Col2, was examined through qRT-PCR (Figures 4.4B). TD14 showed lower Col2 fold expression when compared to the positive control group, T. Nevertheless, it is still significantly higher than in T7A. Interestingly, T7A actually demonstrated a similar Col2 expression to that of A (statistically insignificant difference). On the other hand, cells of T14A and T expressed significantly lower PPAR γ

gene while T7A cells expressed comparable gene level as A (Figures 4.4C). Insignificant influence of 21 days adipogenic induction in T14A samples indicates that 14 days of TGFβ3 treatment was sufficient to direct MSC commitment towards chondrogenic lineage.

Figure 4.4. Optimisation of TGFβ3 treatment duration. (A) Illustration of experiment conditions. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) validations of adipogenic (A) samples (B) Col2 marker and (C) adipogenic marker, PPARγ. Samples were normalized with GAPDH expression using the comparative CT formula $2^{-\Delta\Delta CT}$. (ns: non-significant, * $p < 0.05$, ** $p < 0.01$)

4.3.3. Screening for culture conditions with nanosensors

While RT-qPCR and immunohistochemical staining have enabled screening process of growth factor combinations to achieve proliferative chondrocytes, these processes are disruptive and thus require extensive sample preparations to account for each time point and treatment conditions. In this regards, non-invasive gene expression monitoring as enabled by molecular beacon nanosensor (MB-NP) provides a promising alternative to streamline and enhance efficiency of the screening process. To facilitate monitoring of MSCs differentiation to proliferative chondrocytes, MBs targeting chondrogenic marker (Col2 mRNA) and proliferation marker (Ki67 mRNA) were incorporated into the nanosensors. Hybridisation study was first done to determine the specificity of the MBs to their respective targets. As shown in Figure 4.5, the presence of correct target led to at least 6-fold increase in MB fluorescence relative to untreated solution. Notably, fluorescence recovery following target addition is comparable to when MB solution was digested with DNase I (Supplementary). In short, we expect minimal MB fluorescence recovery from non-target mRNA hybridisation.

Figure 4.5. Hybridization study of MBs used in MB-NPs with its oligonucleotide target sequences and DNase I as control group.

Figure 4.6A shows the longitudinal monitoring of both Col2 and Ki67 markers for T, TF7 and TF14 group through molecular beacon nanosensors that were pre-labelled to the MSCs. At day 0 (prior to induction treatment), MSCs exhibited high Cy3 fluorescence signal representing strong Ki67 expression, with minimum FITC (Col2) signal. Detailed observation was then performed to compare dynamic nanosensor signal change, on MSCs as they underwent T, TF7 and TF14 treatment. At each time point, Col2 and Ki67 MB fluorescence signal were quantified and presented on Figure 4.6B. Along the chondrogenic induction period, there was an increase in

Col2-FITC fluorescence signal for all the groups. On the other hand, Ki67 signal generally declined from that of day 0. Interestingly, we observed that Ki67 expression did not further decreased in TF14 group from day 14, suggesting that FGF2 addition here was advantageous to prevent formation of hypertrophic chondrocytes.

Figure 4.6. The use of molecular beacon nanosensors (MB-NPs) for screening of chondrogenic conditions. (A) Confocal images of chondrogenic differentiated MSC in 3D agarose under different conditions (T, TF7, TF14) at day 0 (D0), day 14 (D14), and day 28 (D28). Scale bar: 100µm. Quantitative fluorescence intensity profile of (B) Ki67 MB and (C) Col2 MB signals.

In addition to stronger Ki67 expression signal, TF14 samples demonstrated considerable amount of Col2 MB fluorescence at day 28 (Figure 4.7A). Crucially, there were more cells expressing positive FITC and Cy3 signals, indicative of cells which possessed both proliferative and chondrogenic phenotype. From the quantitative fluorescence intensity data at day 28 shown in Figure 4.7B, TF14 showed the highest Ki67 MB fluorescence signal compared to T and TF7 (2.31 and 1.55-fold, respectively). In addition, TF14 expressed significantly greater Col2 signal compared to TF7 (2.03-fold), though lower than T (0.72-fold) (Figure 4.7C). Figures 4.7B and 4.7C also compared the fluorescence intensity of MB nanosensors with the fold difference of target genes evaluated through qRT-PCR analysis. Data from qRT-PCR for both Ki67 and Col2 genes coincides with the trend of MBs fluorescence signal. With high expression of Ki67 and considerable amount of Col2 mRNA, the addition of FGF2 from day 14-21 (TF14) was shown to be the optimal condition to obtain MSC-derived proliferative chondrocytes.

