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**NANYANG
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Non-Invasive Nanosensor Monitoring of
mRNA expression for Regenerative
Medicine Application

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2017

Non-Invasive Nanosensor Monitoring of mRNA expression for Regenerative Medicine Application

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A thesis submitted to the Nanyang Technological University
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2017

NANYANG TECHNOLOGICAL UNIVERSITY SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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CHRISTIAN WIRAJA

July 2017

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List of Publications

JOURNAL ARTICLES

- 1) **Christian Wiraja**, David C. Yeo, Xinhong Lim, Chenjie Xu*. Real-Time Imaging of Dynamic Cell Reprogramming with Nanosensors. (*manuscript in preparation*)
- 2) Jin Pan[#], **Christian Wiraja**[#], Jinmin Zhao, Jinlu Zhang, Muyan Li, Li Zheng*, Chenjie Xu*. Nitric oxide nanosensors for monitoring the development of osteoarthritis in rat model, ACS Applied Materials & Interfaces, 2017, <http://dx.doi.org/10.1021/acsami.7b06404>. (co-first author)
- 3) Shiyang Liu, David C Yeo, **Christian Wiraja**, Hong Liang Tey, Milan Mrksich, Chenjie Xu*. Peptide delivery with poly(ethylene glycol) diacrylate microneedles through swelling effect, Bioengineering & Translational Medicine, 2017, DOI: 10.1002/btm2.10070
- 4) Jiayin Fu, Hamizan Muhammad, **Christian Wiraja**, Chenjie Xu, Dongan Wang*. Improvement of endothelial progenitor outgrowth cell (EPOC)-mediated vascularization in gelatin-based hydrogels through pore size manipulation. Acta Biomaterialia, 2017, <http://dx.doi.org/10.1016/j.actbio.2017.06.012>
- 5) Wang, Lifeng; Xu, Keming; Hou, Xiaochun; Han, Yiyuan; Liu, Shiyang; **Wiraja, Christian**; Yang, Cangjie; Yang, Jun; Wang, Mingfeng; Dong, Xiaochen ; Huang, Wei; Xu, Chenjie*. "Fluorescent Poly (glycerol-co-sebacate)-acrylate Nanoparticles for Stem Cell Labeling and Longitudinal Tracking" ACS Applied Materials & Interfaces, 2017, DOI 10.1021/acsami.7b01203
- 6) Tay, Li Min[#], **Christian Wiraja**[#], David C. Yeo, Yingnan Wu, Zheng Yang, Yon Jin Chuah, Eng Hin Lee, Yuejun Kang, and Chenjie Xu. "Noninvasive Monitoring of Three-Dimensional Chondrogenic Constructs Using Molecular Beacon Nanosensors." Tissue Engineering Part C: Methods, 2017, 23(1): 12-20. (co-first author)
- 7) Fu J[#], **Wiraja C**[#], Chong R, Xu C*, Wang D*. Real-time and Non-invasive Monitoring of Embryonic Stem Cell Survival during the Development of

- Embryoid Bodies with Smart Nanosensor. *Acta Biomaterialia*, 2017, 49, 358–367 (co-first author)
- 8) Sivasubramanian K, Mathiyazhakan M, **Wiraja C**, Upputuri PK, Xu C*, Pramanik M*. Near Infrared light-responsive liposomal contrast agent for photoacoustic imaging and drug release applications *Journal of Biomedical Optics* 2016, 22(4), 041007.
 - 9) Tay, H. M., Yeo, D. C., **Wiraja, C.**, Xu, C., Hou, H. W. Microfluidic Buffer Exchange for Interference-free Micro/Nanoparticle Cell Engineering. *J. Vis. Exp.* 2016, 113, e54327.
 - 10) **Wiraja C**, Mathiyazhakan M, Movahedi F, Upputuri P.K, Cheng Y, Pramanik M, Yang L, Becker DL, Xu C*, Near-infrared light-sensitive liposomes for enhanced plasmid 2 DNA transfection. *Bioengineering & Translational Medicine*, 2016, 1(3), 357–364
 - 11) Karahan HE, Wei L, Goh K, **Wiraja C**, Liu Z, Xu CJ, Jiang R, Wei J, Chen Y. Synergism of Water Shock and a Biocompatible Block Copolymer Potentiates the Antibacterial Activity of Graphene Oxide. *Small*. 2016, 12(7):951-62.
 - 12) Kumar N, **Wiraja C**, Palanisamy K, Marsili E, Xu CJ*, Heat Shock Mediated Labelling of *Pseudomonas aeruginosa* with Quantum Dots, *Colloids and Surfaces B: Biointerfaces*, 2016, 142, 259-265
 - 13) Wang M, Hou X, **Wiraja C**, Sun L, Xu Z, Xu CJ*. Smart Magnetic Nanosensors Synthesized through Layer-by-Layer Deposition of Molecular Beacons for Non-Invasive and Longitudinal Monitoring of Cellular mRNA, *ACS Applied Materials & Interfaces*, 2016, 8(9):5877-86
 - 14) **Wiraja C**, Yeo D, Chong SK, Xu CJ*. Nanosensors for Continuous and Non-invasive Monitoring of Osteogenic Differentiation of Mesenchymal Stem Cells. *Small*, 2016, 12(10):1342-50
 - 15) Yeo D, **Wiraja C**, Chuah YJ, Gao Y, Xu CJ*. A Nanoparticle-based Sensor Platform for Cell Tracking and Status/Function Assessment. *Scientific Reports* 2015, 5:14768
 - 16) Yeo D, **Wiraja C**, Zhou Y, Tay H, Hou H, Xu CJ*. Interference-Free Micro/Nano-particle Cell Engineering Using High-Throughput Microfluidic Separation. *ACS Applied Materials & Interfaces* 2015, 7(37):20855-64

- 17) **Wiraja C**, Yeo D, Chew SY, Xu CJ*. Molecular Beacon-Loaded Polymeric Nanoparticles for Non-Invasive Imaging of mRNA Expression. Journal of Materials Chemistry B, 2015, 3, 6148 - 6156
- 18) **Wiraja C**, Yeo DC, Lio D, Labanieh L, Lu M, Zhao W, Xu CJ*. Aptamer technology for tracking cells: status & function. Molecular and Cellular Therapies 2014, 2:33
- 19) Yeo, D.C., **Wiraja, C.**, Mantalaris, A.S. and Xu, CJ*, 2014. Nanosensors for regenerative medicine. Journal of biomedical nanotechnology, 10(10), pp.2722-2746.

BOOK CHAPTERS

Wiraja C, Chong M, Liao Y, Chew S, Xu CJ*, Chapter 7: "Multi-Functional Biomaterials for Bone Tissue Engineering". Smart Materials No. 25, Smart Materials for Tissue Engineering: Applications, Edited by Qun Wang, 2017, © The Royal Society of Chemistry

CONFERENCE SUBMISSIONS

- D.C.L Yeo, **C. Wiraja**, C.J. Xu*, "A Safe, Versatile, Nanoparticle-based Sensor to Monitor Status & Function in Live Cells"; Poster, Materials Today Asia, 2014, P2.50, December, Hong Kong.
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- **L.M. Tay**, **C.Wiraja**, Z.Yang, Y.N. Wu, E.H. Lee, C.J. Xu*. "Micro/Nano-technology for Physal Growth Plate Engineering"; Poster; TERMIS European Chapter Meeting, 2017, June; Switzerland.
- **H.E. Karahan**, **C. Wiraja**, N.F. Binte Nasrun, S.H.J. Toh, M.H. Goh, L. Wei, C.J. Xu, W. Jun, Y. Chen*, "Antibacterial Performance of Graphene Oxide Complemented with Pluronic F-127 on Physiologically Mature Gram-Negative Bacteria"; Oral; IEEE NANO, 2017, July; United States.

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Summary

The field of regenerative medicine focuses on utilizing therapeutic cells either on itself or in conjunction with scaffolds to replace, repair or allow regeneration of injured tissues. Thus far, various approaches involving wide ranges of cell and biomaterial types have been proposed and validated at the bench-side (laboratory). However, their translation towards the clinical side have been minimal, with most approaches failing in pre- or early clinical trial phase. One limiting factor that is very crucial in ensuring successful treatment efficacy and thus translation process is quality control of cells to be injected/implanted. Prior to its re-introduction, cells of various sources need to be validated for their functionality (i.e. phenotype).

Conventionally, such verification is achieved through end-point, population-based methods like polymerase chain reaction (PCR; for mRNA expression) or western blotting (for protein expression). These assays however, necessitate sample disruption for isolation of analytes. As such, it only provides a snapshot of the dynamic processes occurring (i.e. provides no temporal resolution). To this end, incorporation of reporter gene constructs enable continuous, longitudinal tracking of gene-of-interest's expression profile. Nevertheless, it also has limitations including the tedious and lengthy development, and risks of introducing random mutations which make modified cells less clinically-friendly.

In this thesis, application of nanosensor platform as a safe, non-integrative alternative for prolonged monitoring of cellular biomarkers is proposed and demonstrated. Specifically, nanosensors were fabricated by incorporating molecular beacons (MBs), oligonucleotide-based hairpin probes with specific target gene recognition ability, into biocompatible, biodegradable polymeric nanoparticles. Encapsulated within the nanocarriers, MBs can be effectively internalized by cells without requiring transfection procedures. Crucially, sustained release of MBs into the cytoplasmic region prolongs intracellular monitoring window of MBs, which are rapidly digested and cleared from cells otherwise. This was initially proven using MB targeted towards housekeeping gene of β -actin, against pore-inducing bolus MBs delivery method. Subsequently, nanosensor was applied to validate and track

osteogenic (2D) and chondrogenic (3D hydrogel) differentiation of MSCs. In both cases, loading of nanosensors with both functional MBs (against specific differentiation markers) and reference MBs (against housekeeping gene), enable accurate depiction of cellular gene expression with good correlation against results from gold-standard of PCR (R^2 value between 0.8 to 0.9). Finally, nanosensors were adapted to evaluate successful reprogramming induction of somatic fibroblast cells, relative to both PCR and gene-reporter validation.

Aside from its monitoring performance, safety aspect of nanosensor labeling was studied in detail. Labeling concentration was assessed and optimized to ensure minimal influence over cell metabolism and proliferation rate. Meanwhile, multipotency of labeled mesenchymal stem cells was minorly affected towards three differentiation lineages: adipogenesis, osteogenesis and chondrogenesis. At the same time, Oct4 protein expression and reprogramming efficacy were not impaired by nanosensor labeling.

In conclusion, a facile, versatile yet safe nanosensor monitoring platform is introduced in this thesis. Especially for the field of regenerative medicine, such nanosensor can facilitate swifter translation of various therapeutic approaches by providing scientists information regarding dynamic cellular changes with both spatial and temporal resolution. In the future, such tool can be applied for optimization of culture conditions as well as purifying heterogeneous cell populations to optimize treatment efficacy.

Chapter 1 Introduction

1.1 Background and Scope

Regenerative medicine approaches have vastly developed and studied during the past few decades. With projected growth of aging population and incidence rate of organ-system diseases, regenerative medicine aims to replace or promote healing capability of native tissues, which may have been significantly compromised or non-existent.[1] In general, regenerative medicine approaches involve injection of therapeutic cells (cell therapy) and generation of engineered tissue constructs to replace damaged tissues (tissue engineering). These involves the application of various cell sources (e.g. stem cells, reprogrammed somatic cells) and a plethora of biomaterial scaffold designs.[2, 3]

Nonetheless, clinical translation of these approaches is very limited thus far, and mainly focused on skin grafts and orthopedic implants. While plenty bench-site works are ongoing on other tissues/organs, their bed-site translations are comparatively slow to a halt.[4, 5] Primarily, there are numerous optimization required for any given protocol to obtain functional tissue construct, from the choice of cell source and biomaterial design, to expansion and differentiation method, and injection or implantation site. Especially, each variable may need re-consideration when moving to different stages of development (e.g. from *in vitro* to *ex vivo/in vivo* and finally in clinics).[6] Moreover, it is crucial that injected stem, progenitor or mature cells behave as intended upon injection and implantation. During its development, few studies have reported terrible consequences of teratoma formation or altered tissue generation upon stem cell implantation.[7, 8] Typically, stem cells are expanded *in vitro* and directed towards specific lineage first prior to *in vivo* application. However, few undifferentiated stem cells may remain upon the treatment, which can potentially become teratoma or differentiate to cells of distinct lineage when exposed to the microenvironment *in vivo*. On the other hand, injected therapeutic cells may migrate out of tissue of interest and instead reside elsewhere. In this case, the therapeutic cells may not only not function as intended, it may cause disruption to healthy tissues where it eventually reside.[9] In conclusion, quality control is very crucial and is essential to ensure successful regenerative medicine approach.

Given how strict quality controls are necessary in regenerative medicine, multiple validation techniques have been adapted even during *in vitro* cell culture and expansion. Gene and protein expression of therapeutic cells are typically monitored and evaluated throughout and at least prior to *in vivo* implantation. For gene (mRNA) expression, polymerase chain reaction (PCR) and DNA microarray are generally regarded as gold standard with its specificity and ease of adaptation. Meanwhile, protein expressions of cells can be checked through western blot and immunohistochemical staining, followed by microscopic imaging or flow cytometry. These methods are disruptive and necessitate cell isolation (and even lysis) for sample collection. Consequently, the sample cannot be utilized for subsequent analysis and longitudinal tracking cannot be performed.[10, 11] To enable longitudinal tracking of cellular gene expression, fluorescent or luminescent gene reporters have been developed as well. Introduction of such constructs enable highly specific tracking of any gene of interest. Nevertheless, this approach can be highly laborious and costly to develop, with risks of random mutagenesis. [12-14]

Furthermore, such tracking needs to be extended to after cell implantation as well (*in vivo*). Mainly, this is to ensure cells do not migrate away from the intended tissue site, and remain viable and functional at the same time. Conventionally, sample sacrifice for histological analysis is used to evaluate native tissue integration (including immune responses) as well as extracellular matrix (ECM) deposition of the implanted cells / tissue construct. However, again this method is disruptive and experiment termination is necessary for staining purposes.[15] Longitudinal tracking of injected cells *in vivo* is typically enabled by labeling cells with contrast agents prior. These agents include the usage of radio-isotopes, iron oxide nanoparticles etc. which give therapeutic cells detectable contrast as compared to native host cells, when viewed under various imaging modalities such as X-ray/CT-scan and magnetic resonance imaging (MRI).[16, 17] While cell localization can be traced in this manner, these passive agents do not give any indication as to whether cells of interest remain viable and importantly, functional. After all, it is potentially even worse that a detectable signal remains at targeted tissue, but no therapeutic efficacy can be seen from the procedures as the cells lose its viability.[18, 19] Therefore, there is a need for smart nanosensor which can recognize and report on cell status

and functionality, which remains active for a sufficient period of time (typically a few weeks to completely determine the fate of these cells).

To address this need, a smart nanosensor is designed and evaluated in this thesis. Briefly, the proposed nanosensor consists of FDA-approved biodegradable nanoparticle based on poly(lactic-co-glycolic acid) (PLGA) and molecular beacon (MB) probes. Molecular beacon, an oligonucleotide-based sensor which can recognize its specific target sequences, works based on the Förster resonance energy transfer (FRET) mechanism. Initially, these probes retain a hairpin structure with quenched fluorescence due to short proximity between fluorophore and quencher. Upon hybridization with its target RNA/DNA, it changes conformation to a linear structure which then restore its fluorescence. Historically, MB probes were developed for utilization in polymerase chain reaction (PCR) or protein-DNA interaction studies.[20, 21] Recently however, MB has acquired interests as intracellular mRNA sensor, to detect and semi-quantitatively reveal cellular mRNA expression.[22-24] Despite its promising potential, getting MBs inside the cells has been challenging with its relatively large size (≥ 10 kDa) and net negative charges. As such, assistance from electroporation, microinjection, transfection agents or toxins to permeabilize cell membrane (e.g. Streptolysin O) are necessary to facilitate MBs cellular entry. Moreover, oligonucleotides lifespan in the cytoplasm are typically quite short, due to the presence and activity of endonucleases.[25]

With respect to this, nanocarrier encapsulation of MBs was proposed to facilitate efficient cellular internalization of probes, while at the same time enabling prolonged observation period with sustained release of MBs to counterbalance MB degradation by endonucleases. Meanwhile, multiple distinct MBs can be co-incorporated into the nanoparticles, to obtain a more thorough evaluation of cell functionalities.

Comprehensively, the scope of this work includes the design and synthesis of MB nanosensor against functional stem cell markers, validate nanosensor signal generation against gold-standard method, and evaluate their influence to the *in vitro* efficacy of several regenerative medicine approaches.

1.2 Overarching Thesis Goal

Design of Nanosensor for Prolonged, Non-invasive Monitoring of Cellular mRNA Expression for Regenerative Medicine Application

1.2.1 Demonstrating sustained intracellular release of functional molecular beacon probe from polymeric nanoparticle carrier (Chapter 3)

Most nanoparticles (NPs) in clinical / pre-clinical settings are applied to enable cell tracking in a passive manner, to evaluate cell biodistribution and site retention after injection or implantation. However, such tracking thus far does not reveal cell status and functionality, an important factor which determines treatment efficacy. Meanwhile, non-disruptive and longitudinal functional cell tracking can facilitate swifter optimization of therapeutic protocols. Molecular beacon (MB) probes which can semi-quantitatively reveal specific DNA/RNA targets introduce an interesting method to non-disruptively image cellular gene expression. Nevertheless, its intracellular application is thus far limited with the need for assistive method to facilitate entry, and its short lifespan in the cytoplasm due to endonucleases activities. Thus, we firstly studied MB encapsulation within polymeric nanocarrier, which may assist not only in cellular uptake, but also by serving as a depot to facilitate sustained release of intact MB probes. Thereafter, cellular delivery efficiency of MB nanosensor was evaluated using MB targeted against housekeeping β -actin mRNA. Nanosensor cytotoxicity was also gauged by determining viability and proliferation rate of cells labeled with varying nanosensor concentration. Last yet most importantly, MB nanosensor signal retention was compared against bolus delivery of naked MBs, as to assess suitability of proposed MB nanosensor to enable longitudinal intracellular gene tracking.

1.2.2 Application of nanosensor to monitor osteogenic differentiation of hMSCs in 2D settings (Chapter 4)

Following the successful proof-of-concept using MB nanosensor for longitudinal gene monitoring in mesenchymal stem cells (MSCs), the nanosensor was applied

to track a more routine protocol performed on these stem cells (i.e. differentiation). Being a multipotent stem cells, MSCs may undergo differentiation towards few lineages (e.g. osteogenic, adipogenic, etc.). While this means that MSCs are good cell source to expand and obtain various therapeutic cells, strict differentiation cues are required to prevent differentiation towards the unwanted lineage. Thus, MSCs are typically injected / implanted only after being directed towards certain lineage. Nevertheless, few cells may retain their stemness even after exposure to the differentiation protocols, which poses risks of tumorigenesis or development towards another tissue type *in vivo*. Methods to evaluate such successful cell differentiations are typically population-averaged, by pooling together gene or protein content of cells for PCR or western blotting assay. By designing and fabricating MB nanosensor targeted towards osteogenic differentiation marker, we aimed to enable real-time differentiation verification with spatial resolution as well. Moreover, co-loading of both functional MBs (against osteogenic marker Alkaline Phosphatase/ ALP) and reference MBs (against housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase/ GAPDH) allowed direct colorimetric observation on individual cells level. Finally, semi-quantitative signal generated was computed and correlated against the gold standard of polymerase chain reaction (PCR), to measure nanosensor capability to accurately indicate cell differentiation states non-invasively.

1.2.3 Application of nanosensor to monitor chondrogenic differentiation of hMSCs in 3D agarose (Chapter 5)

During *ex vivo* culture, one main challenge is to mimic original / desired tissue settings as close as possible, as to ensure minimal phenotypic alterations and subsequently good adaptation of therapeutic cells during implantation. Over the past few decades, various kinds of scaffold have been developed for cell expansion and differentiation, ranging from soft hydrogels to rigid metallic constructs. While the 3D nature of these scaffolds allows closer imitation of the physiological conditions, it also poses challenges in relation to the heterogeneous cell state depending on its location on the scaffold. The usage of MB nanosensor to non-invasively evaluate

cell behavior in 3D settings was then explored and demonstrated on MSCs undergoing chondrogenic differentiation in soft agarose hydrogel. Utilizing collagen type II (COL2) as a chondrogenic marker and GAPDH as reference marker, the progression of MSCs towards chondrocytes was evaluated. The nanosensor signal ratio was correlated against PCR at weekly timepoints to ensure highly specific / accurate signal generation. Lastly, nanosensor influence towards MSCs adipogenic, chondrogenic and osteogenic differentiation was studied through the respective histochemical staining.