Figure 4.7. MSC chondrogenic differentiation at Day 28 under different conditions. (A) Confocal images of Ki67 MB, Col2 MB and its composite signals of MSC chondrogenic differentiated samples under different conditions. Scale bar: 100 μ m. Combined plots of MB signal and qRT-PCR data for (B) Ki67 and (C) Col2 expressions. (ns: non-significant. ** $p < 0.01$).

4.3. Discussion

Repair of growth plate has remained a challenge due to its complex architecture. The existence of different chondrocytes at distinct zones provides a need to study different culture conditions to achieve regenerative therapy for injured growth plate. Most of the reported work are mainly on the pre-treatment of MSCs during expansion for better chondrogenic potential, the induction and inhibition of hypertrophy reactions. Little was done on attaining zonal growth plate chondrocytes from MSCs, especially proliferative chondrocytes. In addition, many cellular developments, such as proliferation and differentiation, are closely related with growth factors^{123, 136-138}. Therefore, in this study, we focus on formulating optimal culture cocktail consisting of growth factors to achieve MSC-derived proliferative chondrocytes.

TGF β 3 was used in this paper as a positive inducer for MSC chondrogenesis due to its well-established *in vitro* and *in vivo* evidences¹³⁹. On the other hand, FGF2 was reported to play a part in cell proliferation and differentiation. Herein, we studied the time-dependent effects of FGF2 on cell proliferation in early-stage chondrocytes. In particular, the time frame for FGF2 addition was investigated. The hypothesis here is that FGF2 addition at optimal time point, alongside TGF β 3 chondrogenic

treatment, could lead to proliferative chondrocytes with an upregulated col2 and proliferative marker Ki67. Our qRT-PCR data exhibited a high expression of both Col2 and Ki67 only when FGF2 was supplemented from day 14 to day 21(TF14 group), compared to when it was added between day 7 to day 14 (TF7). This was further supported with our immunohistochemical staining of Col2 and immunostaining of Ki67.

Studies have demonstrated that FGF2 treatment during MSC expansion enhanced the kinetics of MSC chondrogenesis which eventually led to early differentiation¹⁴⁰. It also maintained high proliferation capability of MSCs with chondrogenesis potential of at least 46 population doublings^{141, 142}. In addition, Takahashi et al. showed enhanced proliferation in chondrocytes with the synergistic effect of FGF2 and insulin or insulin-like growth factor (IGF)¹³³. Nevertheless, there were also studies suggesting that FGF2 is a potent inhibitor of chondrogenesis and diminished the chondrogenic effect brought by other inducers^{143, 144}. The inhibitory effect from FGF2 was suggested to occur during the initial phase of MSC chondrogenesis¹⁴⁵. Correa et al. demonstrated that FGF9 and FGF18 addition at the beginning of TGF β 3-mediated chondrogenesis, led to inhibited differentiation. However, when they were added at the later phase (≥ 14 days into the induction), anabolic effect took place with it enhanced extracellular matrix deposition, thus promoted cartilage repair¹⁴⁵. Therefore, addition of growth

factors at distinct time point within the chondrogenesis process may lead to considerable expression changes. We speculated that 14-day of TGF β 3 treatment is sufficient to induce chondrogenic commitment of MSCs. Following which, the subsequent addition of FGF2 only enhanced the proliferation of MSC-derived chondrocytes, while maintaining its chondrogenic phenotype.

We then looked further to confirm proliferative marker (Ki67) expression by MSC-derived chondrocytes. The optimal duration of chondrogenesis for MSC to commit towards early-stage chondrocytes was investigated. We were able to demonstrate that 14 days of TGF β 3 treatment (T14A) were optimal for MSC chondrogenic commitment with significant amount of Col2 expression as compared to just 7 days of TGF β 3 treatment (T7A). Moreover, when TGF β 3-treated samples were subsequently induced towards adipogenic lineage, T7A samples exhibited low Col2 expression, with high adipogenic markers (PPAR γ and Leptin) expression, similar to that of non-TGF β 3 treated samples (A). A possible explanation for this is that 7 days of TGF β 3 chondrogenic induction was not sufficient for MSCs to commit to chondrogenic lineages. There is also a possibility that cells underwent de-differentiation following this short period of chondrogenic induction. On the other hand, T14A samples weakly expressed PPAR γ . Though Col2 fold expression in T14A samples was lower than that in

control group T, substantial amount of Col2 were expressed. This indicated that during the 14 days of chondrogenic differentiation in the presence of TGF β 3, most MSCs were already committed to chondrogenic lineage. Thus, subsequent induction to adipogenic lineage was not successful.

There were studies demonstrating high fold-increase in chondrogenic markers when MSCs were subjected to 14 days of TGF β 3 treatment¹⁴⁶⁻¹⁴⁸. Specifically, Mehlhorn et al. investigated the effect of MSC chondrogenesis with different TGF β 3 treatment duration. MSCs were encapsulated in alginate beads and pre-treated with TGF β 3 before culturing in osteogenic differentiation medium. Higher Col2 α 1 gene were exhibited when encapsulated MSCs (3D) were pre-treated with TGF β 3 for 14 days (14d TGF) as compared to 3 or 6 days, prior to 9 days culture with osteogenic medium. 14d TGF was also showed to have lower alkaline phosphatase (ALP) activity¹⁴⁸. Therefore, 14 days of TGF β 3 treatment on encapsulated MSCs was sufficient for full commitment of MSCs towards the chondrogenic lineage, without trans-differentiation occurring significantly.

As previously suggested and shown herewith, screening of culture conditions can be tedious and time-consuming. Specifically, the use of end-point assessments (e.g. qRT-PCR and immuno/immunohistochemical staining) involves disruption of samples and lack of real-time monitoring

aspect of the differentiation process^{100, 149, 150}. For a more efficient and effective screening procedures, molecular beacon nanosensors were utilised. Not only do MB-NPs enable real-time detection of intracellular mRNA, samples to be monitored can be longitudinally studied for a period of 28 days without disruption^{52, 129-131}. Confocal fluorescence images taken at weekly timepoints were quantified to understand the trend of both Col2 and Ki67 signals. Importantly, trend similarity with Col2 and Ki67 mRNA expression from qRT-PCR results confirmed the accuracy of MB-NPs in detecting the intracellular mRNA. Thus, the results proved that MB-NPs is a valuable, highly efficient *in vitro* screening tool.

In the future, we envision additional utilization of such nanosensor platform to perform cell sorting on patients' isolated MSCs. With its heterogeneous phenotype, identification of few subpopulation that has more chondrogenic potential before/during differentiation induction or just prior to re-implantation can significantly promote therapeutic efficacy. In such a manner, personalized cell therapy can be realized more efficiently.

4.4. Conclusion

This paper investigated the optimal time period for the addition of FGF2 to obtain proliferative chondrocytes. FGF2 was introduced at day 7-14 (TF7) and day 14-21 (TF14), while MSCs were treated with chondrogenic medium supplemented with TGF β 3 for the rest of the days till day 28. TF14 samples exhibited high Ki67 and relatively high Col2 expression compared to TF7. 7 days of TGF β 3 treatment was insufficient for MSCs fully chondrogenic commitment. After 7 days of treatment, cells were still able to differentiate or transdifferentiate into adipocytes. It was observed that 14 days of TGF β 3 treatment allowed MSCs to have substantial amount of MSCs to be committed to chondrogenic lineage. MB-NPs was introduced as a non-invasive screening and monitoring platform. Both Ki67 and Col2 were monitored by the hybridisation of intracellular mRNA to MB released from PLGA NPs, without disrupting the samples. Fluorescence signals observed were quantified and the trends matched with the qRT-PCR results. With this promising platform, more combination of growth factors cocktails could be screened, thus one step closer to achieve regenerative therapy for growth plate injury.

Chapter 5: Controlled-released delivery system of cargo-loaded magnetic microbubbles (MMBs) in three-dimensional (3D) hydrogel under ultrasound stimulation

(In progress for submission)

5.1. Introduction

Microbubbles were first discovered as good contrast agents for echocardiography in 1968⁵³. Due to the difference between the acoustic impedance of its gas core and surrounding medium, microbubbles were known to enhance ultrasound images^{53, 60}. Under ultrasound excitation, microbubbles are highly responsive and exhibit dynamic behaviours such as microstreaming, shear forces, inertial cavitation and jetting^{151, 152}. These behaviours of microbubbles drew attention to explore microbubbles as potential delivery carrier¹⁵¹. Many experiments were done with the use of microbubbles to deliver different cargos, such as functional nanoparticles, gene and drugs, for a wide variety of applications (examples: cellular engineering, imaging and even cancer therapies)^{153, 154}.