1.2.4 Application of nanosensor to monitor fibroblasts reprogramming and transdifferentiation (Chapter 6)

In the last part of the thesis project, we aimed to further elucidate the performance of nanosensor and to extend the nanosensor utilization on other therapeutic cells beside MSCs differentiation. Spatio-temporal resolution and influence of nanosensor towards target mRNA translation is assessed on model cells modified with inducible enhanced green fluorescent protein (EGFP) expression. Meanwhile, fibroblast cells which have received much attention after successful reprogramming demonstration to induced pluripotent stem cells (iPSCs) and transdifferentiation to other mature cells were identified as an interesting candidate for monitoring application. Here, the low success rate of both procedures necessitates a non-invasive tool to identify and track the rare cell subpopulation useful for subsequent isolation and usage. As in previous chapters, the observed nanosensor signal ratio was correlated with mRNA expression as evaluated through PCR. In addition, nanosensor performance was also compared against transgenic construct approach, which is typically adapted to confirm the successful procedure of reprogramming / transdifferentiation. Similar resolution down to single cells level is used to evaluate suitability of nanosensor as a clinical-friendly alternative to transgenic construct.

Chapter 2 Literature Review

2.1 Regenerative Medicine

2.1.1 Significance of Regenerative Medicine

In terms of its definition, organ regeneration differs from organ repair in its endpoint result after the injury are healed. Repair is an adaptation to loss of healthy organ which leads to restoration of the damaged parts through the synthesis of scar tissue. Regeneration meanwhile, restores the normal morphological structure and functions of the organ.[26, 27]

As we age however, the extent by which our organs can regenerate (or repair) following injuries diminishes significantly, which may ultimately lead to loss of organ function.[28, 29] Therefore, techniques/approaches which can replace or amplify the body's self-healing mechanism are receiving significant attention from both healthcare and scientific community. This field of regenerative medicine is a multidisciplinary field which combines expertise from biology, chemistry, engineering, materials, as well as medicine, to find multi-faceted solutions to the challenges mentioned above, the insufficient self-healing capability of the body.[30-32]

Regenerative medicine, firstly termed in 1992 by Leland Kaiser, started as a new branch of medicine which attempts to change the progression of chronic diseases by regenerating failing organs.[33, 34] Then, the term began to widespread as William A. Haseltine recognized the unique capability of embryonic stem cells and embryonic germ cells to differentiate to all cell types in the body may contribute significantly to regenerative therapy.[35, 36] In 2008, the first tissue engineered trachea was transplanted by Professor Paolo Macchiarini and his team, using chondrocytes grown from patient's bone marrow stem cells and decellularized human tracheal segment. After four days of ex vivo culture, graft was applied to patient's bronchus. One month later, a biopsy displayed local bleeding, indicating that blood vessels had grown back successfully.[37] More recently on September 2014, surgeons at Kobe, Japan, have transplanted a 1.3 by 3 mm cell sheet of retinal pigment epithelium (RPE cells), differentiated from induced pluripotent stem cells (iPS cells), to an eye of a woman suffering from macular degeneration.[38, 39]

Given its promising preliminary results from its early clinical applications, various approaches have been developed in the past few years, to further improve the treatment efficacy, while minimizing off-target results.[40] Such improvement, is very crucial and desperately needed, given the large and continuously-growing market need for organ/tissue replacement (Figure 2.1).

Figure 2-1: Global healthcare market for regenerative medicine approaches. Figures courtesy of Brightlands.

2.1.2 Approaches in Regenerative Medicine

The central focus of regenerative medicine is to develop functional tissues or organs. For this, several approaches have been proposed and developed over the past few decades, involving the application of only cells (cell therapies), biomaterials for scaffold, or a combination of both (Figure 2.2).

Figure 2-2: Multi-faceted regenerative medicine approaches involve usage of cells and scaffolds, and assisted with nanoparticles and small molecules
Adapted from [41]. Copyright by OAPEN.

Cell therapies represent ways to obtain a significant amount of functional human cells, to be validated and injected back at injury or defect sites. These cells maybe somatic, derived from adult stem cells, embryo-derived cells or pluripotent cells obtained by inducing reprogramming on adult cells (iPS cells).[42, 43] Given the very limited availability of most specialist somatic cells, and ethical issues regarding cells from embryo sources, adult stem cells were firstly identified as potential source for cell therapies.[44, 45]

One notable example of adult stem cells and one that is commonly applied in cell therapies thus far is the multi-potent mesenchymal stem cells (MSCs), which can give rise to cells of adipo-, chondro- and osteo-lineages.[46, 47] In addition to cell replacement, injection and recruitment of MSCs to damaged tissue (e.g. infarcted myocardium, fibrotic liver) have brought therapeutic effects through native

cells 'empowerment'. This mechanism was proposed as although the rate of MSC engraftment and its lifespan tends to be poor, there were therapeutic effects observed in some studies.[48, 49] In general, this 'empowerment' depends on the capacity of MSCs to regulate inflammation and/or homeostasis via immunosuppressive factors, cytokines, growth and differentiation factors (e.g. hepatocyte growth factor, tumor necrosis factor- α).[50, 51] More interestingly, scientists have found this immunomodulation property to be quite plastic. In other words, MSCs can either promote an immune response or inhibit it, in accordance to the dynamics of inflammation at the disease site (i.e. strength of immune system activation, inflammatory cytokines and immunosuppressant present).[52, 53] The treatment of graft-versus-host disease (GvHD) by MSCs highlighted this characteristic, as administration during vigorous inflammation stage yielded successful treatment, but much less efficacy was observed when MSCs were applied on the same day as the marrow transfusion (i.e. prior to inflammation stage).[54]

Endothelial progenitor cells (EPCs) which can differentiate towards mature endothelial cells (ECs), are another example widely studied in the field of cell therapy, for their physiological roles in angiogenesis and vasculogenesis.[55, 56] In one study, injection of endothelial progenitor cells (EPCs) to a murine model with peripheral limb ischemia facilitated significant tissue perfusion, which improved limb salvage rates over 8-fold as compared to control group (58.8% in EPC group vs 7.1% in control group).[56] This and results from other similar studies led to optimism for EPCs application to treat ischemic conditions, as highlighted by the initiation of phase I & II clinical trials utilizing EPCs injections for ischemic myocardia treatment.[57]

Recently however, there are growing interests on the usage of reprogrammed cells as alternatives to adult stem cells. This is related with the restricted expandability of the latter, in addition to its typically heterogenous nature.[44, 58] Therefore, since its first introduction in 2006, various induction techniques have been proposed to improve efficiency and to simplify the original reprogramming protocol, including the usage of miRNA and non-viral vectors to avoid genomic integration of constructs, even relying on purely chemically-defined

procedures using combinations of small molecules.[59, 60] In accordance, more and more functional cells were generated through cells reprogramming method, including but not limited to neuronal cells, cardiomyocytes and osteoblasts.[61, 62]

Extending beyond stem cells which is a promising source for generation of large quantity of functional cells, immune-related cells are also categorized as therapeutic cells to be injected. Isolation and priming/purification of these cells (i.e. dendritic cells, T-cells) followed by re-transplantation holds great significance to solve diseases associated with inflammatory responses, including cancers and auto-immune diseases.[63] As an example, patient's dendritic cells can be isolated and primed against patient's own tumor antigen to subsequently act as antigen-presenting cells once implanted back, triggering further immune response against the tumor cells.[64] Similarly, patient's T cells are also modified with gene constructs encoding chimeric antigen receptor (CAR), to induce their attack mechanism on cancer cells upon infusion.[65, 66]

Aside from cells, another key ingredient for regenerative medicine (especially tissue engineering) is the selection and design of suitable scaffold materials.[67] These scaffolds are required as a template for new tissue formation, either with or without being subjected to cell seeding, growth factors incorporation, and sometimes biophysical stimuli in the form of bioreactors.[68]

So far, numerous scaffolds have been generated from various biomaterials and manufactured through a plethora of fabrication methods. Regardless of the tissue targets, several considerations are crucial to determine suitability of any scaffold design. These includes biocompatibility, biodegradability, mechanical properties and architecture of scaffolds.[69, 70] First and foremost, scaffolds must allow cells to adhere and behave normally, before secreting their extracellular matrices (ECM). Related to this, implanted scaffolds should be biodegradable to eventually be replaced by native cells and their ECM.[71] Regarding its physical properties, scaffolds should have consistent mechanical strength with its implanted site. Sufficient integrity is also required to provide support throughout the treatment period, from the start of implantation until the completion of tissue remodeling.[72,

73] Furthermore, interconnected porosity is necessary to allow metabolites exchange and removal of waste products from within the scaffold.[74, 75]

In general, three groups of biomaterials exist and used to fabricate scaffolds, namely ceramics, natural and synthetic polymers. Given its stiff nature, brittle surface and low elasticity, ceramic scaffolds are excellent and typically applied in osteogenic tissue engineering. In fact, few studies have reported enhanced osteogenic differentiation, proliferation and matrix deposition on these ceramics (e.g. hydroxyapatite, tri-calcium phosphate).[76, 77] Natural polymers like collagen, proteoglycans and chitosan, on the other hand, have poor mechanical properties. Nevertheless, they may promote great cell adhesion and growth behaviors, as they are naturally biologically active. Thus, they are typically applied for cartilage and cardiovascular tissue generation.[78, 79] Lastly, synthetic polymers such as poly-l-lactic acid (PLLA) and poly-glycolic acid (PGA) are another option for scaffold generation, as their mechanical and degradation characteristics, including their architecture can be tailored easily depending on fabrication conditions.[80, 81] However, they do still have their own drawbacks, such as the risks of rejection due to minimal bioactivity of these polymers. Thus, surface modification or combination with natural polymers maybe required to ensure sufficient bioactivity.[82]

An example showing how mechanical properties of the scaffold substrate regulates cellular behaviors was demonstrated on the multipotent MSCs.[83] MSCs grown on firm gels with similar elasticity to muscle, underwent differentiation towards myogenic lineage. Meanwhile, MSCs seeded on rigid gels mimicking pre-calcified bone differentiated towards osteogenic pathway instead.[84] Similarly, cardiomyocytes were shown to retain its functional beating only on substrate with heart-like elasticity. On hard substrates (mimicking post-infarct fibrotic scar), cells tend to overstrain themselves and eventually stop beating.[85]

Composition of scaffolds may also significantly regulate seeded cell behaviors. In one example, osteogenic differentiation of human iPSCs generated from gingival fibroblasts were notably enhanced when nanohydroxyapatite (nHA) content in nHA/chitosan/gelatin 3D construct is 3 wt/vol%.[86]

In recent years, in addition to focusing on scaffold 'passive' properties, scaffolds are being developed to be bioactive, to provide regenerative signals to native cells, thereby harnessing and enhancing innate regenerative capacity of the tissue.[87] To achieve, such bio-instructive properties, scaffolds are integrated with growth factors, peptides and cytokines during their fabrication.[88] Angiogenic growth factors like vascular endothelial growth factor (VEGF) for instance, can improve vascularization throughout the scaffold.[89] Finally, the use of scaffolds to deliver therapeutic genes are also being explored, for local, sustained delivery of plasmid DNAs or interfering RNAs.[90]

2.1.3 Need for Quality Control

While various interesting and promising regenerative medicine approaches may have been proposed as described above, the key underlining question is whether these techniques can eventually be applied successfully in clinical settings. To this end, unfortunately, very few out of the approaches suggested and experimented *in vitro* have made it to the patient's side.[91, 92]

Primarily, this is due to the highly exhaustive characterization and optimization processes required at each stage of therapy development (Figure 2.3). Moving from one stage to the next (in vitro bench-side screening, preclinical experiments with smaller and bigger animals, phase 1 clinical trials and further), treatment efficacy tends to drop significantly, or even nullified with the changes associated with the models / sample populations the experiments were performed on.[93, 94] Meanwhile, risk of toxicity poses additional issues concerning therapeutic scale-up. As one example, directing MSCs differentiation to certain functional lineage maybe well documented for 2D culture settings, but replicating it on a 3D culture system may result in uneven cell differentiation related with diffusional limit of nutrients.[95] In addition, the small subpopulation of cells that remain undifferentiated may turn into non-targeted tissues or even tumorigenesis when injected in vivo, due to unclear differentiation cues presented.[96] Moreover, significant amount of injected therapeutic cells may not integrate well with tissue of interest, and migrate out to reside elsewhere instead. Thus, treatment is effectively

served only by the small fraction of cells that integrates as intended, while the rest may cause disruption to other tissues it eventually resides.[97]

Figure 2-3: Scheme showing the long road-map required for clinical translation of cell therapy. Adapted from [94]. Copyrights by Nature Publishing Group.

In conclusion, quality control is very crucial and is essential to ensure successful regenerative medicine approach. This control should include comprehensive evaluation and characterization of the proposed cells or tissue constructs, with particular regard to its purity, safety and biological activity.[98] The presence of condition that may alter the recipients' regenerative potential or promote potential failures needs to be carefully assessed and subsequently eliminated prior to any implantation.[99] On this note, careful consideration of risk-benefit analysis and certain degree of refinery to the protocols defined previously is needed at every stage, not limited to late preclinical or clinical experiments to ensure swift translation.[100]

2.2. Available tools for validating regenerative medicine approaches

2.2.1 Before in vivo application

Ex vivo cell culture as performed at bench-site, exposes cells to culture conditions that differ greatly from its original niche. Even though various 3D scaffolds and dynamic bioreactors can alleviate this discrepancy, the gap between *in vivo* and *in vitro* cultures still induces phenotypical changes to the therapeutic cells during their expansion or differentiation.[101, 102] Correspondingly, these cells may acquire altered behaviors which may influence overall treatment efficacy. Additionally, as mentioned before there might be cell subpopulation which remains stem-like after differentiation procedures. In view of this, gene and protein expression of therapeutic cells are typically monitored throughout *in vitro* procedures and at least prior to *in vivo* implantation.[103]

For evaluation of gene expression, polymerase chain reaction (PCR) and DNA microarray techniques are generally regarded as gold standard with its specificity, sensitivity and ease of adaptation. Meanwhile, protein expressions of cells can be checked through western blot and immunohistochemical staining, followed by microscopic imaging or flow cytometry. Additionally, in certain cases physiological characterization of therapeutic cells can be a meaningful way of quality control (Figure 2.4).[94, 104] However, these methods are disruptive in nature and necessitate cell isolation (and even lysis) for sample collection. Consequently, the sample cannot be utilized for subsequent analysis. In turn, the culture period in between is commonly treated as a black box with slight possibilities to steer culture conditions.[105]

Figure 2-4: Schematic summarizing quality control tools available for ensuring successful stem cell differentiation. Adapted from [94]. Copyrights by Nature Publishing Group.

Methods which can nondestructively, and noninvasively, assess the performance of tissue-engineered constructs with sufficient spatiotemporal resolution are therefore highly desirable, to achieve faster and more effective culture procedures.[106, 107] A popular way which enable continuous monitoring of gene expression is through incorporation of fluorescent or luminescent gene reporters. Introduction of such constructs enable highly specific tracking of any gene of interest. Nevertheless, there are still some drawbacks with this approach, in that it can be quite laborious and costly to develop, with certain risks of inducing random mutagenesis. [108, 109]

2.2.2 Following *in vivo* application

Moving beyond bench-side work, such tracking needs to be extended to post cell implantation as well (*in vivo*). Mainly, this is to ensure cell retention at the intended tissue site, and ideally remain viable and functional at the same time.[110] Therefore, parameters such as extracellular matrix (ECM) deposition and

inflammatory rejection of the implanted cells / tissue construct are crucial to be assessed.[111, 112]

Conventionally, animal sacrifice for tissue/organ collection is inevitable, to facilitate subsequent immunohistochemical staining of tissue sample. Again, however, such method is disruptive and experiment termination would be necessary. To obtain a more complete longitudinal study (with information regarding its temporal resolution), cells are typically labeled with contrast agents prior to injection.[113] These agents include the usage of radio-isotopes, iron oxide particles, etc. which give therapeutic cells detectable contrast signal as compared to native host cells/tissues, when viewed non-invasively under various imaging modalities including X-ray, CT-scan and magnetic resonance imaging (MRI). To this end, these passive agents permit tracking of cell localization / biodistribution at particular timepoints post implantation.[111, 114] Nevertheless, typically very little information can be acquired with regards to the aforementioned cell properties (viability and functionality).[115, 116] A more complete account of nanoparticle application for regenerative medicine will be reviewed and discussed in the following subchapter.

2.3. Application of nanoparticle in regenerative medicine

As mentioned above, various nanoparticle systems have been proposed to enable cell tracking, either prior to or after cell implantation.

2.3.1. In vitro tracking

In *in vitro* settings, these nanoparticles are mostly aimed to ensure / validate successful therapeutic cell conversion prior to their implantation. These therefore includes monitoring of cell viability, differentiation state, etc. Furthermore, as optimal culture conditions are detrimental to the yield and efficacy of cell expansion and conversion, some nanosensors are prepared as well to monitor bioprocessing parameters (e.g. O₂ level, pH, nutrients concentration). In such case, the non-disruptive indicators enable researchers to aptly adjust these parameters in case required, without the need of trial and error.[117] Especially when experiments performed involve long and complex procedures with bioreactors and scaffolds, such trial and error would be very costly and time consuming.[118]

Oxygen Nanosensors

Varying levels of O₂ tension has been shown to influence pathogenesis of cancer cells[119] as well as stem cell identity.[120] In actively proliferating cells (e.g. stem cell colonies), local O₂ is often rapidly consumed, leading to hypoxia.[121] In scaffold platforms, especially thick ones, monitoring O₂ levels in scaffold microenvironment is critical to assess its availability to the core region. To this end, numerous oxygen-sensitive probes have been developed including ruthenium hexafluorophosphate Ru(dpp), Oxyphore R2 (PdTPCPP), Oxyphore G2 (PdTCPTBP).[122-124] Loading these probes into NPs can ease cell delivery for intracellular monitoring[125] or the reverse, to prevent cellular uptake of nanosensors for prolonged suspension on extracellular environment.[126]

pH Nanosensors

Extracellular pH level is a critical bioprocess parameter commonly exploited to gauge and control operating conditions.[127] Stem cells, in particular have an

arguably greater sensitivity to pH conditions, given the loss of stem cell potency following relatively minor changes in pH (± 0.3). [128] Thus, non-invasive monitoring of pH levels is of prime importance to maintain productive culture processes, as a quality assurance tool. Moreover, it can be utilized as an indirect indicator of cell health and metabolism, since poor viability and changes in metabolism result in lower pH.

In recent years, Chan et al. demonstrated a pH-sensitive system by embedding L-arginine within alginate microcapsules. [129] By virtue of its abundant, exchangeable guanidyl NH protons, L-arginine results in reduced radiofrequency signal contrast, corresponding to decreased pH levels. Such chemical exchange saturation transfer (CEST) agent was shown to detect differences in loss of viability of hepatocytes injected to immunosuppressed or unsuppressed mice, and more importantly well-matched against gene-modified bioluminescence technique. [130, 131]

Another approach for detecting extracellular pH involves the incorporation of pH probes-loaded nanosensors within agarose culture system, [132] or assembly of thin layers containing sensor probes, to which cultured cells can be seeded. For example, Tian et al. assembled thin films from naphthalimide derivatives containing methacrylate pH sensing moieties. Using E.coli as a model, they continuously monitor extracellular pH change induced by cell metabolic activity. [133]

Nutrient nanosensors

Nutrients and metabolites are another crucial parameter of the bioprocess microenvironment. Previously, the importance of regulating metabolite content in bioreactors was demonstrated as accumulated lactate impaired embryonic stem cell self-renewal even in the presence of sufficient growth factors. [134] Kazakova et al. developed a CaCO_3 -based sensor to simultaneously evaluate glucose and lactate concentration, based on reduction of glucose/lactate oxidase which restores signal of Ru(dpp) and dihydrorhodamine 123 (DHR 123). [135] Sandros et al meanwhile demonstrated a maltose sensor using quenched CdSe platform, which regain its intensity upon contact with maltose. [136]

Viability Nanosensors

As mentioned previously, loss of cell viability (which occurs through either necrosis or apoptosis) after implantation can significantly impair therapeutic efficacy. To date, several characteristics can be used to identify non-viable cells, including: 1) extracellular exposure of phosphatidylserine (PS), 2) caspase activity, 3) mitochondrial membrane depolarization and 4) DNA fragmentation and loss of membrane integrity.