Microbubbles have continuously evolved to improve on targeting and controlled-release of cargos. Previously, after intravenous injection of microbubbles into systemic circulation, cargos on the microbubbles were released by localised high energy ultrasound beam at specific sites. This high acoustic energy then triggered the inertial cavitation of microbubbles and drove these cargos into sites across the vessel wall^{61, 154}. However, the

high energy shockwave and microstreaming from inertial cavitation may, at the same time, damage the surrounding tissues. It may also cause toxicity to cells due to the sudden burst release of cargos^{61, 155}.

The incorporation of nanoparticles to microbubbles allows manipulation of microbubbles with multiple functions¹⁵⁵. It was reported in the previous study that integrating magnetic nanoparticles (MNPs) to microbubbles (i.e. magnetic microbubbles, MMBs) helped to stabilise the microbubbles and prevent it from dissolution. The magnetic property of MNPs offered MMBs with targeting capability. Through magnetic manipulation, these MMBs were able to concentrate at target site before trigger the release of cargos. Instead of inertial cavitation, cargos were controlled and continuously released from the MMBs when stimulated at resonant frequency. The size of MMBs decreased upon the detachment of cargos and ceased to exit upon complete detachment of all cargos⁶¹.

This project further explored the possibility of MMBs toward regenerative therapy. We were able to demonstrate oscillation of MMBs when it was encapsulated in three-dimensional (3D) agarose hydrogel. At the same time, we were also to observe the release of cargos from the encapsulated MMBs. Figure 5.1 shows the schematic diagram of the oscillation and cargo release of MMBs in 3D MSC-agarose construct. To demonstrate the

capability of MMBs delivery system in 3D MSC-agarose construct, we integrated molecular beacon-based nanoparticles (MB-NPs). The molecular beacon used target housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which is present in MSCs. The release of cargos can be triggered multiple times till the detachment of all cargos. The release can also be ultrasound-activated at certain time-points to suit the application. This delivery system in 3D microenvironment holds prospective for regenerative therapy, especially when sustainable and time-points release were needed.

Figure 5.5. Schematic diagram of magnetic microbubbles (MMBs) releasing cargos while encapsulated in three-dimensional (3D) cell-agarose constructs.

5.2. Experimental: Materials and Methods

Chemicals and reagents were acquired from Sigma-Aldrich except specifically mentioned. High glucose (4.5 g/L) Dulbecco's modified eagle medium (DMEM) supplemented with L-glutamine was purchased from Lonza. Trypsin-EDTA (0.05%), fetal bovine serum (FBS), Glutamax™, penicillin-streptomycin (P/S) (10,000 U/mL) were purchased from Life technologies (USA)

Fabrication of Magnetic microbubbles (MMBs)

The synthesis of MMBs was according to previous paper⁶⁰. Magnetic nanoparticles (MNPs) comprised of iron oxide, Fe_3O_4 , of approximate 50-100 nm in size were added to deionized (DI) water to attain 2 mg/mL stock concentration. To allow a more homogeneous dispersion, the stock solution was subjected to ultrasonic for 30 min. For a 700 μL working mixture, 400 μL of MNP stock solution was mixed with 150 μL of 10 mM sodium dodecyl sulfate (SDS), as well as 150 μL of DI water. This working mixture was then subjected to mechanical mixing through either vortex or probe homogenizer for 30 sec. Different mechanical mixing and different homogenization speed will result in MMBs of various sizes. After the mechanical agitation, the

solution was left overnight for MMBs to stabilize before replacing it with DI water.

Loading of different cargos on MMBs

Silica NPs-loaded MMBs: Mesoporous silica NPs (SiO_2) of 200 nm size (Aldrich, Singapore) were pre-treated with fluorescence dyes, specifically rhodamine B isothiocyanate (RITC) and Cy5. 5mg/ml fluorescence dyes were added to 1mg of silica NPs and agitated at 4°C overnight. Excess fluorescence dyes were removed by centrifugation at 15,000 rpm for 5 min. Similar to the MMB without any cargos, 150 μL of fluorescence silica NPs were mixed with 400 μL of MNPs and 150 μL of SDS before vortex for 30 sec.