Conjugation of fluorescent probes and IOnp with molecules which have affinity to PS such as Zn (II) di-2-picolyamine (DPA) and synaptotagmin IC2 domain have shown positive correlation with number of apoptotic cells as determined through traditional terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining, after application of staurosporine (apoptotic agent).[137] Using caspase specific cleavage principle, viability nanosensor could also be generated to regain fluorescence only on apoptotic cells expressing caspases. For example, quenched Cy5.5-DEVD26-PEI-DOCA20 nanoparticles were developed to emit near infra-red (NIR) signals only after cleavage of DEVD sequence by caspase 3/7. Such sensor was able to show 12-fold fluorescent intensity over background from HeLa cells following exposure to Tumor Necrosis Factor (TNF)—related apoptosis-inducing ligand (TRAIL) for 30 mins.[138]

Yet another design applies LEHD enzyme substrate synthesized with 7-amino-4-methylcoumarin (AMC) fluorophore which can be cleaved based on caspase 9 activity, increasing its fluorescence emission.[139] Meanwhile, detection of cells undergoing necrosis is more straightforward by fluorophore or ¹¹¹In-DOTA radionuclide usage to monitor DNA damage response protein (γH2AX) which binds and localizes at double strand DNA breaks, following increased accessibility of plasma membrane.[140]

Differentiation nanosensors

So far, instances of nanosensors applied to track cell differentiation are still limited with invasive protein/mRNA analysis being the gold standard for validation (i.e. PCR, immunostaining and/or enzyme-linked immunosorbent assay-ELISA).[141] However recent advances in DNA nanotechnology, including design of aptamers which can recognize specific cell biomarkers are promising for

development of differentiated/stem cell-specific nanosensors. For example, single walled-carbon nanotubes (SWNT) functionalized with insulin-binding aptamers could be applied to detect insulin production by INS-1 cells, a functional indicator of pancreatic cell differentiation.[142]

Aside from detection of extracellular markers, various nanosensors have been proposed to recognize the presence of intracellular mRNAs and reveal its target expression. Such example is the spherical nucleic acid (SNA), a platform introduced and developed by Mirkin's lab which involves assembly of releasable flare strands on gold nanoparticle (AuNP) core. Upon cellular internalization and specific target mRNA hybridization, these flares are detached from the AuNP core and become fluorescence, thereby acting as indicators of its expression level.[143] Until now, SNAs have undergone multiple improvements including multiplexing and been applied to detect circulating tumor cells (CTCs) from human blood, through evaluation of epithelial-mesenchymal transition (EMT) marker genes namely vimentin and fibronectin.[144, 145]

More recently, nanoconstructs made entirely from oligonucleotide have also been proposed as intracellular nanosensors, given its biocompatibility, efficient cellular internalization and specificity towards its target sequence. As an example, DNA tetrahedrons were demonstrated to reliably detect the expression of tumor-related mRNAs in live cells (e.g. TK1 mRNA, GalNAc-T mRNA).[146, 147] By designing specific recognition and flare sequence to match specific differentiation markers, signal generated from these two nanosensor constructs could be adapted to monitor the progression of cell differentiation down a certain lineage.

2.3.2. In vivo tracking

To date, nanoparticles are most commonly used in the field of regenerative medicine to track/evaluate biodistribution of therapeutic cells post-infusion. For such purpose, nanosensors to emit sufficiently strong contrast signal to be differentiated from noises originated from host tissues. These signals need to be compatible with imaging modalities including fluorescence/luminescence imaging, magnetic resonance imaging (MRI), ultrasound and positron emission tomography (PET).[148, 149]

Rosen et al. for example, labeled MSCs with fluorescent quantum dots (QDs) to enable visualization of MSCs within canine ventricle for an 8-week period.[150] Fluorescent nanodiamonds have also been adapted for lung stem cells tracking on mice with healthy or injured lung.[151] Such optical imaging are typically performed at the near infra-red (NIR) region to avoid major signal attenuation by body fluids and blood. Additionally, they are commonly accompanied with multi-photon platform for better penetration depth.

Additionally, super paramagnetic iron oxide (SPIO) particles are widely utilized for MR-based cell tracking. In one study, co-labeling of dendritic cells with SPIO and ^{111}In -oxine allowed dual-modal tracking on rabbit muscle and brain for at least 8 weeks period.[152] In another examples, the incorporation of SPIO within pancreatic islet cells-containing alginate capsules allowed evaluation of cell engraftment in large animals (e.g. pigs).[153]

More recently, development of multi-modal contrast agents is gathering significant attention with the purpose of acquiring *in vivo* images with better specificity and sensitivity, as to facilitate clearer evaluation of processes occurring following cell implantation. As example, optical-based probes with its high resolution but low penetration depth can be utilized in conjunction with MRI probes which have good penetration capability.[154] In such case, localization of cells obtained through non-invasive MR imaging can later be validated with fluorescence signal from isolated histological samples at the end of the experiment.

Unfortunately, all the aforementioned nanoparticle probes only facilitate passive tracking of implanted therapeutic cells, without revealing their status and functionality. In other words, injected cells may have lost their viability due to harsh environmental conditions, clearance by immune cells, etc. but signal from these contrast agents may remain and keep persisting until they are eventually cleared from the body. Such false-positive signal results in great discrepancy between non-invasive cell signal and eventual therapeutic efficacy, when observable signal is not carefully analyzed and interpreted.[155, 156]

Therefore, nanosensors which have capability to report actual cell status (i.e. differentiation and viability) as described in previous section would be ideal.

However as thus far activatable nanoprobe rely and emit detectable signal for optical imaging, limitations associated with tissue penetration depth are hindering widespread development and utilization. Nevertheless, the development of cutting edge technology imaging system (e.g. dual-photon system) alongside inserted fiber optics are alleviating this problem.[157, 158]

Chapter 3 Sustained intracellular release of functional molecular beacon probe from PLGA nanoparticle carrier

Wiraja, C., Yeo, D.C., Chew, S.Y. and Xu, C., 2015. Molecular beacon-loaded polymeric nanoparticles for non-invasive imaging of mRNA expression. *Journal of Materials Chemistry B*, 3(30), pp.6148-6156.

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3.1 Introduction

Messenger RNA (mRNA) as we know conveys genetic information from nuclei to ribosomes which then specify the amino acid sequence of proteins to be generated. Non-invasive assessment of intracellular mRNA expression help to understand many biological questions, including the identification of oncology disease stage [159, 160] and as quality control during the *ex vivo* expansion and differentiation processes required for therapeutic cells.[161, 162] Furthermore, as compared to the gold-standard of quantitative polymerase chain reaction (PCR) method which requires disruptive sample collection, non-invasive mRNA monitoring is more efficient in terms of cost and time required for analysis.[163, 164]

Molecular beacon (MB) is an oligonucleotide-based probe which recognizes the presence of its specific target sequences by involving the Förster resonance energy transfer (FRET). MBs have originally been developed as a versatile sensing platform for genetic studies, including for PCR and protein-DNA interaction analyses. [20, 21] This remains until quite recently, when MB technology has undergone transition in its application as a non-invasive, convenient solution to evaluate intracellular mRNA instead. [23, 165]

However due to its relatively large size and negative charge (from the phosphate groups), MBs cannot readily diffuse through plasma membrane and require external assistance for intracellular delivery. These includes, but not limited to microinjection, electro-/microporation, and membrane pore inducer (streptolysin-O/SLO).[166, 167] In addition, MBs are still subjected to endogenous nuclease digestion and clearance which makes them effective for a limited period (i.e. few hours).[168, 169]

Over the past decade or so, nanotechnology has begun to reveal its full colors to both drug delivery and diagnostic imaging fields, from enhancing efficacy to reducing toxicity.[170, 171] Several noteworthy examples include the self-assembly of lipid-conjugated MBs into a micelle structure and conjugation of multiple MBs onto gold NPs.[172, 173] In general, the nanoparticle formulation assisted cellular internalization efficiency, as compared to delivery of unconjugated MBs.

Nevertheless, such surface-bound MB nanoparticles still have its limitation in that its protective mechanism relies on steric hindrance to resist nucleases degradation. In this regards, internal encapsulation of MBs within nanoparticle carrier may enable even slower intracellular MB release, which translates to longer monitoring window. Hence, we first sought to formulate, synthesize, and characterize MB nanosensor from incorporation of MB probes inside FDA-approved poly(lactic-co-glycolic acid) nanoparticles (PLGA NPs, Figure 3.1). In addition, PLGA NPs were shown previously to be effectively internalized by Mesenchymal Stem Cells (MSCs) and well-retained in the cytoplasm when their size is about 500-1000 nm.[174]

Figure 3-1: Schematic illustration of the synthesis and performance of the mRNA nanosensors. Nanosensor is prepared by loading MB probes inside PLGA nanocarrier. Upon cellular internalization, sustained release of MBs enable prolonged mRNA expression monitoring.

3.2 Materials and Methods

Materials except when specifically-mentioned were purchased from Sigma-Aldrich. Dulbecco's modified eagle medium (DMEM) high, fetal bovine serum (FBS), trypsin-EDTA (0.05%), and penicillin-streptomycin were purchased from Lonza. DiB cytoplasmic membrane stain was purchased from Biotium. Trizol reagent was

purchased from Invitrogen. M-MLV reverse transcriptase was obtained from Promega and iQ SYBR Green Supermix was obtained from Bio-Rad.

Synthesis of MB Nanosensors

MB targeted towards the housekeeping gene β -actin was designed with fluorescein dye and black hole quencher 1 ([Fic]-CCCGA-GCGGCGATATCATCATCCAT-TCGGG-[BHQ1]). MB nanosensors were synthesized using a double-emulsion method. Briefly, 100 μ l MB solution in RNase/DNase-free distilled water (10 mM) were mixed with 600 μ l chloroform containing 10 mg PLGA (50:50, Mw:30–60kDa) and 1% (v/v) span 80 with a homogenizer (30 s at 30k rpm, Scilogex D160) to assemble the first emulsion. Then the mixture was mixed into 6 ml of 3% poly-vinyl alcohol (PVA) to form the double emulsion particles in homogenization. Then, the solution was stirred and left inside a fume hood for 4 hours to evaporate chloroform content. Excess PVA was then removed by rinsing the particles thrice with DNase/RNase-free distilled water. Finally, the particles were freeze-dried and kept in -80°C.

Characterization of MB Nanosensors

Hydrodynamic diameter of MB nanosensors was analyzed using Zetasizer nano Z (Malvern), by measuring 1mg of nanosensors dispersed in 10ml distilled water at least thrice. Surface morphology of MB nanosensors was visualized with JSM-6700F (JEOL) field emission scanning electron micrography (FESEM) (at 5 kV), following plasma-coating of particles with gold.

To quantify encapsulation and release profile of MB from nanosensor, calibration curve correlating maximum MB fluorescence and its concentration was first generated. Briefly, solutions containing known MB concentration (0.01-0.2 μ M) were first treated with excess DNase I enzyme (40 U/ml) for cleavage of hairpin structure before measurement with fluorescence microplate reader.[25, 175]

To evaluate encapsulation efficiency of MB probes within the nanosensor, un-encapsulated MBs in the supernatants during the fabrication processes were collected and similarly exposed to DNase I digestion, for measurement with

fluorescence reader. The concentration obtained was finally deducted from the total MB used (1nmol).

Release profile of MBs from the nanosensor was then studied at 37°C in 3 distinct conditions: DMEM + 10% FBS (cell culture medium), PBS, and pH 5 buffer. Briefly, 2mg nanosensor was dispersed well in 1ml of above-mentioned solution. At designated time points, supernatants were collected and again subjected to DNase I treatment for quantification. Additionally, functionality of MB probe following nanosensor incorporation and release was examined by measuring their fluorescence change before and after the addition of excess target sequence (500nM).

Cell culture and labeling of nanosensors

hMSCs harvested from normal bone marrow (Lonza) were cultured in DMEM containing L-glutamine supplemented with 10% FBS in humidified condition of 37°C, 5% CO₂. Culture medium was replaced every 2/3 days. Cells to be labeled were seeded at ~80% confluency one day prior to treatment. Naked MBs were delivered into MSCs during incubation through a mixture of 6 U/ml activated Streptolysin-O in serum-free DMEM. 10 minutes after, cells were rinsed thrice with culture medium and given 30 minutes' recovery period prior to any further experiment. Meanwhile for nanosensor labeling, lyophilized nanosensors were incubated in poly(L-lysine) (PLL) solution (MW 70-150kDA, 0.01% w/v) for 20 minutes. Excess PLL were removed through centrifugation, before PLL-coated nanosensors were re-dispersed in culture medium for overnight incubation. The following day, cells were rinsed thrice with PBS to remove excessive nanosensors.

Cytotoxicity study of nanosensors

hMSCs were incubated with PLL-coated nanosensors at distinct concentrations between 0.1 and 5 mg/ml in cell culture medium. Following which, cells were rinsed thrice with PBS and stained with trypan blue for counting on haemocytometer for viability examination. Proliferation rate of nanosensor-labeled hMSCs was studied in the same manner by examining cell number at day 3 and 6 post-labeling, as compared to unlabeled control hMSCs.

Nanosensor uptake mechanism

To understand the mechanism by which the nanosensors were internalized, hMSCs were pre-treated with various endocytosis-inhibitors, with concentrations adapted from previous publications.[176-178] Briefly, cells were pre-incubated for an hour with 10mM sodium azide with 20mM 2-deoxy-D-glucose, 20μM genistein, 10 μg/ml chlorpromazine, 5 μg/ml cytochalasin D or 50 μg/ml amiloride. Then, nanosensors were added to the cell medium for 4 hours. After which, cells were rinsed thrice and kept for another 4 hours in fresh culture medium before fluorescence imaging. Then, fluorescence intensity of treated cells was quantified through ImageJ (≥150 cells per sample) and normalized against that of unlabeled cells and nanosensor-labeled cells with no treatment (100%).

Fluorescence imaging

Fluorescence images were captured using an LX71 inverted microscope (Olympus), equipped with a Retiga-2000R CCD camera. All images were taken with a fixed exposure time of 500ms and gain of 5, followed by background reduction through the ImageJ software. Fluorescence images taken were then analyzed with ImageJ to quantify cellular fluorescence intensity (≥150 cells per sample).

Confocal imaging

Cell samples for confocal were prepared based on a previous protocol. (46) Briefly, nanosensor-labeled MSCs were rinsed thrice with ice-cold PBS and resuspended at 10^6 cells per ml, subjected to membrane dye staining (DiB, 5mM) for 5 minutes. Subsequently, cells were rinsed with warm culture medium prior to re-seeding on fibronectin-coated slips. 20 minutes after, cells were fixed with ice-cold 4% paraformaldehyde solution. Confocal images were taken with LSM710 (Zeiss) and processed using Zen 2009 software.

Flow cytometry

LSR Fortessa™ X-20 (BD Biosciences) was used to perform flow cytometry analysis, with its corresponding FACSDiva software (BD Biosciences). Data

obtained were then analyzed and plotted using FlowJo (TreeStar). For any sample, about 1 million hMSCs were screened in PBS.

3.3 Results and Discussion

Synthesis and characterization of MB nanosensors

The size of nanosensor was controlled around 400nm to 1 μ m to allow prolonged cellular retention (Figure 3.2A).[174, 179] Through generation of standard curve and quantification of unencapsulated MBs following DNase I treatment, the encapsulation efficiency of MB within nanosensor was determined to be $81.79 \pm 10.91\%$ (Figure 3.3). This is equivalent to be 8.42 μ g MBs per 10mg nanosensor.

Subsequently, MB release profile was studied by immersing nanosensors in three solutions (PBS, culture medium, acetate pH5 buffer) at 37°C. After sampling at designated timepoints (till week 4), the amount of MBs in all supernatant were accumulated together. Overall, the nanosensor displayed sustained release of MBs in all solution (Figure 3.2B). In pH5 buffer, the release was the fastest (complete release by 8 days), most likely due to accelerated PLGA degradation.[180] Nanosensor release was the slowest in cell culture medium, where only ~25% of MBs were released after a period of 4 weeks.

Next, we evaluated whether the MBs still retain their original specificity to β -actin mRNA, following nanosensor incorporation and release. Supernatants obtained from the release study at various time points were treated with its complementary target sequence and examined for their fluorescence recovery. As shown in Figure 3.2C&D (for culture medium and PBS release, respectively), the hybridization of matching target sequence results in 7-8 fold fluorescence recovery. This demonstrated that MB functionality was well-maintained following nanosensor preparation.

Figure 3-2: Physical characterization of MB-loaded nanosensors. A) Hydrodynamic diameter of nanosensors and corresponding SEM image (inlet). (B) Release profile of MBs from nanosensors in PBS, cell culture medium, and acetate buffer at pH 5. (C) Hybridization assay of MBs released from nanosensors in cell culture medium with complementary target sequences. (D) Hybridization assay of MBs released from nanosensors in PBS with complementary target sequences. $n \geq 4$, *** indicates $p < 0.001$ and error bars denote standard deviation.

Figure 3-3: Encapsulation characterization of MB-loaded nanosensors: A) Calibration curve of fluorescence intensity against MB concentration. B) Encapsulation efficiency for five different batches.

Encapsulation efficiency of MB nanosensors for 5 fabrication batches. $n \geq 4$ and error bars denote standard deviation.

Successful cell labeling with MB nanosensors

Applying the fabricated nanosensors to the hMSCs, firstly we evaluated their cytotoxicity to determine suitable labeling conditions (i.e. concentration in culture medium). Initially, cell viability after 24 hours nanosensor incubation was evaluated, with concentrations ranging between 0 to 5 mg/ml. As shown in Figure 3.4A, only the highest concentration tested (5mg/ml) shows significant influence to cell viability. Subsequently, the proliferation of MSCs following nanosensor labeling was studied. Figure 3.4B reveals a similar pattern, that nanosensor labeling at concentration lower than 5mg/ml did not adversely impede stem cell proliferation.

Encapsulating green fluorescence MB probes targeted against β -actin mRNA, the nanosensor delivery efficiency was compared relative to control bolus delivery via SLO-mediated pore permeation. As shown in Figure 3.5A, comparable green fluorescence signal could be observed from the two groups, significantly stronger than when non-targeted nanosensors was used (encapsulating scrambled MBs with no known mRNA target). Thereafter, MSCs were incubated with nanosensors in the same concentration range as in cytotoxicity assay and fluorescence intensity following which were quantified. Figure 3.4C shows that signal intensity reached a plateau as nanosensor concentration exceeds 2.5mg/ml. Based on these results (cytotoxicity and cellular nanosensor loading studies), the concentration of 1 mg/ml was chosen for all subsequent experiments due to low cytotoxicity and reasonable fluorescence signal.

To further quantify nanosensor loading, labeled MSCs were trypsinized and analyzed with flow cytometry (Figure 3.5B). Cells labeled with bolus naked MBs through Streptolysin O-reversible permeabilization method showed 75.5% positively-expressing cells. Nanosensor resulted in comparable labeling efficiency with 74.4% fluorescent cells. High signal specificity of nanosensor was further verified with the low percentage of MSCs showing positive signal when scrambled

MB was encapsulated instead (1.21%). Given that nanosensor surface chemistry does not change regardless of loaded MBs, such low signal is believed due to the lack of mRNA target. Without proper hybridization, released MBs remained weakly fluorescent.

Intracellular localization of MB nanosensors after internalization was confirmed through confocal imaging, in which plasma membrane was stained beforehand with lipophilic fluorescent dye (DiB). As shown in Figure 3.5C, green MB fluorescence mainly resided within the blue boundary which demarcates the cytoplasmic region.

At this stage, a critical question was brought up regarding internalization mechanism of these nano/microparticles. To address this, various inhibitors targeted at distinct uptake pathways were added prior and during the labeling process. Briefly, Sodium azide and 2-deoxy-D-glucose prevents all active uptake. Chlorpromazine and genistein block clathrin-mediated and caveolae-mediated endocytosis. Amiloride inhibits micropinocytosis while cytochalasin D blocks phagocytosis. Figure 3.6 and Figure 3.7 shows that sodium azide and 2-deoxy-D-glucose blocked the particle internalization almost completely (96.36%), which indicates an energy-dependent internalization. Of the individual pathway, cytochalasin D resulted in the greatest signal decline (87.41%), suggesting that phagocytosis is the main mechanism of nanoparticle uptake.[176, 177] Nonetheless, all the other pathways are contributors to internalization, as observed with signal decrease following treatment with specific inhibitors.

*Figure 3-4: Cytotoxicity study of nanosensors. A) Viability assay of MSCs after 24hr nanosensor labeling. B) Proliferation assay of nanosensor-labeled MSC at day 3 and 6. C) Fluorescence intensity of MSCs following nanosensor labeling at different concentrations. $n \geq 4$, * indicates $p < 0.05$, N.S indicates not significant ($p > 0.05$) and error bars denote standard deviation.*

Figure 3-5: MSC labeling with MB-NPs. (A) Representative images of unlabeled MSCs, MSCs treated with β -actin MB-SLO, β -actin nanosensors or scrambled nanosensors. B) Flow cytometry analysis of A). C) Confocal image showing intracellular localization of nanosensor

Figure 3-6: Normalized intensity of MSCs, following nanosensor incubation with or without specific endocytosis inhibitors. $n \geq 4$, *** represents $p < 0.001$ and error bars denote standard deviation.

Figure 3-7: Representative images of MSCs following nanosensor labeling with or without specific endocytosis inhibitors. Scale bar: 100 μ m.

Prolonged MB monitoring window from nanosensor delivery

Following successful demonstration of nanosensor labeling, we then evaluated nanosensor signal persistence or intracellular retention. MSCs labeled with β -actin nanosensors were examined longitudinally as compared to MSCs transfected with bolus MBs through SLO delivery method (same quantity of MBs was delivered) (Figure 3.8). At the beginning, similar fluorescence signal and labeling percentage was observed in both groups (Figure 3.9A).

Nonetheless as time goes to day 8, signal retention from nanosensor group was evident. When compared to that of SLO group which showed dramatic decrease in labeled percentage (from 75.5% to 3.5%) with about 75% decrease in intensity, nanosensor group retained significantly better labeled percentage (from 74.4% to 55.8%) and signal intensity (20% intensity reduction) (Figure 3.9B).

Subsequently, to confirm whether observed fluorescence was specific and not due to intracellular disruption by nucleases, the same experiment was repeated using scrambled (non-targeted MBs). As shown in Figure 3.10, minimal signal was observed in cells treated with nanosensor or SLO group. Therefore, fluorescence signal observed in Figure 3.8 is validated to be a result of correct hybridization of released MBs with β -actin mRNA.

Figure 3-8: Representative images of MSCs labeled treated with β -actin nanosensor (A) and β -actin MB SLO (B) at day 1, 4 and 8. Scale bar: 100 μ m.

Figure 3-9: Prolonged signal retention of nanosensors. (A) Flow cytometry result of β -actin nanosensor (left) or MB-SLO treated MSCs at 4 hour and 8 day post-

treatment. (B) Signal intensity analysis. ***: $p < 0.001$ between nanosensor and MB-SLO group at individual

Figure 3-10: Longitudinal imaging of MSCs treated with scrambled nanosensors and MB-SLO. Representative images of MSCs at day 1, 4 and 8 after labeling with scrambled nanosensors (A) or treatment with scrambled MB-SLO (B). (C) Signal quantification of A&B. Scale bar: 100 μ m. Error bars denote standard deviation.

Discussion

Taking β -actin mRNA target for proof-of-concept, the development of nanosensor to enable continuous, long-term tracking of mRNA expression is reported. Aiming towards wide adaptability and biocompatibility of nanosensor, MB probes were encapsulated within FDA-approved PLGA nanocarrier. Prepared nanosensor showcased sustained release of MBs for a 4-week period under three buffer conditions. In culture medium, only 25% MBs were liberated from the nanoparticles even after 4 weeks. This slow release minimizes the loss of MBs from nanosensors during the labeling stage. Crucially, encapsulated and released MBs

maintained specificity to β -actin target, allowing accurate representation of target mRNA expression intracellular.

The advantage of using nanocarrier instead of introducing bolus naked small molecules is the prolonged residence of nanocarrier within the cytoplasm.[172, 173] In this study, comparison between bolus, naked MBs and nanosensor delivery confirmed such hypothesis. 8 days post-labeling, nanosensor-treated MSCs retained its fluorescent well in comparison to that of naked MBs group. This is likely due to rapid clearance of MBs from intracellular space by nucleases degradation followed by exocytosis. In turn, bolus delivery of naked MBs would require repeated probes introductions[22], which is not only disruptive but also cytotoxic.[168]

It is worth mentioning that there was a decline in fluorescence signal from nanosensor-labeled MSCs during the longitudinal monitoring (Fig 3.9), albeit to a much lesser degree than that in naked MBs group. This is likely due to a reduced concentration of MB probes available for hybridization in the cytosolic region, as nanosensors loading would be compromised after some period of time by cell division and exocytosis. Photo-bleaching effect from repeated imaging could also contribute to this decline. Nonetheless, given the steady intensity drop over time, signal normalization strategy could be employed for an accurate comparative study (i.e. one experimental and one control target).

3.4. Conclusion

In conclusion, we have demonstrated the usage of polymeric nanocarrier to encapsulate MB probes and enable prolonged monitoring of cellular mRNA expression. The biodegradable polymeric carriers act as MB reservoir, which facilitates sustained release of intact and functional MBs for 4 weeks. Using β -actin housekeeping gene as target mRNA, longitudinal observation of fluorescence intensity showcased prolonged availability of MB probes within the cytosolic for at least 8 days in self-renewing MSCs. In the future, such mRNA nanosensor can be applied to track various cell phenotype changes such as differentiation processes.

3.5. Acknowledgement

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Chapter 4 Nanosensors for Continuous and Noninvasive Monitoring of Mesenchymal Stem Cell Osteogenic Differentiation

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4.1 Introduction

Mesenchymal stem cells (MSCs) have been increasingly adopted for cell therapy and regenerative medicine approaches. Often coupled with custom-tailored scaffolds which provide structural support, MSCs are prime cell source for bone tissue engineering and repair.[181, 182] One important aspect which determines the efficacy of MSC therapeutics is the state and functionality of transplanted cells. These include but not limited to recruitment and homing to injury sites, and differentiation towards osteo-lineage cells (e.g. osteoblasts).[183]

Conventionally, differentiation status of stem cells is determined through evaluation of protein and gene expression through disruptive antibody staining or polymerase chain reaction (PCR). As such, animal subjects sacrifice is required to obtain tissue of interest. The same is true for cell dislodgement from scaffold system culture.[184, 185] Consequently, these analyses only provide a single snapshot, and the same sample cannot be longitudinally tracked.

Alternative methods involve utilization of fluorescent or luminescent reporter constructs which can be integrated into the cells to non-invasively report/validate the expression of gene markers in real time. Nevertheless, such technique is costly, tedious to develop and may introduce random mutagenesis.[186-188] With regards to this, development of a facile and non-integrative platform to provide real-time information on MSCs differentiation status greatly accelerates the development as well as optimization of MSCs-based regenerative approaches, inclusive of bone tissue engineering.[22]

Molecular beacons (MBs), oligonucleotide-based molecular probes which can hybridize and report the presence of specific mRNA sequences are a promising tool to meet such criteria to enable non-invasive, real-time gene expression tracking.[23, 189] However, its applications are typically limited due to the short monitoring window, with respect to nucleases degradation inside the cells.[167] In a previous effort of implementing MBs to monitor MSCs osteogenesis in a longitudinal fashion, multiple rounds of MB introduction were required (i.e. once every 2-3 days).⁹ In this regards, the repeated transfection procedures may compromise cell viability significantly.[190]

On this note, polymeric nanoparticles have in the past decade gained significant attention as carriers for sustained intracellular release/delivery. One particular example is the biocompatible and biodegradable nanoparticles from poly (lactic-co-glycolic) acid (PLGA) which are approved by US Food & Drug Administration (FDA) and have been widely adapted for *in vitro* and *in vivo* delivery of drug or imaging probes. Notably, small interfering RNAs (siRNAs) and iron oxides have been used in tandem with PLGA nanoparticles for cancer treatment or magnetic resonance imaging (MRI)-based cell tracking.[174, 191]

As the PLGA shell gradually degrades within the cytosolic region, sensor or drug moieties are liberated to the cytoplasm to subsequently perform its function. Incorporation of MBs within such PLGA nanoshell (termed nanosensor), enable sustained release of MB probes into the cytoplasm as compensation for MBs which are lost due to cellular elimination. In turn, prolonged yet accurate tracking of mRNA expression could be performed.[192]

Stemming from our previous characterization work, this study evaluates such nanosensor application in monitoring MSC osteogenic differentiation (Figure 4.1). Herein, the proposed osteogenesis nanosensor contains two types of MB probes. One is designed to target a housekeeping gene of gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), while another is aimed at the early osteogenesis marker, alkaline phosphatase (ALP). By obtaining signal ratio between the functional ALP MBs and the reference GAPDH MBs, the extent by which cells are undergoing osteogenic differentiation could be monitored throughout the induction period. To demonstrate its versatility in reporting osteogenesis processes, nanosensors are applied to investigate osteogenic differentiation of MSCs on a conventional 2D culture as well as on tricalcium-phosphate / polycaprolactone (TCP/PCL) films.

Figure 4-1: Utilization of osteogenic nanosensor for tracking early stage osteogenesis in MSCs. Co-incorporation of two MB types allow simultaneous monitoring of functional biomarker ALP mRNA and reference GAPDH mRNA. Signal tracking of both MBs throughout the differentiation period allows confirmation of early osteogenesis.

4.2 Materials and Methods

Materials, except those mentioned otherwise, were purchased from Sigma-Aldrich. Primers used in PCR study were verified using basic local alignment search tool (BLAST) and obtained from AIT Biotech (Table 4.1). Dulbecco's modified eagle medium (DMEM) with high glucose (4.5 g/L) and MSC Osteogenic Differentiation Bullet Kit medium were purchased from Lonza. Fetal Bovine serum (FBS), trypsin-EDTA and penicillin-streptomycin (PS) were obtained from Gibco. TRIzol reagent was purchased from Invitrogen. Moloney Murine Leukemia Virus (MLV) Reverse Transcriptase was purchased from Promega while iQ Sybr Green Supermix was from Bio-Rad.

Synthesis of Nanosensors:

Sequences of GAPDH and ALP MBs were adopted from a previous study and verified for its specificity using BLAST. The MB sequences are listed in Table

4.1. Nanosensors were prepared adopting our previous study.[192] Briefly, water solution containing 1 nmol of ALP MBs or ALP & GAPDH MBs (0.5 nmol each) was homogenized into PLGA-chloroform solution (2mg/ml concentration). Following which, the mixture was homogenized again into 3% polyvinyl alcohol solution (PVA, 1:5 v/v ratio). Finally after chloroform evaporation, nanosensors were collected through centrifugation (10krpm) and lyophilized for storage at -80°C.

Table 4.1 Sequences of MBs, MB targets and primers used in this study

MBs, MB targets and primers	Sequences (5'–3')
ALP MB ^{a)}	[Flc]-CGCTCC-AGAGTGCTCTCCGAGGAG-GTCAA-GGAGCG-[BHQ1] ^{b,d)}
GAPDH MB ^{a)}	[Cy3]-CGACG-GAGTCCTCCACGATACCA-CGTCC-[BHQ2] ^{c,d)}
ALP target	TTGACCTCCTCGGAAGACACTCT
GAPDH target	TGGTATCGTGGAAGGACTC
ALP primers	Forward: ACGTGGCTAAGAATGTCATC Reverse: CTGGTAGGCGATGTCCTTA
GAPDH primers	Forward: GAAGGTGAAGGTCGGAGT Reverse: GAAGATGGTGATGGGATTTC

^{a)}MB: molecular beacon; ^{b)}Flc: fluorescein; BHQ1: black hole quencher 1; ^{c)}Cy3: cyanine 3; BHQ2: black hole quencher 2; ^{d)}Stem regions of MBs are underlined, flanking the middle loop regions.

Characterization of Nanosensors:

MB fluorescence calibration curve was generated for ALP and GAPDH MBs by treating solutions with known MB concentrations with excess DNase I (40U/mL) for 15 min of hairpin cleavage & fluorescence restoration. Fluorescence intensity was assessed using Spectramax M5 microplate reader and plotted.[25] Morphology of nanosensors was visualized using JSM-6700F (JEOL) scanning electron micrography (SEM at 5kV) after plasma gold coating.

MB encapsulation efficiency inside the nanosensor was calculated by measuring leftover MBs in the supernatant following nanoparticle preparation (remaining unencapsulated MBs). Briefly, all solutions during nanosensor synthesis were treated with excess DNase I (40U/mL, 15 min) and read for their fluorescence. Then, measured intensity was converted to MB concentration by fitting back against the calibration curve. Encapsulated MBs were finally calculated by deducting these MB concentrations from the initial amount used. Supernatants from at least five independent fabrication batches were analyzed.

Release profile of MBs from nanosensors were determined by dispersing single/dual-loaded nanosensors in PBS or culture medium at 37°C and collecting the supernatants at designated timepoints (e.g. day 1, 2, 3, 4, 6, 9, 12, 15, 21 and 28). MB concentration at each timepoint was determined as described above by applying DNase I and back-fitting against the calibration curve. Importantly, retention of MB functionality following encapsulation and nanosensor release was evaluated by examining release supernatants' fluorescence, before and after the addition of correct oligonucleotide sequences (0.5 μ M).

Labeling of MSCs with nanosensors:

Bone marrow-originated MSCs (Lonza) were cultured in high glucose DMEM supplemented with 10% FBS and L-glutamine in 37°C, 5% CO₂ condition. Cell labeling was performed when seeded MSCs reach >80% in confluency. Nanosensors were premixed with poly-L-lysine (PLL) solution (0.01% w/v) for 20 min and centrifuged to remove excess PLL. Coated nanosensors were added to culture medium at 1mg/ml and incubated with MSCs for 18hr. Excess nanosensors were subsequently rinsed with PBS prior to further analysis or imaging. Cytotoxicity of nanosensors was evaluated in terms of viability and proliferation rate directly or few days post-labeling (trypan blue counting, N $\geq$ 6).

Fabrication of TCP-containing PCL films:

TCP-containing PCL films were prepared in accordance with previous protocol.[193] Briefly, PCL and TCP powder (65:35 weight ratio) was placed between two stainless steel on Carver system. Then, 25 kN loading and 80°C heating was applied for 30 min to press the powders into a thin film. Pressed film was given sufficient time to cool down via normal room temperature convection. Thickness of generated film was controlled about 30-60 µm.

Nanosensor-facilitated monitoring of MSCs osteogenesis on normal plates and TCP/PCL films:

For cell seeding, PCL-TCP films were treated for 3 hr with 5M NaOH, before being glued to 12mm glass coverslips using silicone sealant and sterilized. 40k MSCs were seeded and left for 24hr incubation prior to nanosensor labeling and osteogenic induction with differentiation medium. At particular time points (day 0, 1, 3, 7, 10, 14 & 18), cells were imaged under fluorescent microscope. For comparison purposes, the same procedures were repeated on normal 24-well plate.

Fluorescence microscopic imaging:

Fluorescent images of cells were obtained using LX71 (Olympus) inverted microscope with Retiga-2000R camera. Exposure settings were fixed at 500ms and gain 5x for all images taken. Subsequently, ImageJ was used to process and quantify signal intensity of cells.

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR):

MSCs undergoing osteogenesis were collected at specific time points (day 0, 1, 3, 7, 10, 14 & 18) into the differentiation and lysed using TRIzol. cDNA conversion was done using RT kit M-MLV RNase H(-) Mutant. Primers used in qPCR (CFX Connect, Biorad) are listed in Table 4.1. CT values obtained for ALP of individual samples were translated to fold expression through $2^{-\Delta\Delta Ct}$ formula.

Von Kossa validation staining:

3 weeks post osteogenic differentiation, MSCs were rinsed with PBS, fixed with 10% formalin and washed thoroughly with water. Cells were then stained with fresh 2% silver nitrate solution for 10min in the dark, before 15min exposure to strong light. Bright field images of stained samples were taken with LX71 (Olympus) inverted microscope with Retiga-2000R camera at 100x magnification.

Statistical analysis:

Analysis of variance (ANOVA) one way was performed to evaluate significance of signal and RT-qPCR fold differences (p values). Suitable post-hoc analysis was also performed on SPSS statistics software (IBM). Data presented here are all average values of ≥ 3 independent experiments.

4.3 Results and Discussion

Nanosensor Characterization

Synthesized nanosensors encapsulating two types of MBs targeting both ALP and GAPDH mRNA were firstly characterized. Following coating with PLL, nanosensors were roughly about 1 μ m in diameter with surface potential of 16.3 $\pm$ 5.7 mV (Figure 4.2A). Linear calibration curves to correlate MB concentration with fluorescence intensity was generated and subsequently used to determine its encapsulation efficiency within the nanosensors (Figure 4.3A&B). Encapsulation of MBs was observed to be between 75-80% for both types (Figure 4.3C).

Then, release profile of both MBs was studied by dispersing nanosensors in PBS and culture medium, following aforementioned MB concentration quantification. In accordance to our hypothesis, release profiles were similar for both MBs given their similar molecular weight, in both solution (Figure 4.2B). In PBS, ~30% MBs were liberated during the first 48hr before additional 40% were released

over the next 19 days. Meanwhile in culture medium, only 30% MBs were released throughout the 3 weeks. Such retardation of release is probably due to adsorption of serum proteins on nanosensors' surface, which prevented water interaction and thus hydrolysis.^{21,22} In both cases, nanosensor served its intended purpose for sustained cytosolic delivery of MBs, sufficient to cover the osteogenic induction period (3 weeks).

One important matter that we later checked is whether MB functionality was maintained following the encapsulation and release. For this, supernatants collected at particular timepoints was mixed with the complementary oligonucleotides and checked for their fluorescence. As shown in Figure 4.2C&D, hybridization of correct target sequences resulted in 5.13 and 5.03-fold greater fluorescence intensity (for ALP MB: 485/515nm and GAPDH MB: 550/570nm, respectively). Furthermore, it is noteworthy that addition of one target sequence influences only its corresponding MB, without affecting the other MB. (Figure 4.3D) This confirmed the preservation of MB hairpin structure during nanosensor encapsulation, which are followed by linearization and signal restoration upon specific hybridization.

Figure 4-2: Physical characterization of nanosensors. A) SEM images of fresh nanosensors (i) and nanosensors after 30-day release study (ii), scale bar: 2 μm . B) Release profile of ALP and GAPDH MB from nanosensors in PBS and culture medium. Hybridization assay of ALP (C) and GAPDH nanosensor (D) release supernatant with respective complementary oligonucleotides. $n \geq 4$ and error bars denote standard deviation.

Figure 4-3: Characterization of nanosensors loading. Fluorescence calibration curve versus DNase I-treated ALP MB (A) and GAPDH MB (B) concentration. C) Nanosensor encapsulation efficiency of 5 batches. D) Fluorescence intensity of day 28 nanosensor release supernatant with or without target oligonucleotides addition. $n \geq 4$ and error bars denote standard deviation.

Nanosensor labeling of MSCs

Following its physical characterization, nanosensor was loaded to MSCs following overnight incubation and evaluated. With successful hybridization of liberated GAPDH MBs and constitutively-expressed GAPDH mRNA, labeled MSCs expressed strong Cy3 fluorescence signal directly after labeling. In contrast, minimal FITC-channel fluorescence was seen from ALP MB, indicative of weak ALP mRNA expression in undifferentiated MSCs (Figure 4.4A).

In order to achieve most effective monitoring of osteogenesis process, labeling conditions were optimized. Firstly, it is worth noting that PLL coating enhanced nanosensor internalization, presumably from facilitating electrostatic interaction between the positively-charged nanosensor and negatively-charged cell membrane (Figure 4.5A&B). Influence of labeling concentration to signal generation

was studied alongside its effect on cell viability and proliferation rate. As shown in Figure 4.5C, steady increase in GAPDH MB fluorescence was seen when labeling concentration was varied between 0.1-2 mg/ml. Beyond 2mg/ml however, fluorescence intensity plateaued, most likely due to saturated nanosensor uptake. In addition, cytotoxic effect was also observed from 4 & 6mg/ml concentration in >10% loss of cell viability and inhibited proliferation rate (Figure 4.5C&D).