Polystyrene (PS) microsphere-loaded MMBs: Rhodamine B-labelled PS microsphere with particle size of 0.1 μm was added to the mixture of MNPs and SDS at a concentration of 100 ng/mL MMBs were formulated by 30 sec of vortex.

GPI (Glycosylphosphatidylinositol) anchored proteins-loaded MMBs: RedGlow Molecular Painting Reagent was GPI anchored proteins tagged with red fluorescence protein (tdTomato) (Anovasia, Singapore). 50 ng/mL of solution was used to formulate MMBs in the presence of MNPs and SDS by vortex the mixture for 30 sec.

Molecular beacon nanoparticles (MB-NPs)-loaded MMBs: GAPDH molecular beacon (MB) sequence ([Cy3]-CGACG-GAGTCCTTCCACGATACCA-CGTCTG-[BHQ2]) was adapted from previous studies¹⁵⁶. The synthesis of MB-NPs was as according to previous published protocol⁵⁰. MBs was emulsified in biodegradable poly(lactic-co-glycolic acid) (PLGA) NPs through double emulsion method before MB-NPs underwent lyophilisation. Before labelling, poly-L-lysine (PLL) solution (Mw 70,000-150,000, 0.01% w/v) was added to MB-NPs and vortex for 20 min at 4°C. Excess PLL was removed through centrifugation. PLL-treated MB-NPs were then re-dispersed to 0.1 mg/mL with nuclease-free water. Similarly, 150 µL of MB-NPs solution was added to 400 µL of MNPs and 150 µL of nuclease-free water before vortex for 30 sec. The mixture was then stored in 4°C fridge overnight. Solutions with unbound MB-NPs were

removed by using strong magnetic to attract the MB-NP-loaded MMBs to a side. Fresh nuclease-free water was added.

MMBs in 3D agarose construct

3% agarose hydrogel (HispanLab) was diluted into 1.5% and 0.75% concentration with MMBs. The mixture was added to the 8 wells cell culture chamber slide (SPL, Bio Laboratories Pte Ltd, Singapore) to form a thin gel.

3D construct with MSCs and MBNPs-loaded MMBs

Human bone marrow MSCs were acquired from Lonza and expanded in high glucose (4.5 g/L) DMEM supplemented with L-glutamine, 10% FBS and 1% P/S. Medium was changed every 2-3 days. At about 80% confluency, MSCs were treated with 0.25% trypsin-EDTA to obtain cell suspension. 120 μ L of 2×10^5 cells /mL MSCs in PBS solution was first added to MBNPs-loaded MMBs, before mixing with 40 μ L of 3% agarose hydrogel (HispanLab). A final concentration of 0.75% agarose construct was loaded to one of the 8 wells cell culture chamber slide (SPL, Bio Laboratories Pte Ltd, Singapore) to form a thin gel.

Oscillation of MMBs in microfluidic chip: Polydimethylsiloxane (PDMS) microfluidic channel chip with a dimension of 1 cm x 1 mm x 0.2 mm was first attached to a glass slide with a piezo transducer. MMBs of approximately 200 μm were then injected into the microchannel before trigger with ultrasonic frequency of 41 kHz, 500 mVpp and with amplification of 50.0%. Videos were acquired with SAX1 monochromatic high speed camera.

Oscillation of encapsulated MMBs in 3D construct on microwell cell culture chamber chip: A piezo transducer was attached 8 microwells cell culture chamber slide (SPL, Bio Laboratories Pte Ltd, Singapore). MMBs that were encapsulated in agarose hydrogel were loaded on these wells. Upon encapsulation, MMBs in agarose hydrogel were subjected to ultrasound frequency of 44 kHz, 500 mVpp with amplification of 50.0%. Videos were acquired with SAX1 monochromatic high speed camera. Fluorescent microscope was used to capture the post-released images of fluorescent polystyrene NPs and MB-NPs.