Considering these facts, cell labeling condition at 2mg/ml was used in subsequent studies, to maximize signal generation while preserving healthy cell condition. At such concentration, flow cytometry analysis showed 81.8% of cells were successfully labeled (positive GAPDH MB fluorescence). Interestingly, only 1.6% of cells expressed positive FITC channel (ALP MB), underlining the specific signal generation in undifferentiated MSCs (Figure 4.4B).

Based on previous studies,[174, 179] nanoparticles in size range between 500nm to 2 μ m can be retained within MSCs for 3 weeks. This is confirmed here by the relatively stable GAPDH MB fluorescence throughout the 18-day culture period (Figure 4.4C). Importantly, minimal difference was observed between GAPDH MB signal on undifferentiated and osteo-induced MSCs (Figure 4.4D). As such, GAPDH MB can serve as internal reference to provide a more accurate readout (i.e. taking into consideration possibility of unequal nanosensor internalization in individual cells). ALP MB signal was then normalized to that of GAPDH MB as a way to semi-quantitatively evaluate osteogenic differentiation of labeled MSCs.

As revealed in Figure 4.6A&B, normalized ALP MB/GAPDH MB signal ratio showed a sudden jump at day 7 into the differentiation induction, with 3-fold signal as compared to day 3. Following which, signal ratio declined and finally stabilized at 2-fold signal of day 3. To validate such signal profile, gold-standard analysis of RT-qPCR was done to obtain $2^{-\Delta Ct}$ expression in differentiating group over the induction period (Figure 4.6C). Briefly, ALP mRNA was confirmed to peak at day 7 with double the expression at day 3, before returning steadily to just above day 3.

Crucially, when nanosensor signal ratio (ALP/GAPDH) at specific time point was plotted against its corresponding mRNA expression from RT-qPCR, good correlation value was obtained between the two parameters ($R^2 = 0.812$; Figure

4.6D). This signifies sensitivity and specificity of the proposed osteogenic nanosensor to monitor dynamic early osteogenesis marker in ALP mRNA. Specificity is further demonstrated by the consistent low ALP MB signal on undifferentiated MSCs throughout the 18-day period (Figure 4.7; minimal non-target unquenching of MBs).

Figure 4-4: Nanosensor labeling and retention in undifferentiated and osteo-induced MSCs. A) Fluorescence from nanosensor-labeled undifferentiated MSCs (i: ALP FITC channel, ii: GAPDH Cy3 channel, iii: phase contrast, iv: merged), scale bar: 10µm. B) Flow cytometry analysis of unlabeled and nanosensor-labeled MSCs. Representative fluorescence images (C) and GAPDH Cy3 signal quantification (D) of nanosensor-labeled osteo-induced and undifferentiated MSCs (scale bar: 100µm). Error bars denote standard deviation.

Figure 4-5: Optimization of nanosensor labeling. A) Representative images of MSCs labeled with uncoated or PLL-coated nanosensors at 2 mg/ml (red: GAPDH Cy3 MB, blue: Hoechst33342 nuclear staining, scale bar: 100 μ m). B) Fluorescence quantification following labeling with uncoated or PLL-coated nanosensor. Viability / fluorescence intensity (C) and proliferation rate (D) of MSCs after PLL-coated nanosensor labeling at different concentration. $n \geq 4$ and error bars denote standard deviation.

Figure 4-6: Monitoring of early osteogenesis by non-invasive nanosensor tracking. Representative images (A) and normalized signal quantification (B) of nanosensor-labeled MSCs undergoing osteogenic differentiation, scale bar: 100 μ m. C) $2^{-\Delta Ct}$ expression of ALP to GAPDH mRNA by RT-qPCR. D) Correlation plot between normalized nanosensor intensity with $2^{-\Delta Ct}$ expression at corresponding timepoints. Error bars denote standard deviation.

Figure 4-7: Monitoring ALP mRNA expression on osteo-induced MSCs and undifferentiated MSCs. A) Representative fluorescent images (scale bar: 100 μ m).

B) Signal quantification. *** represents $P < 0.001$ and error bars denote standard deviation.

Nanosensor tracking of Osteogenic Differentiation of MSCs on PCL-TCP Films

With the background that most tissue engineering approaches (including bone) involve the usage of scaffold constructs to either direct or promote stem cell differentiation into functional mature cells,[181, 182] we then tested nanosensor ability to report early osteogenesis of MSCs on osteo-inductive tricalcium phosphate-containing poly caprolactone (PCL-TCP) films.

Based on both normalized nanosensor signal and mRNA expression profile by RT-qPCR, MSCs undergoing osteogenesis on PCL-TCP films showed earlier ALP peak timing as compared to MSCs seeded on normal 2D culture plate (Figure 4.8A, 4.9 & 4.10). On PCL-TCP films, peak timing was observed on day 3 instead, albeit at a lesser value (1.21-fold to 1.49 of that on 2D plate; Figure 4.11). This trend is confirmed through RT-qPCR assay, with good correlation obtained when normalized nanosensor intensity was plotted against mRNA expression ($R^2=0.939$; Figure 4.8B).

Such observation is in accordance with previous literatures, which showed enhanced MSCs osteogenesis and faster mineralization on TCP-containing PCL films (35% w/w) than on normal PCL films.[193] TCP additives play an important role in enhancing cellular- extracellular matrix (ECM) interactions through $\alpha_2\beta_1$ integrins, which in turn activates mitogen-activated protein kinase (MAPK) / extracellular-related kinase (ERK) pathway, leading to upregulation of transcription factors involved in osteogenesis such as Runx2.[194] In consequence, this translates to stimulation of ALP promoter which substantiates our result.[195]

Finally, von Kossa staining was used to evaluate calcium deposition from MSCs undergoing osteogenesis. This served as a validation tool of successful osteogenic differentiation, and whether nanosensor labeling have hindered the

differentiation process. As shown in Figure 4.12, both unlabeled and nanosensor-labeled groups exhibited significant ECM calcification, while negligible calcium deposition was seen on undifferentiated MSC groups. This suggests that nanosensor labeling and routine imaging did not significantly interfere the osteogenesis of MSCs.

Figure 4-8: Monitoring of osteogenic differentiation on TCP-containing PCL films. A) Normalized nanosensor intensity (left axis) and corresponding $2^{-\Delta C_t}$ expression from RT-qPCR (bars, right axis). B) Correlation plot of the two parameters in A. $n \geq 4$ and error bars denote standard deviation.

Figure 4-9: Representative images of osteo-induced MSCs seeded on TCP-containing PCL films (scale bar: 50 μ m).

Figure 4-10: Representative images of osteo-induced MSCs seeded on normal culture plates (scale bar: 50 μ m).

Figure 4-11: Comparison on MSCs undergoing osteogenic differentiation on normal culture plates and on TCP-containing PCL films. A) Normalized nanosensor intensity. B) $2^{-\Delta C_t}$ expression from RT-qPCR. $n \geq 4$ and error bars denote standard deviation.

*Figure 4-12: Von kossa staining verification of MSCs osteogenesis, on either unlabeled or nanosensor-labeled cells. Representative images (A) and quantification of staining (B). *** represents $p<0.001$. $n\geq 4$ and error bars denote standard deviation.*

Discussion

Stem cell differentiation procedures typically result in heterogenous cell population. Therefore, to achieve maximum cell therapeutic efficacy without undesirable risks/ effects (i.e. teratoma formation, incorrect differentiation), it is important to ascertain successful differentiation. One popular way for this is through verification of biomarker expression (e.g. through RT-qPCR).[196, 197] However, such technique is destructive as it requires mRNA extraction. Additionally, as a

population-averaged assay, it is impossible to distinguish the presence of a minor subpopulation of cells which remained undifferentiated. The development and incorporation of gene-reporter systems provides a mean to non-invasively track the expression of differentiation gene markers.[198] Nonetheless, such technique is also limited with its laborious and technically-demanding development, alongside risks of introducing random mutagenesis.[199, 200]

The proposed nanosensor is easy to prepare and can enable versatile tracking applications given its effective internalization. Thus, it provides a facile alternative to non-destructively verify stem cell differentiation.[201, 202] In particular, we focused on verifying successful early osteogenesis of MSCs, by utilizing the versatile nanosensor to track early osteogenesis marker (ALP mRNA) with a direct comparison with housekeeping gene (GAPDH mRNA). Linear correlation observed between nanosensor signal ratio and mRNA expression from gold-standard qPCR assay indicates the accuracy of this tracking platform.

Correspondingly, the osteogenic nanosensor were applied to verify MSC osteogenesis on biomaterial substrates. In particular, TCP-containing PCL films (35% w/w) which was reported to promote osteogenic differentiation was chosen as a proof-of-concept. Here, while not directly reflected through the observable peak nanosensor signal ratio, faster timing to peak expression was noted for MSCs seeded on TCP-containing PCL films. The ability to non-invasively report osteogenesis warrants nanosensor potential application as a tool enabling high-throughput screening for optimization of culture scaffolds and/or conditions.

Moreover, such nanosensor strategy can be readily extended to track and validate later-stage differentiation markers (e.g. osteocalcin for osteogenic differentiation).[203] By multiplexing more MB types into the nanosensor, it is conceivable to perform multi-staged differentiation monitoring. Lastly, incorporation of MBs at infrared window can empower deeper observation for MSCs on thicker bio-constructs, or even for *in vivo* implanted constructs (e.g. cranial defects).

4.4 Conclusion

In summary, this present work reports a MB nanosensor to enable non-invasive, real-time monitoring of early-stage osteogenesis of MSCs. As the rate and thus extent of differentiation differs significantly in accordance with culture conditions given, it is important to verify stem cell commitment towards the osteogenic lineage prior to implantation of construct. Unlike conventional methods which are disruptive (e.g. western blot, RT-qPCR) or highly laborious to develop (gene-reporter systems), the proposed nanosensor provides a rapid, facile yet safe alternative.

Importantly, nanosensor was shown to have sensitivity and specificity comparable to that of RT-qPCR, from the close to linear correlation plot between nanosensor signal intensity and cellular mRNA expression. Furthermore, the presence of an internal reference signal prevents mis-interpretation of signal intensity (e.g. interference from background signal). In the future, it is expected that such nanosensor monitoring can be extended to even in vivo-implanted constructs, as to evaluate and verify cell-based regenerative approaches.

4.5 Acknowledgements

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Chapter 5 Nanosensors for Non-invasive Monitoring of 3D Chondrogenic Constructs

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5.1. Introduction

Cartilage tissue engineering (TE) consists of techniques to regenerate injured / damaged articular cartilage. This concept typically involves constructs implantation, incorporating scaffold, chondrogenic cells, with or without bioinductive-factors.[204] Until now, only a few TE-based strategies have reach clinical investigation stage such as the implantation of autologous chondrocytes in conjunction with fibrin glue or minced cartilage supported by polymers.[205]

Autologous cells are preferred for cartilage tissue reconstruction to ensure minimal immune rejection. Nonetheless, such approach necessitates highly invasive cartilage biopsy which implicates donor morbidity. Furthermore, collected chondrocytes are often insufficient in number thus requiring *ex vivo* expansion beforehand.[205] Meanwhile, culture of mature chondrocytes is tedious with its slow proliferation rate and is susceptible to cell de-differentiation.[206, 207] These issues have led to the development of mesenchymal stem cells (MSCs)-based chondrogenesis approaches, involving cues from cell microenvironment and/or growth factors.[208] For example, three-dimensional (3D) hydrogel scaffolds which resemble native cartilage environment (e.g. agarose gel) supports chondrocyte-like rounded morphology and allows deposition of neo-cartilage matrices.[209, 210] At the same time, factors like transforming growth factors super family (TGF β 3) are commonly employed to induce MSCs chondrogenesis and collagen deposition.[211]

Conventionally, quantitative polymerase chain reaction (qPCR) is adopted to evaluate cellular expression of chondrogenic markers (e.g. collagen 2/ Col2 mRNA). Meanwhile, histology followed by immunohistochemical staining are usually done to assess deposition of cartilage extracellular matrices. With these end-point analyses however, sample disruption is inevitable and no possibility for real-time monitoring of chondrogenesis.[212, 213] One alternative involves incorporation of reporter gene technology, in which fluorescent or luminescent signal is generated upon activation of gene-of-interest.[214] Risks of insertional mutagenesis following gene integration however, render transformed cells unsuitable for clinical usage.[215]

To this end, nanosensors hold potential to provide a safe, non-disruptive and highly efficient method for continuous monitoring of MSCs chondrogenesis. Flexibility and tunable properties of nanocarriers allows loading of wide molecular probes to facilitate real-time cell tracking, and over a longer observational period as compared to when naked probes are utilized.[216] Additionally, nanosensors enable visualization in various imaging modalities, including but not limited to optical imaging and magnetic resonance imaging (MRI).[216, 217] Nonetheless, majority of nanosensors application are focused on providing insight to cells' spatial localization or biodistribution, and very few have tapped on observing stem cell differentiation.[218]

Recently, our group has explored the development of smart nanosensor to non-invasive and continuously monitor cell differentiation over a 4-week period.[219] Such smart nanosensor is prepared by incorporating molecular beacons (MBs) inside biodegradable poly(lactic-co-glycolic acid) (PLGA) nanocarriers (MB-NPs). Following cell internalization, PLGA degrades gradually to release MBs into the cytoplasm.[192] Released MBs would then hybridize with target intracellular mRNA to regain fluorescence from their pre-quenched state and provide semi-quantitative report on its expression. Importantly, the sustained release of MBs from nanocarriers overcomes the previously reported limited intracellular monitoring window of MB due to rapid cellular degradation and/or clearance.[220, 221] In consequence, precise long-term monitoring of mRNA expression is enabled, including a 3-week observation of MSCs osteogenesis.[222]

In the current study, we explore the application of this nanosensor to monitor MSCs chondrogenesis in a 3D culture setting (agarose hydrogel). In particular, MBs carrying distinct fluorophores and targeting mRNAs of articular cartilage-specific collagen II (Col2) and housekeeping glyceraldehydes-3-phosphate dehydrogenase (GAPDH) were loaded into PLGA particles. Dual MB encapsulation (Col2 MB: FITC channel & GAPDH MB: Cy3 channel) enables accurate assessment of chondrogenic Col2 marker expression. Herein, taking into consideration heterogeneity in nanosensor internalization and retention, cell labeling was performed prior to 3D hydrogel encapsulation and chondrogenesis induction (Figure 5.1). At weekly timepoints, Col2 mRNA expression were monitored through

fluorescence imaging and signal quantification, and compared to gold-standard PCR analysis.

Figure 5-1: Schematic showing adaptation of molecular beacon (MB)-containing nanosensors for longitudinal monitoring of MSCs chondrogenesis in 3D hydrogel condition. Cells were labeled with nanosensors co-encapsulating Col2 MBs and reference GAPDH MBs prior to 3D agarose hydrogel seeding. Continuous monitoring of gene expression is then performed with confocal microscopy.

5.2 Materials and Methods

All chemicals except when specifically mentioned otherwise were obtained from Sigma-Aldrich and applied without additional purification. High and low glucose Dulbecco's modified eagle medium (DMEM; 4.5g/L or 1g/L) with L-glutamine, penicillin-streptomycin (PS, 10kU/mL), fetal bovine serum (FBS), TrypLE™ express, sodium pyruvate, insulin, proline, SYBRGreen mastermix and TRIzol reagent were purchased from Life technologies. DNase I was from Invitrogen, while ITS premix

culture supplement was purchased from Corning. Transforming growth factor $\beta 3$ (TGF $\beta 3$) protein was obtained from R&D System. SensiFast™ cDNA synthesis was from Bioline while RNeasy Kit was from Qiagen. Endothelial growth media (EGM™) was purchased from Lonza. Mouse monoclonal antibody (anti-Col2) was from Merck Millipore and Ultravision™ Quanto Detection HRP DAB kit was obtained from Thermofisher Scientific.

Synthesis and characterization of MB-NPs:

MB sequences targeted towards GAPDH & Col2 mRNA (Table 5.1) were adapted with modification from previous studies and validated with basic local alignment search tools (BLAST).[22, 223] Nanosensors were prepared through double emulsion method. In brief, 100 μ l of MB water solution (0.5 nmol each) was added to a mixture containing 10mg PLGA in 0.5ml chloroform and 5 μ l span 80 for 30s homogenization at 30krpm (Scilogex D160). This mixture was then added into 5ml of polyvinyl alcohol (PVA) solution (3% w/v) and homogenized again. Remaining chloroform was then allowed to evaporate before nanosensors were rinsed and centrifuged. Finally, nanosensors were stored at -80°C following freeze drying.

In calculating encapsulation efficiency, leftover un-encapsulated MBs in the supernatant during nanosensor preparation were quantified. In brief, these supernatants were treated with DNase I digestion (40 U/ml; 15 min) followed by fluorescence measurement. Concentration of MBs within these solutions were obtained by fitting back the fluorescence reading into MB fluorescence calibration curve. Finally, these values were deducted and normalized against initial MB concentration.

Morphology of nanosensors was examined through field-emission scanning electron microscope (FE-SEM, JEOL JSM 6007F) at 5kV after gold plasma coating. Hydrodynamic diameter and zeta potential of nanosensors in PBS buffer were evaluated through Zetasizer nano Z (Malvern).

Release profile of MBs from nanosensors

Dual-loaded nanosensors were incubated in PBS at 2mg/ml concentration and stored in 37°C for a total period of 30 days. Supernatant collection was performed at day 0, 0.5, 1, 2, 3, 4, 6, 9, 12, 15, 21, 30, before new 1ml PBS buffer was added. These supernatants were digested with DNase I treatment (40U/ml) and read with fluorescence microplate reader to similarly quantify concentration of GAPDH or Col2 MBs within each solution.

Hybridization study of chondrogenic MBs

In order to examine the specificity of both MBs, GAPDH and Col2 complementary oligonucleotides (sequence in Table 5.1) were added at the same concentration (0.5 μ M) and allowed to incubate at 37°C for 15min. Fluorescence intensity reading at corresponding MB channels (FITC: 485/517 nm & Cy3: 550/570nm) was then taken for these samples and normalized against DNase I-treated MBs (positive control) and PBS-treated MBs (negative control).

Cell culture and nanosensor labeling

Human MSCs were harvested and isolated from bone marrow samples of consented patients at National University Hospital (Singapore) following the guidance from Institution Review Board (IRB)-approved protocol.[224] Purified MSCs were expanded in low glucose DMEM containing sodium pyruvate and supplemented with 10% FBS and 1% PS. Culture medium was replaced every 2-3 days until confluency reached 90%.

Human umbilical vein endothelial cells (HUVECs; Lonza) was cultured on collagen I-coated flasks (50 μ g/ml in 20mM acetic acid; Gibco). EGM™ (Lonza) was replaced every 2-3 days.

Conditions for nanosensor cell labeling were in accordance to our previous work.[225] Briefly, 50k MSCs were seeded in each well of a 6-well plate and allowed to grow until 80% confluency. 4mg nanosensors was coated with 50 μ l poly-L-Lysine (PLL) solution (0.01%) for 20min, before dispersion in culture medium at 1mg/ml

concentration. Cells were then incubated with nanosensor solution overnight and rinsed thrice with PBS prior to further usage. Flow cytometry analysis (LSR Fortessa™ X-20; BD Biosciences) with FACSDiva software and FlowJo (Treestar) was performed to check nanosensor labeling efficiency.

Two-dimensional (2D) adipogenic and osteogenic differentiation of MSCs

100k MSCs were seeded on each of a 12-well plate and were given 24hr incubation period prior to 2D differentiation induction. Adipogenic differentiation medium consisted of high glucose DMEM with 10% FBS, 1% PS, 0.1µM dexamethasone, 10µg/ml insulin, 1mM sodium pyruvate, 200µM indomethacin and 0.5 mM 3-Isobutyl-1- methylxanthine (IBMX). Osteogenic differentiation medium comprised of low glucose DMEM with 10% FBS, 1% PS, 1% Glutamax™, 1mM sodium pyruvate, 0.1µM dexamethasone, 50µg/ml ascorbic acid and 10mM β-glycerophosphate. Cell differentiation medium was replaced every 2-3 days.

Three-dimensional (3D) Chondrogenic differentiation in agarose hydrogel

125k MSCs in 25µl culture medium were mixed with 25µl 3% agarose solution (w/v; Agarose type VII) to obtain a concentration of 2.5×10^6 cells/ml in 1.5% final agarose gel. Three hydrogel droplets (50µl) were then transferred to one well of a 12 well-plate and left to solidify for 5-10 min prior to culture medium addition. One day later, medium was replaced with chondrogenic medium which consisted of high glucose DMEM, 1% PS, 1% Glutamax™, 50 µg/ml ascorbic acid, 0.1µM dexamethasone, 1mM sodium pyruvate, 4mM proline and ITS and 10 ng/ml TGF-β3. These chondrogenic constructs were cultured for 4 weeks with chondrogenic medium replacement every 2-3 days. For comparison purposes, some chondrogenic constructs were cultured in expansion medium instead.

Confocal fluorescence imaging

Chondrogenic hydrogel constructs were imaged at weekly interval with confocal laser microscopy (LSM710; Carl Zeiss) to evaluate nanosensor signal intensity (Col2 MB FITC: 485/517nm and GAPDH MB Cy3: 550/570nm). 10µm fluorescent slices of 3D constructs were captured using Z-stack, processed with LSM Image Browser (Zeiss) and quantified through ImageJ.

To evaluate nanosensor signal localization in MSCs, cells were labeled with nanosensors carrying GAPDH MB only and counter-stained in suspension with 5µM Calcein AM (485/517nm) and 2 drops/ml of Hoescht33342 (nuclear staining: 385/461nm) for 15min before fixation with 10% formalin (while half-attached), rinsed with PBS and taken for confocal imaging.

Quantitative polymerase chain reaction (qPCR) assay

After 3 weeks of chondrogenic induction, 3D agarose cell constructs were homogenized using Trizol reagent. Following thorough mixing with chloroform, mixtures were centrifuged at 12krpm for 15min at 4°C and RNA in the aqueous phase was extracted. RNA was subsequently purified with RNeasy Mini Kit and quantified for concentration through NanoDrop 2000 UV-Vis Spectrophotometer (ThermoFisher Scientific). Reverse transcription of RNA samples was performed with SensiFast™ cDNA Kit. Finally, quantitative PCR step was performed using SYBRGreen Mastermix kit on StepOnePlus Real-Time PCR (Life Technologies). RNAs underwent 10min denaturation at 95°C, and 40 cycles of amplification (15 sec denaturation at 95°C and 1 min extension at 60°C per cycle). Sequences of primers are listed in Table 5.2. mRNA fold expression is reported after normalization with GAPDH mRNA, using C_T comparative method.

Histological and Immuno-histological analysis

Samples following differentiation procedures were fixed with 10% formalin and rinsed thoroughly with PBS. Then, samples were embedded in FSC22 Mounting Medium (Leica) and left to freeze at -20°C. Cryo-sectioning was then done with Leica CM1900 cryostat, and 7µm section slices were collected.

Immunohistochemical staining of chondrogenic samples was performed with UltraVision™ Quanto HRP DAB kit and mouse monoclonal anti-Col2 antibody.

Meanwhile, adipogenic and osteogenic 2D differentiation samples were fixed after 3 weeks and subjected to Oil Red O and Alizarin Red staining, respectively. Lipid droplets for adipogenic samples were stained with Oil Red O solution for 30s (6ml of 0.5% stock solution with 4ml distilled water). Meanwhile, mineralized osteogenic samples were stained with Alizarin Red S solution for 5min.

Statistical analysis

Each experiment reported herein was repeated at least thrice. Statistical difference in experimental groups was tested using one-way analysis of variance (one-way ANOVA) followed by Tukey HSC post-hoc test. Differences were significant when p-value is less than 0.05 (* p<0.05; ** p<0.01).

Table 5.1 Sequences of MBs and complementary MB targets used

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

Table 5.2 Primer sequences used in quantitative polymerase chain reaction (qPCR) analysis

<i>Gene</i>	<i>Forward primer sequence (5'-3')</i>	<i>Reverse primer sequence (5'-3')</i>
<i>GAPDH</i>	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
<i>Col2</i>	CCAGATGACCTTCCTACGCC	TTCAGGGCAGTGTAAGTGAAC
<i>Leptin</i>	TGCCTTCCAGAAACGTGATCC	CTCTGTGGAGTAGCCTGAAGC
<i>PPARγ</i>	TACTGTCGGTTTCAGAAATGCC	GTCAGCGGACTCTGGATTTCAG

5.3 Results and Discussion

Characterization of chondrogenic nanosensors

Nanosensors synthesized in accordance to our previous work¹⁹ results in similar nanoparticle size of 500nm-1 μ m, regardless of the change in loaded MBs (Figure 5.2A). Based on encapsulation efficiency calculation, we estimated that each nanosensor carried in average 140 strands of MB (Figure 5.2B). These nanosensors had almost-neutral surface charge (-0.06 mV) initially, but can be coated with PLL to become positively charged (16.6 mV).[222]

Firstly, we examined MB release from nanosensors in PBS for a period of 30 days, a sufficient time frame for MSC chondrogenesis. As shown in Figure 5.3A, release occurred through a two-stage mechanism. In the first stage, about half of encapsulated MBs were released in the first 4 days. Later, steady release continued until 30 days with ~30% encapsulated MBs further liberated.

Supernatant at day 30 was then hybridized with complementary oligonucleotide targets (Table 5.1). As revealed in Figure 5.3B, restoration of pre-quenched MB signal (~5-fold for Col2 MB & ~4.5-fold for GAPDH MB) demonstrates retention of MB functionality throughout the whole experimental period. When fluorescence intensity of untreated day 30 supernatant's was compared against fresh MB samples, a slight increase in fluorescence signal revealed that some MBs have lost its original hairpin structure upon NP preparation and hydrolysis (Figure 5.3B & 5.2C&D). Nonetheless, this is not expected to alter monitoring procedures significantly, as overall MB functionality is retained.

Figure 5-2: Physical characterization of chondrogenic nanosensor. A) Hydrodynamic diameter with representative SEM image. B) Encapsulation efficiency of MB probes within nanosensor. C) Hybridization study of fresh MBs with its complementary target oligonucleotides (normalized to that treated with excess DNase I). D) Fluorescence signal comparison of fresh MBs and MB supernatant at day 30. * represents $p < 0.05$. $n \geq 4$ and error bars denote standard deviation.

Figure 5-3: Characterization of chondrogenic nanosensor and labeling in MSCs. A) Release profile of MBs from nanosensor. B) Hybridization assay of day 30 MB nanosensor supernatant (normalized against DNase I-treated supernatant). C) Orthogonal confocal fluorescence image of MSCs labeled with nanosensor. D) Flow cytometry analysis showing efficient nanosensor internalization by MSCs. $n \geq 4$ and error bars denote standard deviation.

Labeling of MSCs with nanosensors

Intracellular localization of nanosensors and origin of fluorescence signal was verified by labeling MSCs with GAPDH nanosensors and counter-staining with Hoescht33342 (nuclei) and calcein AM (cytoplasm). Figure 5.3C reveals that GAPDH MB Cy3 signal was specifically localized within the cytoplasmic region, outside of the blue nuclei but within the cell membrane boundary (green).

At the same time, nanosensor labeling efficiency was analyzed by running labeled MSCs on flow cytometry. As summarized in Figure 5.3D, strong Cy3

fluorescence (~150-fold of unlabeled) was evident from most MSCs (91%) signifying successful intracellular hybridization of liberated GAPDH MBs. This observation echoes the stable, strong GAPDH mRNA expression in MSCs.[226]

Tracking MSC chondrogenesis in agarose hydrogel with nanosensors

Next, we evaluated nanosensor sensitivity in distinguishing cells with distinct Col2 mRNA expression. Specifically, we looked at three 3D construct groups (undifferentiated MSCs/UD, chondrogenic-differentiated MSCs/TGF β 3, & human umbilical vein endothelial cells/HUVECs) at day 28 into gel encapsulation. Figure 5.4A shows that TGF β 3 group exhibited strongest FITC fluorescence (Col2 MB), followed by UD group. Meanwhile, little to no FITC fluorescence was observed for HUVECs even at day 28. Of note, all three groups presented a stable and strong Cy3 fluorescence from GAPDH MB. By normalizing Col2 MB signal against that of GAPDH MB, Col2 expression was semi-quantified (Figure 5.4B). At day 28, nanosensor signal from TGF β 3-induced group was about twice of undifferentiated group, while it was practically undetectable in HUVECs. These expressions were supported by a comparable trend in qPCR analysis showing significantly stronger Col2 expression in TGF β 3 group relative to UD group (Figure 5.4C).

We also evaluated non-invasive, dynamic monitoring properties of nanosensor over the 4-week chondrogenesis (Figure 5.5A). FITC fluorescence from Col2 MB increased gradually from day 7 to 28 while GAPDH MB stayed consistent throughout. Importantly, both fluorescence signals were confined within cells' boundary (Figure 5.5A&B), indicative of mRNA hybridization-induced fluorescence restoration instead of non-specific MB degradation. When normalized nanosensor signal ratio (Figure 5.5C) was plotted against qPCR result at each time point (Figure 5.5D), good correlation ($R^2=0.9301$) between the two parameters indicates accurate nanosensor performance.

Figure 5-4: Nanosensor-enabled identification of MSCs undergoing chondrogenesis. A) Confocal fluorescence images of undifferentiated MSCs (UD), chondrogenically-induced MSCs (TGFβ3) and HUVECs on day 28; Scale bar: 100μm. B) Signal quantification and normalization of nanosensor fluorescence in A). C) Quantitative polymerase chain reaction (qPCR) analysis of Col2 mRNA expression in UD and TGFβ3-treated MSCs. mRNA expression was normalized using the comparative C_T formula. ** represents $p < 0.01$. $n \geq 4$ and error bars denote standard deviation.

Figure 5-5: Tracking of MSC chondrogenesis with nanosensor. A) Representative confocal fluorescent images of chondrogenically-differentiating MSCs labeled with nanosensor at weekly timepoint. B) 3D fluorescence reconstruction of MSCs undergoing chondrogenesis in 3D agarose at day 28; scale bar: 100µm. C) Normalized fluorescent signal of nanosensor-labeled MSCs undergoing chondrogenesis in A). D) Correlation plot between normalized nanosensor signal and its corresponding Col2 mRNA expression by qPCR at each timepoint. $n \geq 4$ and error bars denote standard deviation.

Influence of nanosensor labeling towards functionality of MSCs

Finally, influence of nanosensor labeling towards the multi-potency of MSCs was examined. In particular, MSCs were allowed to undergo its three common differentiation lineages (chondrogenesis, osteogenesis, & adipogenesis) with or

without nanosensor labeling. Upon completion of differentiation procedures, samples were fixed and stained for the respective differentiation markers. As represented by Figure 5.6, there were minimal differences between unlabeled or nanosensor-labeled samples. Further validation was done by performing qPCR for MSCs undergoing adipogenesis with or without nanosensor labeling. Insignificant differences found on both adipogenic markers, following nanosensor labeling (Leptin: $P>0.2$, Peroxisome proliferator-activated receptor gamma/PPAR γ : $P>0.1$; Figure 5.7) confirmed that nanosensor-labeling did not alter MSCs multi-potency.

Figure 5-6: Immunohistochemical and histological staining of MSCs undergoing chondrogenesis, adipogenesis and osteogenesis before or after nanosensor labeling. Scale bar: 500µm.

Figure 5-7: Quantitative polymerase chain reaction (qPCR) analysis of MSCs undergoing adipogenesis at day 14 after or before nanosensor labelling. Readings were normalized through the comparative CT formula. NS represents non-significant. $n \geq 4$ and error bars denote standard deviation.

Discussion

Combination of hMSCs, scaffold constructs and bioinductive growth factors is a typical tissue engineering approach adapted to address articular cartilage defects/damages.[227] To validate successful chondrogenic differentiation, disruptive analyses like qPCR or histological staining are usually executed. Given the invasive nature of the assay, temporal expression of chondrogenesis biomarkers in induced MSCs cannot be completely comprehended.

To monitor differentiation processes in real-time, scientists employ reporter gene techniques. These reporter genes are regulated by differentiation-specific promoters and introduced into live cells through various transfection methods.[228, 229] In general, these methods involve either non-viral or viral-based delivery. In an example, non-viral liposomic-based transfection was used to deliver osteocalcin-2 reporter for osteogenic differentiation tracking.[228] Lentiviral delivery was applied by Bagó et al to introduce luciferase-fluorescence chimeric reporter to MSCs for vascularization and osteogenic differentiation monitoring.[230] Nevertheless, there are limitations associated with such assay. Namely, cytotoxicity is a major concern

for many transfection reagents, including liposomal-based. Meanwhile, usage of viral vectors risks insertional mutagenesis to target cells' genome.[215]

With respect to this need, the current study proposes application of nanosensors for non-invasive, real-time tracking of MSCs chondrogenesis in 3D agarose hydrogel. MB nanosensors were fabricated by co-loading Col2 and GAPDH MBs within biodegradable PLGA nanocarrier. In this case, ratiometric assessment of both MBs fluorescent signal is important to obtain accurate depiction of Col2 mRNA expression, given certain degree of heterogeneity in nanosensor internalization and cellular retention.

Firstly, we demonstrated successful loading, and retention of MBs' specificity and integrity following nanoparticle encapsulation and 30 days hydrolysis exposure. As these nanosensors were applied for MSCs labeling, fluorescent signal originated from the cytoplasm region. This is in agreement with our previous observation, in which 1 μ m nanosensors are pre-dominantly internalized by MSCs through phagocytosis (i.e. thus resulting in direct delivery of nanosensors to the cytoplasm region). This translates to better intracellular retention of nanosensors, given significantly lesser nanosensor dilution through exocytosis. Furthermore, direct release into the cytoplasm also ensure better stability of released MBs, by avoiding rapid degradation of MBs in the acidic condition of endo/lysosomal pathway.[221]

MB nanosensor capability to report Col2 mRNA was tested on three cell types expressing distinct levels of Col2 expression (undifferentiated MSCs, chondro-induced MSCs and HUVECs). Normalized Col2 MB fluorescence demonstrated nanosensor specificity, with chondrogenically-induced MSCs showing stronger signal as compared to undifferentiated MSCs and negligible signal on HUVECs. Crucially, when MB signal ratio was compared against qPCR result at the same timepoint, a good correlation between the two parameters was obtained throughout the differentiation period of 28 days ($R^2=0.9301$). From in-solution hybridization study of liberated MBs, we observed non-specific signal generation at the final release sample (day 28, Figure 5.3B). Such non-specific MB opening is somewhat expected for MBs released during the later time points, and have

probably compromised the correlation value (R^2) obtained here. Nonetheless, this correlation value corroborates nanosensor ability to reflect MSCs' Col2 mRNA expression, in a non-invasive manner while cells are undergoing differentiation within the agarose gel.

Our study (Figure 5.6 & 5.7) also showed that multipotency of MSCs was minimally compromised with nanosensor labeling. This is in conjunction to our prior study on MSCs with molecular dyes-loaded PLGA nanoparticles. Upon differentiation induction to three lineages (i.e. adipogenesis, chondrogenesis and osteogenesis), no significant differences were observed in their respective histochemical staining (relative to unlabeled control group).[219]

Given that MBs release profile from nanosensors occurred over 30 days, accuracy of nanosensor signal generation would decline reaching or extending beyond this time point, as there would be minimal to no further supply of functional MBs into the cytoplasm. Notwithstanding, our 28 days monitoring period is a notable improvement from previous reports on MB utilization for 3D non-invasive tracking. Previously, Illeva M. and Dufva M. deliver Sox2 and Oct4 MBs through transfection to achieve monitoring of neural stem cells (NSCs) differentiation in the form of 3D neurospheres.[231] In their study, the observation period was limited to a week due to dilution of MB concentration intracellular. On this topic, multiple MB introductions can be performed to maintain sufficient MB concentration longer, as implemented by Desai et al in their osteogenic differentiation tracking.[22]

In near future, beside working on fine-tuning MB release period, nanosensor signal specificity can be significantly improved through the usage of two MBs which work in tandem to report one target mRNA (i.e. employing Förster resonance energy transfer/ FRET mechanism).[220] Simultaneously, backbone-modified MBs can improve signal specificity further by reducing non-specific MB degradation, especially for longer culture monitoring period.[232] We envision adaptation of such nanosensor as a screening platform to design optimum differentiation conditions for various tissue engineering. In such application, signal expression of cells or aggregates facilitates functional readout to identify best combination of growth factors in a high-throughput manner (e.g. concentrations, treatment periods).

Additionally, versatile nature of this nanosensor can enable simultaneous tracking of multiple gene markers for distinct differentiation stages (e.g. Col2 and Col10 for chondrogenesis), as to identify suitable factors to best replicate native tissue differentiation which occurred to different extents depending on localization.

5.4 Conclusion

This study reports the usage of nanosensor platform based on dual-encapsulation of MBs in biodegradable PLGA nanocarrier for longitudinal evaluation of MSCs chondrogenesis in 3D agarose hydrogel. Different from conventional assessment methods which is disruptive in nature, sustained release of Col2 and GAPDH MBs from nanosensor allows facile yet accurate continuous assessment of Col2 mRNA expression over the 4-week period. Throughout this induction period, increasing normalized Col2 signal signified successful chondrogenesis on induced MSCs, relative to undifferentiated ones. Most importantly, observable signal profile correlated well against results from gold-standard analysis of qPCR. Additionally, nanosensor labeling resulted in no significant compromise over MSCs' differentiation potential. With better spatio-temporal understanding of chondrogenic gene expression upon differentiation induction, researchers are empowered to identify best combinations of factors for growth plates development *in vitro*.

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Chapter 6 Nanosensors for Non-invasive Monitoring of Fibroblasts Cells Reprogramming and Transdifferentiation

Manuscript submitted

6.1. Introduction

Cell reprogramming involves transforming adult somatic cells to pluripotent stem cells by means of external factors introduction, such as transcription factors (TFs) and small chemicals.[233-235] Over the years since its first introduction, reprogramming concept has blossomed into a diverse subject with the purpose of generating various therapeutic cells (e.g. neuronal cells, cardiomyocytes).[236] Of late, such transformation has also led to the notion of transdifferentiation (inducing transformation of cells towards another lineage without passing the pluripotent state).[237] Cell reprogramming allows the production of pluripotent cells from highly accessible mature cell sources, which otherwise can only be collected from embryos. As such, cell reprogramming can play a major role in advancing cell therapies, regenerative and even personalized medicine.[238]

Typically, cell reprogramming procedures last for about 2-3 weeks for the case of transfected transcription factors and longer than a month for protocols using set of small molecules. Moreover, successful rate of transformation is very low, typically between 0.1%-1% of the cell population.[239, 240] These are problematic to conventional assays which are population-based in nature (e.g. western blot, polymerase chain reaction), due to inaccuracy for evaluating cell conversion rate and efficiency. Additionally, while immunostaining and fluorescence *in situ* hybridization (FISH) probes may facilitate differentiation of cells undergoing reprogramming, these methods require cell fixation, thus only a single timepoint of the dynamic cell process can be evaluated. In other words, temporal resolution can only be obtained by multiple sample preparations.[241, 242]

To this end, introduction of reporter gene constructs with gene-of-interest promoter has been suggested to achieve continuous, non-disruptive tracking of reprogramming marker expression at single cell resolution. However, preparation of such plasmid constructs is costly and usually involve long procedures to design, ensure and optimize stability. In addition, as these constructs require genomic integration, it introduces risks of mutagenesis as well as immunogenicity, which renders it unsuitable for clinical usage.[188, 243]

One solution to enable non-invasive gene evaluation is through the utilization of nanoparticles carrying switchable oligonucleotide probes (which achieve signal generation upon recognition of specific mRNA markers). As examples, spherical nucleic acids (SNAs) rely on assembly of reporter strands acting as probes around gold nanocore, which facilitates effective uptake and achieves activatable fluorescence generation following target hybridization and displacement of reporter strands.[143, 145] Otherwise, these oligonucleotide probes can be tuned to self-hybridize and assemble into nano-constructs in solution, which in turn enhances cellular internalization and intracellular stability from enzymatic degradation, ideally with minimal compromise to its sensing performance.[146, 147] Despite both approaches meeting the requirement to enable non-invasive, intracellular biomarkers evaluation, rapid elimination of nucleic acid-based probes from the cytoplasm still pose a serious challenge which limits probes' monitoring window.[244]

Our group focuses on developing nano-platform to facilitate longitudinal, sustained evaluation of cellular biomarkers expression. Proposed nanosensors are synthesized by incorporating molecular beacon (MB) probes within biocompatible, biodegradable polymeric nanoparticles. Such formulation allows effective cell uptake and sustained retention, thereby extending MBs intracellular monitoring window, by providing protection from nucleases degradation and slow release of probes to the cytoplasmic region.[192] Before, compatibility and monitoring capability of this platform have been demonstrated in Mesenchymal Stem Cells (MSCs) undergoing both 2D and 3D differentiations (osteogenesis and chondrogenesis, respectively).[222, 245]

The current work aims to explore nanosensor platform ability to track cell reprogramming process which has a significantly lower conversion rate compared to MSC differentiation. Resolution provided by nanosensor monitoring (i.e. spatial and temporal) was firstly checked on a model Doxycycline (Dox)-inducible HEK293 cells, in which expression of EGFP can be easily regulated with the addition of Dox into culture medium. Then, nanosensors were applied to track reprogramming of murine embryonic fibroblasts to a pluripotent state. In particular, we verified mesenchymal-epithelial transition (MET) and performed longitudinal observation of

pluripotent Oct4 marker (Figure 6.1). To evaluate sensitivity and specificity of nanosensors cell reprogramming monitoring, nanosensor signal trend was compared to gold-standard qPCR analysis and gene reporter constructs introduction. Nanosensor results showed good correlation with the two assays mentioned above, with $R^2 > 0.9$ for both. In culture conditions necessitating observation of shorter mRNA regulation however, the nanosensor strategy improves the temporal resolution limit of reporter-based constructs, which is dependent also on the lifespan of reporter protein.