5.3. Results and Discussion

5.3.1. Synthesis of magnetic microbubbles (MMBs)

With reference to previous study⁷, MMBs were fabricated using magnetic NPs to stabilise the microbubbles. The size of MMBs can be controlled through the different mechanical preparation methods, the duration of emulsification and mixing speed. Figure 5.2 showed the variation of MMB sizes when subjected to different preparation parameters. When MMBs were fabricated using vortex (Scilogex) with a speed of around 2500 rpm for 30 sec and 2 min, it gave an average of 200 μm and 100 μm respectively. On the other hand, with the use of homogenizer probe, it produced smaller MMBs. Different homogenising speeds of 8000 rpm (speed 1) and 20000 rpm (speed 4) produced MMBs of different sizes with an average of 25 μm MMBs and 15 μm respectively. It was observed that the greater the homogenising speed or longer stirring duration, the smaller the MMBs produced. In addition, MMBs prepared by homogeniser probe were more homogenous in size as compared to that of vortex mixing. This is likely due to the different mixing mechanisms of the two techniques. The current limitation is to obtain homogenous size of MMBs and this can potentially be resolved by utilising microfluidics. Hashimoto et al. previously

reported the successful formation of uniform bubbles with microfluidic system¹⁵⁷.

Figure 5.2. Variation in size of magnetic microbubbles (MMBs) when subjected to different mechanical mixing, duration and speed of mixing. MMBs were fabricated using vortex for 30 sec, 2 min (top, left to right) and using homogenizer probe of speed 1 and 4 (bottom, left to right)

Different cargos loaded on MMBs were observed with the presence of fluorescence signals as shown in Figure 5.3. These MMBs were prepared

by vortex for 30 sec to 1 min. We were able to synthesis MMBs with 200 nm silica NPs loaded with two different fluorescent dyes (i.e. RITC and Cy5), protein (GPI-anchored protein) and 0.1 μ m polystyrene (PS) microsphere. The successful loading of different cargos with different sizes demonstrated the diverse potential of MMBs for different applications.

Figure 5.3. Different cargos loaded on MMBs. The cargos are (Starting from top left, clockwise) silica NPs loaded with RITC; Glycosylphosphatidylinositol (GPI)-anchored proteins labelled with

tdTomato; polystyrene microspheres labelled with rhodamine; silica NPs loaded with Cy5.

5.3.2. Oscillation of magnetic microbubbles (MMBs)

Oscillation of approximately 200 μm MMBs was observed when subjected to ultrasound stimulation in microchannel. Figure 5.4 showed the oscillation of MMBs when subjected to 41 kHz ultrasonic frequency but different amplitudes. A volume mode oscillation was observed when 500 mVpp was applied to the MMB. While the diameter or volume of the MMB changed during the oscillation, the MNPs were also released as shown from the particles around the MMB. With the increase of amplitude to 1 Vpp, the MMB oscillated with a dramatic change in shape.

Figure 5.4. Oscillation of MMBs under different amplitudes. When subjected to 500 mVpp, the MMB oscillated by volume mode with the change in diameter. The increase in amplitude to 1 Vpp resulted in shape mode oscillation.

5.3.3. Oscillation of MMBs encapsulated in 3D agarose hydrogel

While MMBs have shown its capability to oscillate and release cargos at resonant frequency, experiments were mainly done in 2D environment or when MMBs are in solution. Little was done on the oscillation of MMBs in 3D agarose hydrogel. The red lines in Figure 5.5 was of equal length and

thus, illustrated oscillation with a change in diameter of the MMBs for MMBs in 0.75% agarose. However, no change was observed when MMBs were encapsulated in 1.5% agarose. With the increase concentration of agarose, the high the viscosity, and thus, hindered the movement of MMBs.

Figure 5.5. Oscillation of MMBs in different concentration agarose hydrogels, specially 0.75% and 1.5%.

The release of cargos during MMB oscillation in 3D agarose were also studied and compared to samples that did not underwent ultrasound activation. The cargo used for this experiment was polystyrene microspheres (0.1 μm). Figure 5.6 displayed the release of polystyrene

microspheres that were pre-loaded on MMBs under 2 different conditions. Under no ultrasound stimulation, no release of polystyrene microspheres were observed. On the other hand, under ultrasound stimulation, clouds of fluorescence signals (labelled with white arrows) were observed only for MMBs encapsulated in 0.75% agarose and not for 1.5% agarose. This further verified our observation in Figure 5.5. Thus, 0.75% agarose concentration will be used in subsequent experiment to encapsulate MMBs.

Figure 5.6. Release of polystyrene microspheres from MMBs in 0.75% and 1.5% agarose, with and without ultrasound stimulation. White arrows

showed the dispersion of polystyrene microsphere in agarose after ultrasound stimulation.