Figure 6-1: Schematic of nanosensor principle for intracellular target recognition and monitoring application in cell reprogramming. Incorporating MBs targeted against biomarkers for pluripotency (OCT4, E-cadherin), fibroblasts responsive to the reprogramming induction can be identified from non-responsive fibroblasts.

6.2. Materials and Methodology

Materials

Materials were purchased from Sigma-Aldrich, except specifically mentioned. High glucose Dulbecco's modified eagle medium (DMEM, 4.5g/L) and non-essential amino acids were from Lonza. Leukemia Inhibitory Factor and basic Fibroblast Growth Factor were from BioBasic Inc. Knockout DMEM & Serum Replacement, and β -mercaptoethanol were from Life Technologies. AlexaFluor647-tagged antibodies were obtained from Santa Cruz Biotechnology. CMV-tight eGFP plasmid was obtained from Addgene. Penicillin/streptomycin, fetal bovine serum, and trypsin-EDTA were purchased from Gibco. Moloney Murine Leukemia Virus reverse transcriptase (RT) was purchased from Promega. Lastly, iQ SYBR Green was from Bio-rad and Trizol reagent from Invitrogen.

Hybridization study of MBs

To examine EGFP MB intensity restoration, EGFP mRNA was allowed to hybridize at concentration of 0.5 μ M. Kinetic fluorescence assay was tested to evaluate the rise in fluorescence signal, relative to when PBS or DNase I (40U/ml) digestion was performed. Similar hybridization study was performed for reprogramming MBs, in which MBs were treated with complementary or non-complementary oligonucleotides (Table 6.1). Following 15 min incubation at 37°C, fluorescence intensity of these mixture solutions was read and normalized against solutions digested with DNase I treatment (40U/ml).

Table 6.1 List of MBs, targets and primers in this study

MB and Primers	Sequences (5' → 3')
EGFP MB	(Cy3) <u>ACGCC</u> -TTCTCGTTGGGGTCTTTGCTC- <u>GGCGT</u> (BHQ2)
GAPDH MB	(FAM) <u>CTTCGG</u> -TGTAGACCATGTAGTTGAGGT- <u>CCGAAG</u> (BHQ1)
OCT4 MB	(Cy3) <u>CGTCGT</u> -CATACTCGAACCACATCCTTC- <u>ACGACG</u> (BHQ2)
Thy1 MB	(Cy3) <u>CGCTT</u> -CGGGACACCTGCAAGACTGAGAG- <u>AAGCG</u> (BHQ2)
E-cad MB	(FAM) <u>CGCTT</u> -CGGTAGACAGCTCCCTATGACTGG- <u>AAGCG</u> (BHQ1)
GATA4 MB	(Cy3) <u>CGCGA</u> -CCTGCTGGCGTCTTAGATT- <u>TCGCG</u> (BHQ2)
GAPDH target	ACCTCAACTACATGGTCTACA
OCT4 target	GAAGGATGTGGTTCGAGTATG
Thy1 target	TCTCAGTCTTGCAGGTGTCCC
E-cad target	CAGTCATAGGGAGCTGTCTACC
GATA4 target	AATCTAAGACGCCAGCAGG
EGFP primers	Forward: CATGGTCCTGCTGGAGTTCGTG
	Reverse: CGTCGCCGTCCAGCTCGACCAG
GAPDH primers	Forward: CATCACCATCTTCCAGGAGC
	Reverse: ATGCCAGTGAGCTTCCCGTC
Oct4 primers	Forward: GGCTTCAGACTTCGCCTCC
	Reverse: AACCTGAGGTCCACAGTATGC
Thy1 primers	Forward: GGA ACTCTTGGCACCATGAAC
	Reverse: GGGACACCTGCAAGACTGAGA
E-cad primers	Forward: CAGGTCTCCTCATGGCTTTGC
	Reverse: CTTCCGAAAAGAAGGCTGTCC
Gata4 primers	Forward: CCTGGAAGACACCCCAATCTC
	Reverse: AGGTAGTGTCCCGTCCCATCT
Tnnt2 primers	Forward: GCGGAAGAGTGGGAAGAGACA
	Reverse: CCACAGCTCCTTGGCCTTCT

Nanosensors fabrication

MB oligonucleotides are summarized in Table 6.1 and verified for specificity through basic local alignment search tools (BLAST). Nanosensors preparation was modified from previously published protocol.[192] Briefly, water solution containing 1 nmol MBs (either single or with two types of MB) was added dropwise into poly(lactic-co-glycolic acid)-chloroform solution under homogenization at 30krpm. Following which, solution was mixed into 3% polyvinyl alcohol solution under secondary homogenization at 30krpm. Whole mixture was then stirred in fume hood

for 4 hours. Nanosensors were then collected through centrifugation at 10krpm, lyophilized and stored at -20°C.

Culture of Dox-activatable HEK293 cells

Dox-activatable EGFP construct-containing HEK293 was prepared by performing lentiviral transfection with pLenti CMV-tight eGFP Hygro (Addgene #26585). Modified HEK293 cells were expanded following clonal selection after Dox addition and removal. Cells were maintained in high glucose (4.5 g/L) DMEM supplemented with 10% FBS and L-glutamine, with replacement every 2-3 days. To control periodical EGFP expression, Dox was included in culture medium at 250 ng/mL for 6 hours period, followed by 42 hours removal before another cycle.

Culture of MEFs and reprogramming protocols

Unmodified murine embryonic fibroblasts (MEFs) and modified to carry Oct4 Promoter/EGFP plasmid were cultured in high glucose (4.5g/L) DMEM supplemented with 10% FBS and L-glutamine, with replacement every 2-3 days. Reprogramming protocol was from previous study.[235] In short, reprogramming medium was composed of knockout DMEM, 10% knockout serum replacement, 10% FBS, 1% nonessential amino acids, and 0.1 mM 2-mercaptoethanol. Reprogramming medium is then freshly supplemented with small molecules of VCRTF (in Table 6.2) until day 20. Following which, reprogramming medium was mixed with VCRTFZN until day 32. From then, cells were given pluripotency maintenance medium for 8 days (reprogramming medium with 1 μ M PD0325901, 3 μ M CHIR, and 1000 U/mL leukemic inhibitory factor-LIF). Throughout the procedure, medium replacement was performed every 4 days with freshly prepared small molecules mixture.

Table 6.2 Chemical compounds used in reprogramming induction

Full Name	Abbreviation	Final concentration	Molecular weight
Valproic acid sodium salt	VPA (V)	500 μ M	166.19
CHIR99021	CHIR (C)	10 μ M	465.34
616452/ Repsox	R	10 μ M	400.34
Tranylcypromine	Tranyl (T)	5 μ M	182.23
Forskolin	FSK (F)	10 μ M	410.50
3-deazaneplanocin A	DZNep (Z)	0.05 μ M	262.26
TTNPB	N	1 μ M	348.48
PD0325901	P	1 μ M	482.00

Nanosensor labeling for longitudinal study

Cells (modified HEK293 or MEF) were seeded in well-plates until it reaches >80% confluency. To enhance cell uptake, nanosensors were firstly coated with poly-L-lysine (MW 70-150 kDa) solution (0.01% w/v) for a period of 20 min. Dox-inducible modified HEK293 cells were labeled with 1 mg/mL nanosensors for 18hr. Meanwhile, for the extended monitoring of MEF reprogramming or transdifferentiation procedures, nanosensors were introduced to culture medium at 0.25 mg/mL concentration every second media replacement.

Fluorescence imaging and intensity quantification

Images were taken with LX71 inverted fluorescence microscope and infinity3-1 CCD camera. Capture settings (exposure and gain) were kept consistent for all samples of the same experiment. Fluorescent images were then processed with ImageJ to obtain intensity of individual cells or colonies (i.e. in reprogramming/transdifferentiation study).

qPCR assay

Dox-inducible HEK293, MEFs undergoing reprogramming or transdifferentiation at various points were collected and lysed with TRIzol for RNA extraction, in comparison to corresponding control group. cDNA conversion was

performed with SensiFast cDNA Kit. Then, quantitative PCR was performed on CFX Connect™ to obtain CT values of markers, using primers in Table 6.1. Delta delta CT formula was taken to evaluate fold-expression of these genes. For each treatment, 6 independent samples were used and analyzed for RT-qPCR.

Antibody staining

Cell samples undergoing reprogramming and transdifferentiation for immune-cytochemical staining was fixed in 10% neutral-buffered formalin (NBF) for 10 min period. Following PBS rinsing, cells were permeabilized through treatment with 0.1% Triton X-100 for 10 min. Subsequently, protein blocking process was done through 1% bovine serum albumin (BSA) in PBS for 30 min. Then, cells were treated for 1hr with Alexafluor647-antibodies solution for Oct4, CD9, or Gata4 (1/200 in 1% BSA) at room temperature, before DAPI staining in 1% BSA for an additional 10 min.

AlamarBlue analysis

AlamarBlue analysis was used to check nanosensors cytotoxicity when labelling is performed for 4-day period (i.e. matching media replacement period in reprogramming and transdifferentiation). Briefly, murine embryonic fibroblasts were seeded & allowed to reach 90% confluency. Nanosensor was then introduced to culture medium at designated concentrations for 4 days. After PBS rinsing remove excess nanosensors, AlamarBlue reagent was introduced to culture medium in 100 dilution ratio for 24-hour incubation. Fluorescence reading at 570/585nm was taken against unlabeled cells to evaluate influence on viability.

Statistical analysis

Analysis of variance (ANOVA) One-way or student T-test were applied to check significance in various groups differences (i.e. p-values). Suitable post-hoc analysis was chosen accordingly on SPSS Statistics software. All data are

presented as mean plus/minus standard deviation of more than independent samples.

6.3. Results and Discussion

Characterization of nanosensors applied on Dox-inducible HEK293

In this project, we firstly aimed to further evaluate temporal resolution limit of nanosensors and to address an important concern over nanosensor influence towards the expression level and translation rate of target mRNA. Taking Dox-inducible HEK293 as model cells, we validated nanosensor capability to report the dynamic EGFP mRNA expression upon Dox activation, relative to its translation to green fluorescent proteins.

EGFP MB, modified and verified from previous study [246] recognizes base 622-642 of EGFP mRNA. MB recognition ability towards EGFP mRNA was checked by reading its fluorescence restoration kinetics upon hybridization, relative to when DNase I was added to degrade the MBs. Figure 6.2A reveals that MB fluorescence begin to recover from 2 minutes of target addition, before plateauing at 75% normalized fluorescence intensity after around 20 mins.

For the first cell experiment, MB-containing nanosensors were introduced to human umbilical vein endothelial cells (HUVECs) modified or not modified with EGFP plasmid (Figure 6.2B). A 5-6 fold stronger Cy3 nanosensor signal was observed when nanosensor was introduced to HUVECs transfected with EGFP plasmid, as compared to that in unmodified HUVECs (i.e. not expressing EGFP; Figure 6.2C).

Subsequently, nanosensor was used to report dynamic EGFP mRNA expression in Dox-responsive model cells, by periodically applying Dox treatment (as summarized in Figure 6.3A). Representative images in Figure 6.3B show that EGFP nanosensors fluorescence peaked at 6 hour after the introduction of Dox in each cycle, and declined along the Dox-removed period. Meanwhile, GAPDH nanosensors expressed relatively stable fluorescence throughout these Dox cycles

(Figure 6.3C). By normalizing EGFP nanosensors fluorescence with that of GAPDH nanosensors, signal ratio also peaked at approximately 6 hr post Dox addition, and at 6-7 fold of the starting value of each cycle (Figure 6.3D). Crucially, the dynamic change of normalized nanosensor signal matches well with mRNA trend from qPCR (Figure 6.3D). Notably, when nanosensor signal at each timepoint was plotted against its mRNA expression, a linear correlation was seen on the cycles tested (with $R^2 > 90\%$, Figure 6.3E & 6.4).

Noteworthy, this study showed that green fluorescence from EGFP increased at a similar pace until reaching a plateau for both groups (i.e. cells with EGFP or GAPDH nanosensors labeled; Figure 6.3F). This result is dramatically different from that derived from nanosensors. As mentioned above, Dox was discontinued at hour 6 and re-added at hour 48. In this aspect, nanosensor accurately monitor changes in EGFP mRNA, which was not represented through the EGFP reporter protein. This shows attractiveness of proposed nanosensor in tracking highly dynamic gene, surpasses that of plasmid constructs which incur lag-time related to protein translation delay and lifetime.

One concern about using mRNA-targeted sensor probes is the possible influence over target mRNA expression and their eventual translation into proteins. Beside physically blocking assembly of ribosomal subunits during the start of translational process, binding of DNA probes to mRNA possibly trigger ribonuclease H cleavage activities on the complex. As a result, mRNA expression declined.[247] For this, cellular EGFP mRNA before or after nanosensor labeling was evaluated by RT-qPCR (Figure 6.5). Here, we observed that nanosensor or blank nanoparticle labeling did not significantly influence mRNA relative to unlabeled cells. Similar EGFP fluorescence signal in both groups indicates minimal impact on mRNA translation (Figure 6.6A). This is confirmed through western blot evaluation of EGFP production (Figure 6.6B&C).

*Figure 6-2: EGFP nanosensor signal specificity. A) In solution hybridization kinetics of MBs with mRNA target or DNase I-digestion. Representative images (B) or signal quantification (C) for MB nanosensor application in normal or GFP-expressing HUVECs. Scale bar: 100μm. * represents $p < 0.05$. $n \geq 4$ and error bars denote standard deviation.*

Figure 6-3: EGFP nanosensor performance validated on model Dox-inducible HEK293 cells. A) Schematic representation of performed experiment. Representative EGFP nanosensor (B) and GAPDH nanosensor (C) signal on Dox-inducible HEK293, with two cycles of Dox application. D) Normalized EGFP nanosensor signal on Dox application cycles. E) Correlation plot of nanosensor signal against mRNA expression for 1st and 4th cycle. F) Green cellular fluorescence in response to Dox application cycles. Error bars denote standard deviation.

Figure 6-4: Correlation plot from nanosensor normalized signal against mRNA expression on the four Dox application cycles. Error bars denote standard deviation.

Figure 6-5: Safety aspect of nanosensor labeling on EGFP mRNA expression following Dox application and removal. *: $p < 0.05$ to unlabeled control. $n \geq 4$ and error bars denote standard deviation.

Figure 6-6: Safety aspect of nanosensor labeling on EGFP translation rate and expression, through fluorescence quantification (A) or western-blotting (B&C). $n \geq 4$ and error bars denote standard deviation.

Tracking of chemically-induced reprogramming process with nanosensors

In this project, we studied transformation of fibroblasts to become pluripotent cells via a three-stage small-molecules induction mechanism (Figure 6.7A).[235] 20 days into the induction using valproic acid (VPA), Forskolin, Tranylcypromine, Repsox, and CHIR99021, the presence of colonies with positive green fluorescence indicated true transformation towards pluripotency (positive Oct4 expression; Figure 6.8). Following which, the addition of TTNPB and DZNep to medium cocktail induced further growth of the colonies.

Mesenchymal-epithelial transition (MET), a shifting of mesenchymal cells' phenotype to that of epithelial, is an essential starting step in cell reprogramming.[248] During such transition, fibroblast cells which are typically loosely associated in nature and required extracellular matrix interaction to mediate

their substrate adhesion, become more epithelial-like with closely inter-connected cells that are much less mobile.[249]

In this regards, Thy1, a fibroblastic marker is downregulated during this transition towards epithelial cells. Therefore, nanosensor targeting Thy1 mRNA can be applied to track MET procedures. Matching our hypothesis, Thy1 nanosensor signal gradually became weaker (figure 6.7B). This is in accordance to the mRNA expression trend obtained via RT-qPCR study (figure 6.7C&D). 2.4-fold decline in Thy1 nanosensor signal over the reprogramming was matched with $R^2 = 0.925$ when correlated against mRNA expression at particular timing. (figure 6.9)

At the same time, to actively track and validate successful reprogramming induction, nanosensor non-invasive reporting ability was applied with Oct4 mRNA as a target. Being a pluripotent cell marker, Oct4 mRNA expression has been widely evaluated to ascertain successful pluripotent cells generation.[250, 251] Firstly, the ability of designed MBs to recognize the presence of Oct4 target was tested by comparing the extent of fluorescence recovery following correct or incorrect oligonucleotides addition. As represented in figure 6.10A, whereas non-matching GAPDH oligo resulted in very minor fluorescence increase (<1%), addition of correct Oct4 oligo led to ~95% signal recovery, when normalized against DNase I-treated group (positive control).

For cell tracking study, as the nanosensor labeling protocol was revised to match the media exchange timing, cytotoxic effects of nanosensors on MEFs was re-tested through the alamarblue metabolism assay (Figure 6.10B). With the 4 days labeling, although previous concentrations of nanosensors (0.5 & 1 mg/ml) became slightly cytotoxic and affected cell metabolism at about 5%, insignificant toxicity was observed at 0.25 mg/ml. Thus, it was deemed as a safe concentration for eventual reprogramming study.

Further examination of nanosensor-enabled reprogramming tracking was done through comparison of nanosensors signal with that from reporter construct (encoding for Oct4-driven EGFP; Figure 6.11A-C). Here, gradually increasing Oct4 nanosensor signal along the induction period correlated well with EGFP fluorescence intensity ($R^2=0.93$, Figure 6.11C). Notably, there was ~4.2-fold

increase in nanosensor signal for cells successfully reprogrammed by day 40, relative to MEFs control. Meanwhile, cells failed to undergo reprogramming presented a similar fluorescence profile to MEFs (Figure 6.11B). Important to note also is that nanosensor signal trend matched the change in Oct4 mRNA well (Figure 6.11D&E). This was shown through the linear correlation between the two parameters when they are plotted against each other ($R^2=0.97$).

Given the originally low reprogramming efficacy, checking nanosensors influence over the reprogramming processes is of significant interest as well. To check for such effect, we evaluated Oct4-driven EGFP signal as well as colonies formation amount. As shown in Figure 6.12, minimal effect was evident by the insignificant change in both parameters.

Figure 6-7: Thy1 nanosensor on tracking chemically-induced cell reprogramming. A) Reprogramming protocol with small molecules. Representative images (B) and quantified signal (C) of Thy1 nanosensor fluorescence at different induction timepoints. D) Corresponding mRNA expression. $n \geq 4$ and error bars denote standard deviation.

Figure 6-8: Formation of GFP-positive cell colonies from reprogramming induction.