5.3.4. Labelling MSCs with MB-NPs using MMBs in 3D agarose

MMBs exhibited its potential to oscillate and release cargos in 3D agarose in the above. This technology was further integrated with molecular beacon-based nanoparticles (MB-NPs) to illustrate the delivery of cargos into the cells. MB-NP-loaded MMBs of size of approximately 25 μm were used to encapsulate with MSCs in 0.75% agarose. The 3D constructs were then subjected to two conditions, with and without ultrasound stimulation of with a range from 125 to 150 kHz (500 mVpp, 50.0% amplification). Figure 5.7 exhibited the presence of Cy3 fluorescence signals in MSCs when stimulated with ultrasound. This demonstrated successful delivery of MB-NPs into MSCs as only with the presence of GAPDH mRNA, the GAPDH MBs will then regain its fluorescence. This also means that MMBs required to drive these MB-NPs to penetrate into MSC cytoplasm before GAPDH MBs can hybridise with the intracellular GAPDH mRNA in MSCs.

Figure 5.7. Delivery of molecular beacon-based nanosensors (MB-NPs) into MSCs with magnetic microbubbles (MMBs) in 0.75% agarose with and without the presence of ultrasound stimulation.

This unique phenomenon of cargos release when MMBs were encapsulated in 3D agarose construct can be further investigated for more potential applications. Not only the release of cargos can be triggered multiple times till the detachment of all cargos, it can also be ultrasound-activated at certain time-points to suit the application. This provides the delivery system with flexibility for cargo release. MMB delivery system in 3D microenvironment holds prospective for regenerative therapy, especially when sustainable and time-points release were needed.

5.4. Conclusion

The project utilised magnetic microbubbles (MMBs) to develop controlled-released delivery system in 3D microenvironment. Different preparation methods (i.e. different mechanical stirring machine, stirring speeds and duration) have exhibited to provide MMBs of different sizes. Different cargos ranging from NPs, microspheres and proteins have been loaded to MMBs for potential applications. While we exhibited oscillation of MMBs in microfluidic channel when stimulated at resonant frequency, oscillation was also presence when MMBs were encapsulated in 0.75% agarose hydrogel. The release of polystyrene NPs and successful MSC labelling with MB-NPs illustrated the delivery of cargos from MMBs in agarose encapsulated environment. This platform holds many potentials for regenerative therapy for us to explore.

Chapter 6: Conclusion and Future Work

6.1. Overview

Injuries and disorders in cartilage often lead to some degree of disability and deformity. The lack of regenerative capability of cartilage and limitations in current treatments have attracted the attention of researchers. MSCs have been looked upon as potential cell source for cartilage engineering. Nevertheless, effective tracking of transplanted cells and screening of MSC chondrogenic differentiation conditions are still lacking. In addition, to induce chondrogenic differentiation, growth factors are typically added to culture medium for interaction with MSCs to occur. However, there is a need for constant replenishment of growth factors into the medium due to its degradation. The process will be made even more tedious if it involves a wide range of growth factors to be added at different time points. Therefore, this thesis aims to develop nanosensors to track and screen MSC chondrogenic conditions for cartilage regeneration. The thesis also explores potential controlled-release delivery system in 3D hydrogel construct with ultrasound-activated magnetic microbubbles.

Firstly, PCL-DPP-PCL NPs are used for long-term tracking of MSCs. Some of the current nanoparticles which are used for cell tracking experience

photobleaching and dye leaching, while others show loss of signals due to cell division, diffusion and exocytosis. On the other hand, the covalent conjugation of dye with polymer enhances the photostability of PCL-DPP-PCL NPs. The use of biodegradable and biocompatible PCL polymer matrix minimizes any potential cytotoxicity. The internalization of these NPs demonstrates little negative impacts on MSC viability and proliferation. With the labelling of these NPs for 3 days, we are able to attain good retention and thus strong fluorescence signals even till 28 days. Little negative effect is observed on MSC adipogenic and chondrogenic differentiations. However, osteogenic differentiation is observed to be compromised. Nevertheless, these NPs have shown to be an efficient fluorescent probes for long-term tracing of MSCs during adipogenic and chondrogenic differentiations.

Other than tracing MSCs, monitoring the expression of critical biomarkers during MSC differentiation will greatly benefit the assessment of optimal chondrogenic conditions for cartilage regenerative. In the thesis, we have developed a nanosensor platform based on co-encapsulation of MBs in biodegradable PLGA NPs for real-time monitoring of MSC chondrogenic differentiation in 3D agarose hydrogels. MBs are released in a sustained manner with specific targeting of Col2 (chondrogenic marker) and GAPDH (housekeeping gene). An increase in intracellular Col2 mRNA expression of

MSC chondrogenic-induced samples is noninvasively assessed for a period of 28 days. The fluorescence signal profile is well correlated against readings from standard qRT-PCR analysis.

The potential of MB-NPs is further explored for optimizing MSC chondrogenic conditions for cartilage regeneration. Specifically, this thesis aims to obtain proliferative chondrocytes, which can be found in the proliferative zone of growth plate. The project investigates how treatment duration of growth factors (i.e. TGF β 3 and FGF2) influences the differentiation of MSCs to form proliferative chondrocytes. High expression of Ki67 (proliferative marker) and Col2 genes are observed when MSCs are pre-treated with TGF β 3 for 14 days before 7 days of FGF2 treatment. The trends of the quantified fluorescence signals from Col2 MBs and Ki67 MBs match with that of qRT-PCR results.

As the additions of TGF β 3 and FGF2 occur at different time points, it requires the development of a controlled-released delivery system in 3D microenvironment. Ultrasound-activated magnetic microbubbles is studied as the potential delivery system. MMBs have been fabricated in different sizes and loaded with various cargos. Our study has observed a unique phenomenon in which embedded MMBs in agarose hydrogel are able to undergo oscillation and released cargos.

6.2. Future Work

While substantial works have been studied, more can be done towards clinical translation and regenerative medicine. For cartilage engineering in specific, more chondrogenic conditions can be screened using MB-NPs platform by varying the type of growth factors, concentration and treatment period. Till date, the optimal differentiation conditions to obtain different zonal chondrocytes in growth plate have not been finalized. MB-NPs will be beneficial as detection and monitoring platform for this application. As the nanosensor is versatile, MB probe sequences can be designed depending on the application. By encapsulating more than one MB probe into a nanoparticle, this will provide the platform with multiplexing advantages. The large permutation of possible differentiation conditions can be more effectively screened with real-time monitoring without the need for huge sample sizes. Upon successful screening of differentiation conditions for each zones of the growth plate, regenerative medicine for patients with growth plate arrest can be provided. In addition, behaviors of MSCs tend to vary among patients. Hence, the optimal treatment time for differentiation may differ. MB-NPs offer a platform for real-time monitoring of the mRNA expressed which allows for personalized treatment to be made at the optimal duration. Though MB probes are currently used within the visible

light region, it can be customized to be use in infrared region (e.g. Cy5 and Cy7) or for other imaging modalities (e.g. positron emission tomography). This will offer better tissue penetration for *in vivo* imaging in small animals. By tuning of MB probe, it can assist as monitoring tool for *in vivo* studies.

Having the optimal treatment conditions screened through MB-NPs, MMBs can then help to controlled-release the growth factors for regenerative therapy. For instance, we have observed from our study that MSCs differentiate into proliferative chondrocyte-like cells when they are subjected to TGF β 3 and FGF2 treatments at different time points and duration. MMBs can play a part as a flexible yet controlled-release delivery system in 3D microenvironment. TGF β 3 and FGF2 can be loaded onto MMBs of different sizes and embedded in 3D agarose construct with MSCs. As resonant frequency of MMBs is size-dependent, ultrasonic irradiation can trigger the release of different growth factors at different resonant frequencies. Not only can we control the time to release the different growth factors, we can also provide multiple dose release for different treatment duration. *In vivo* studies using MMBs can be done on small animal model, such as mouse and rabbits, with the aid of ultrasound focused transducer to irradiate at target site. Ultrasound imaging and magnetic resonance imaging (MRI) can be performed to locate the position of MMBs.

The integration of MB-NPs and MMBs can possibly achieve *in vivo* theranostic applications. Both MB-NPs and therapeutic agents can be loaded to MMBs of different sizes and encapsulated in 3D hydrogel. MB-NPs can first be released after transplantation to monitor the status of cells. The release of therapeutic agents can subsequently be triggered at the optimal time point. Thus, unification of these technologies potentially bring about clinical translation for cartilage regeneration.

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