Figure 6-9: Correlation plot of Thy1 nanosensor signal and Thy1 mRNA expression.
 $n \geq 4$ and error bars denote standard deviation.

Figure 6-10: A) Oct4 MB hybridization assay. B) Alamarblue assay to evaluate nanosensor cytotoxicity on MEFs after 4 days labeling period. * represents $p < 0.05$, ** represents $p < 0.01$. $n \geq 4$ and error bars denote standard deviation.

Figure 6-11: Oct4 nanosensor monitoring of reprogramming induction. Representative images (A) and signal quantification (B) of Oct4 nanosensor fluorescence. C) Correlation plot between nanosensor signal and reporter gene construct signal. D) Oct4 mRNA expression from qPCR. E) Correlation plot between nanosensor signal and corresponding mRNA expression in D). $n \geq 4$ and error bars denote standard deviation.

Figure 6-12: Influence of nanosensor labeling on successful reprogramming induction. A) Signal quantification of Oct4-driven EGFP production. B) Amount of cell colonies formed following induction. N.S represents non-significant difference. Error bars denote standard deviation.

Discussion

Cell reprogramming, the process by which mature somatic cells are transformed into a pluripotent state by introduction of factors or small molecules, is a versatile way of generating significant quantity of therapeutic cells. Its process involves long culture period with ≤ 0.1 -1% of cell population successfully reprogrammed.[239] This heterogenous population renders population-based assays (e.g. ELISA, RT-qPCR) mostly inaccurate. Histochemical staining, while enabling visualization of protein markers at individual cells, is also an invasive method which disrupt experiment progression thus providing only a single snapshot. To counter this, scientists typically incorporate reporter constructs for longitudinal assessment of pluripotent markers. Nonetheless, modification of cells to stably express these reporters can be not only time-consuming but also tedious. Moreover, such process may risk insertional mutagenesis. In this regards, non-integrative monitoring platform is highly promising given its clinical-friendliness. As such, herein we proposed utilization of biocompatible MB-nanosensors to track MEFs reprogramming induction.

Both spatial and temporal resolution of nanosensor was initially tested in HEK293 cells modified with Dox-inducible EGFP. Notably, Dox-induced cells expressing EGFP fluorescence also expressed Cy3 nanosensor signal, which then reduced in intensity upon Dox removal from culture medium, to a similar intensity to that of un-induced cells. Importantly, normalized nanosensor signal showed good linear correlation (R^2 value greater than 0.9) with mRNA expression from RT-qPCR. Of note, slower response was seen from EGFP reporter signal with longer time lag to activated signal and occurrence of signal plateau despite when Dox was removed from the system. Such observable lag period in the expression of reporter construct is to be expected, as delay time is required for mRNA translation.[252] At the same time, persistence of already-generated reporter proteins with its longer turnover rate resulted in signal plateau even though Dox is removed.[253] In summary, our results showed that proposed nanosensor has single-cell resolution in spatial aspect, with better temporal resolution as compared to gene reporter constructs (down to few hours). Additionally, nanosensor biocompatibility was validated through both insignificant changes in cellular metabolism and mRNA expression and protein translation rate.

Following deeper characterization study, nanosensor was then adapted to track the chemically-induced reprogramming. Unlike conventional means of reprogramming in which gene constructs encoding for pro-pluripotent transcription factors are inserted into cells to achieve either constitutive or activatable expression of these factors, chemical induction method is more promising towards clinical translation, with no risks of insertional mutagenesis and a well-defined induction process.[254] Given its importance in cell reprogramming initiation, cellular MET process was firstly studied through the declining fibroblastic marker Thy1 expression. As reported through nanosensor signal intensity, downregulation of Thy1 between day 12 to 20 of induction process proved successful MET. Meanwhile, RT-qPCR corroborated the trend of proposed nanosensor signal, as proven with the good correlation between nanosensor intensity and mRNA expression ($R^2 = 0.925$ for Thy1 expression).

Moving deeper into the reprogramming procedures, a pluripotent marker in Oct4 mRNA was used as target for nanosensor monitoring throughout the entire

length of induction period. Matching our expectation, Oct4 nanosensor showed gradual fluorescence signal increase in cell colonies undergoing or successfully reprogrammed. In this study, Oct4 nanosensor signal was validated by comparing its trend against the mRNA expression from qPCR and reporter construct-controlled fluorescent protein. In both comparison evaluation, good correlation was observed with R^2 greater than 0.9. Furthermore, it is noteworthy that minimal signal was observed from MEFs failed to undergo reprogramming even by day 40. Instead, they expressed comparable fluorescent signal to nanosensor-labeled MEFs, without undergoing induction treatment. These findings substantiate nanosensors' capability in accurately and non-invasively report Oct4 expression level.

Prior to this study, several other probes involving nanoparticles have been suggested and explored for non-disruptive visualization of gene expression, including spherical nucleic acid technology assembled on gold nanoparticles and self-hybridized origami of nucleic acids. However, their ability to represent cellular mRNA are so far limited between hours to a few days. For instance, although SNA targeted towards GAPDH resulted in 56.2% positively-expressing cells by day 1, it reduces by about two-fold each day and only about 12.3% cells remained fluorescence at day 3. As such, iPS colonies to be selected for most optimum pluripotency had to be introduced with SNA only two days before the fluorescence-assisted sorting timing.[244] Longer period of nanosensors fluorescence can allow complete analysis of performed monitoring, with fewer re-labeling of sensor probes. With its larger particle diameter avoiding early exocytosis and its incorporated MB probes unexposed from nucleases in the cytoplasmic region, proposed nanosensor here is well-matched for sustained cellular monitoring.

6.4. Conclusion

In a nutshell, MB-nanosensor proposed provides an easy, multi-versatile and reliable evaluation of cellular mRNA for verification of cellular transformation. Its cyto-compatibility and non-genomic integrative nature allows it to be a promising alternative to reporter constructs, for assisting development and optimization of

cellular therapeutics toward clinical translation, including in cellular reprogramming approaches.

6.5. Acknowledgement

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Chapter 7 Summary and Future Outlook

7.1 Summary

Regenerative medicine involves various approaches to restore healthy and functional tissues. One popular way in regenerative medicine is to utilize therapeutic cells to be injected into host's body. Cells applied in this way can either be immune-related cells (e.g. dendritic cells, T cells for immunotherapy), stem cells or even mature functional cells to reside and reconstitute damaged tissues, as to promote its healing or at least allow these tissues to regain physiological functionality.

All of these however, depends closely on whether such functional cells could be obtained (i.e. isolated, purified and expanded in *in vitro* settings) without significant alterations to its phenotypical properties and subsequently whether they are still efficacious following *in vivo* implantation (i.e. whether they adhere, survive and integrate with native tissue to function as intended). Should one of these steps does not occur or even worse as cells behave opposite of their original function, efficacy of cell therapeutics would drop significantly or even be rendered useless. Thus far, unfortunately, such subpar regeneration efficacy is what most researchers working on cell therapy have met.

To improve and optimize therapeutic procedures (both during *in vitro* preparation steps and actual *in vivo* application), better understanding of what actually causes the treatment failure (i.e. what went wrong) is highly crucial and desirable. Conventionally, isolation of small subpopulation of cells and later on host's tissue are necessary to obtain such information. PCR, DNA microarray and antibody staining assay are common tools typically adapted for validation of successful *in vitro* cell preparation, with regards to their gene and protein expression. Meanwhile, histological staining and evaluation of tissue samples impart crucial information whether injected cells actually manage to integrate with targeted tissue, or instead trigger major immune rejection and be cleared from tissue vicinity.

While these methods are gold-standards as to provide such conclusive information, being invasive and disruptive in nature meant that each sample only provides a snapshot of the dynamic processes. In turn, data obtained lacks important temporal resolution (e.g. when these cells are eliminated from injection

site). Furthermore, multiple sample preparations and sacrifices would have to be made, should one intend to obtain time-dependent information of the processes.

As such, non-invasive / non-disruptive validation method is greatly desirable, where monitoring of sample progression does not require termination of the ongoing experiment. To this end, modification with gene reporter techniques permit highly specific and sensitive evaluation of cellular gene expressions. Such method however necessitates viral transduction of plasmid constructs of interest, as to achieve genomic integration. In turn, modified cells are no longer clinically applicable, with risks of random mutagenesis and un-recognized phenotypical changes.

Alternatively, various nanoparticles have been proposed, especially for tracking of cell migration post *in vivo* implantation. Nonetheless, these nanoparticles are typically passive contrast agents in that they do not provide information with regards to cell functionality. Meanwhile, some functional nanosensors (notably spherical nucleic acids; assembly of DNA probes on gold nanoparticles) have been proposed for at least *in vitro* experiments, to reveal cell viability status and even expression level of certain biomarkers. However, intracellular monitoring window of these nanosensors are limited between several hours to 1/2 days given nucleases activity in the cytoplasm.

This motivates this project to develop a nanosensor platform which can be utilized to reveal intracellular gene expression for a sustained period of time. Molecular beacon probes, hairpin-like DNA oligonucleotides with specific recognition towards certain sequences have been developed initially to evaluate PCR amplification. In recent years, these MBs have been proposed for intracellular application. Nonetheless, bolus delivery of MBs through transfection agents or induced membrane porosity is met with the same problem of nucleases delivery. In this regards, nanocarriers which can efficiently encapsulate, deliver and release MBs in a prolonged manner are highly promising. For this purpose, PLGA nanoparticles prepared through double emulsion method were chosen and its combination with MB probes were extensively studied in this project.

Firstly, characterization studies were performed on fabricated nanosensors (as described in chapter 3). In brief, encapsulation efficiency of MB probes, size and surface charge of nanosensors, as well release profile of MBs from nanosensors were firstly evaluated. Importantly, integrity of MB probes following release from nanosensors was also addressed. Following which, nanosensors were studied for actual cellular application on housekeeping gene target. Here, cytocompatibility of nanosensors, means of internalization and eventual MB delivery effectiveness were studied and compared against that of streptolysin O toxin-mediated pore permeation. By choosing MBs targeted towards gene that is stably expressed, reduction in nanosensor signal can be correlated with availability of MBs present in the cytoplasm. In the same manner, longitudinal availability of MBs from sustained nanosensor release was checked relative to bolus introduction of MBs.

Following successful demonstration that proposed nanosensors can effectively and safely deliver and prolong intracellular MB monitoring window, we then proceeded to apply such nanosensor in monitoring functional gene marker. In particular, MSCs multipotency and differentiation towards osteogenic and chondrogenic lineages were non-invasively tracked using the nanosensors. As MSCs are typically heterogenous in nature, spatial resolution of nanosensor signal in addition to its temporal resolution facilitates thorough validation of the differentiation processes, as to prevent stealth undifferentiated cells which maybe tumorigenesis during its further utilization *in vivo* (unlike conventional population-averaged PCR analysis). Chapter 4 describes our efforts in developing and demonstrating nanosensor's capability in tracking early osteogenesis of MSCs on 2D culture plate and film scaffold. Here, alkaline phosphatase mRNA was targeted as functional marker with a reference marker in GAPDH mRNA. By establishing such dual-colored & targeted tracking, more accurate information about the differentiation processes occurring on individual cells was obtained in a ratiometric fashion. Importantly, trend in nanosensor signal ratio was correlated with trend obtained from gold-standard RT-qPCR analysis, as a measure of specificity in signal generation. Lastly, staining for calcium deposits was performed to evaluate whether the differentiation process was impeded by nanosensor labeling.

Similarly, chapter 5 reports nanosensor application in MSC chondrogenesis, with the same GAPDH reference signal but Col2 functional marker instead. In such study, nanosensor monitoring was performed on a 3D hydrogel which encapsulated labeled MSCs, facilitated by confocal microscopy. Signal trend obtained on weekly timepoint was correlated as well with RT-qPCR. In conjunction with the notion that MSCs undergo differentiation procedures in a heterogenous rate especially in 3D culture settings, 3D spatial resolution of fluorescence signal obtained further enable examination of possible diffusional limit of these growth factors.

Lastly in chapter 6, nanosensor application was thoroughly examined for its spatio-temporal resolution limit. Temporally, the nanosensors were tested in Dox-activatable cell model in which expression of target mRNA can be finely-controlled with the addition of Dox molecules. By utilizing Dox constructs encoding for EGFP, nanosensor signal generation at cycles of Dox addition/removal could be correlated with EGFP fluorescence as well as EGFP mRNA expression from RT-qPCR. Meanwhile spatially, the platform application was extended to include reprogramming and transdifferentiation procedures on somatic fibroblastic cells. While both methods have blossomed into a multidisciplinary field given its promising potential to generate therapeutic cells from highly accessible and diverse cell sources, successful transformation occurred at much lesser efficiency at less than 1%. Nonetheless, positive signal expression was observed and successfully tracked in cells undergoing successful transformation (i.e. forming aggregates). Moreover, these positively-expressing cells were stained with respective antibodies to validate true cell transformation.

In conclusion, proposed nanosensor applications in non-invasive tracking of various cell transformation protocols (i.e. differentiation, reprogramming and transdifferentiation) proposed have been demonstrated and confirmed using gold-standard PCR method. Additionally, nanosensor labeling has not resulted in significant alteration of cell phenotypes (i.e. viability, potency, etc.). On this note, such nanosensor platform has great potential in the future to assist the field of regenerative medicine, as will be elaborated in the following section.

7.2 Future Directions

As hypothesized and briefly demonstrated herein, capability of nanosensor to track transformation progression of individual cells non-invasively and in a clinical-friendly manner. Such technology can benefit the translation of regenerative medicine approaches in various ways, both for *in vitro* culture optimization as well as allowing more comprehensive pre-clinical studies performed. Several ways in which nanosensor utilization could be adapted are listed below.

Optimization of culture protocols to resemble *in vivo* niche and maximize transformation efficiency

As mentioned previously, *in vitro* culture conditions differ significantly from actual *in vivo* niche of therapeutic cells. And while various scaffolds, molecules and even co-culture systems may alleviate this differences, inevitably phenotypical changes will happen on therapeutic cells of interest. Nanosensors could be adapted in doing the screening process, as to which combinations of molecules and scaffold materials/conformations result in least changes in cells' functional gene expression relative to when therapeutic cells were first isolated. In a similar way, it could be used to screen for optimal differentiation protocol from stem cells, which results in cells with the closest gene expression and thus functionality as targeted therapeutic cells. As compared to conventional optimization method which is disruptive in nature, such non-invasive evaluation greatly reduces the amount of resources which must be spent. More importantly with multiplexed monitoring enabled by nanosensor, expression of distinct differentiation markers could be evaluated simultaneously to more thoroughly validate successful differentiation.

Extending beyond just screening, nanosensors-labeled therapeutic cells can be suspended and sorted through fluorescence-assisted cell sorting (FACS). In this way, the heterogenous cell populations following *in vitro* treatment can be purified prior to any subsequent usage. Additionally, in a multi-step differentiation procedures, such isolation of successfully-differentiated cells after each round to be subjected to the next round of differentiation can greatly enhance the overall extent of differentiation and purity of eventual cell population.

In vivo monitoring of implanted therapeutic cells

Following successful expansion, derivation and characterization/validation study at *in vitro*-side, therapeutic cells of interest can be labeled with nanosensors to facilitate initial tracking of cells upon injection. In particular, cell viability and retention of functionality is of key tracking interests, aside its biodistribution. Using functional nanosensors targeted towards certain lineage markers, decline or even loss of observable nanosensor signal could be attributed to loss of cell viability and/or de-differentiation of therapeutic cells. While such correlation needs careful validation, it is very promising to potentially reduce the amount of animal subjects required in the study performed.

Thus far, *in vivo* optical imaging is limited to near surface depth given auto-fluorescence and significant signal attenuation from blood and surrounding tissues. Nonetheless, *in vivo* monitoring windows do exist in infra-red and far infra-red regions. At these wavelengths, there is markedly less attenuation from blood. Coupled with development of two-photon microscopy, signal penetration may reach to around 8-10mm from surface. Especially in small scale animal samples like mice, this is sufficiently deep to reach most injection location.

Development of targeted nanosensors

The current version of nanosensors only utilize positively-charged poly-L-lysine coating to non-specifically enhance nanosensors interaction with cell membrane and thus its internalization efficiency. For the purpose of labeling a single cell type in *in vitro* settings prior to treatment and tracking study, such nanosensor formulation is facile, versatile and yet meets the criteria. Nonetheless, in a more complex culture system consisting of multiple cell types and more so for direct nanosensor injection / labeling in *in vivo* settings, nanosensor internalization by specific cells of interest would be beneficial.

Such selectivity can be achieved by decorating nanocarriers with targeting moieties, including but not limited to antibodies and aptamers (oligonucleotides with specific hybridization towards proteins- in this case cell surface marker). While it may potentially alter release profile of MB probes encapsulated within, conjugation

with these targeting moieties may also facilitate greater cellular internalization efficiency.

7.3 Conclusion and Outlook

Since its first introduction, the concept of regenerative medicine has vastly developed over the past few decades due to shortage of available transplantable tissues/organs to replace or compensate for insufficient tissue self-recovery following injuries. Nevertheless, translational process of regenerative medicine approaches has been somewhat lack-luster with many studies stopped at late-stage preclinical or early clinical study. In most of these studies, injection or implantation of therapeutic cells was not followed with significant recovery of tissue function over control groups. Typically, this is a direct consequence of cells not integrating well with host tissue, thus rapidly lose its viability & functionality, and cleared by host's immune system. Validating phenotypical properties of therapeutic cells at every stage of the development is crucial for more comprehensive understanding of cell fate, as to avoid further progression of half-optimized, half-validated treatment procedures. Conventionally, this is performed via protein and/or gene expression analysis (e.g. western blot, PCR, etc.). However, such disruptive methods necessitate termination of ongoing experimental procedures, which is associated with significantly greater resource requirement (i.e. time, materials, animal subjects).

In this project, novel nanosensor formulation was proposed and demonstrated to enable long term, non-invasive tracking of intracellular mRNA expression. Following its physical characterization, nanosensor was utilized to track osteogenesis and chondrogenesis of MSCs, as well as reprogramming and cardiogenic transdifferentiation of somatic fibroblast cells. With its minimal cytotoxicity, versatility and highly specific ratiometric signal generation, we envision further application of proposed nanosensors especially for developmental and optimization process during *in vitro* preparation of therapeutic cells. At this stage, rigorous screening of molecules/factors combination and treatment time can be appreciably simplified with nanosensor non-invasive monitoring. Additionally, such

formulation can be readily adapted to track therapeutic cells' functionality following injection in small animals for early-phase preclinical studies.

In conclusion, while direct in vivo translation of proposed nanosensor might be quite limited due to low penetration depth of optical imaging, its application can greatly simplify early studies of various regenerative medicine approaches, thus accelerating clinical translation of these approaches.

References

1. Duscher, D., et al., *Stem cells in wound healing: the future of regenerative medicine? A mini-review*. Gerontology, 2016. **62**(2): p. 216-225.
2. Wobma, H. and G. Vunjak-Novakovic, *Tissue engineering and regenerative medicine 2015: a year in review*. Tissue Engineering Part B: Reviews, 2016. **22**(2): p. 101-113.
3. Kang, Y.J., *Regenerative Medicine Research: striving to better serve the emerging field*. Regenerative medicine research, 2016. **4**.
4. Allickson, J., *Emerging Translation of Regenerative Therapies*. Clinical Pharmacology & Therapeutics, 2017.
5. Gardner, J. and A. Webster, *The social management of biomedical novelty: Facilitating translation in regenerative medicine*. Social Science & Medicine, 2016. **156**: p. 90-97.
6. Sadtler, K., et al., *Design, clinical translation and immunological response of biomaterials in regenerative medicine*. Nature Reviews Materials, 2016. **1**: p. 16040.
7. Yea, C.-H., et al., *In situ label-free quantification of human pluripotent stem cells with electrochemical potential*. Biomaterials, 2016. **75**: p. 250-259.
8. Mora, C., et al., *Clinical potentials of human pluripotent stem cells*. Cell Biology and Toxicology, 2017: p. 1-10.
9. Gupta, V., et al., *Stem Cell Biology and Its Clinical Application*, in *Basic and Applied Aspects of Biotechnology*. 2017, Springer. p. 333-349.
10. Li, K., et al., *Small molecules facilitate the reprogramming of mouse fibroblasts into pancreatic lineages*. Cell stem cell, 2014. **14**(2): p. 228-236.
11. Holdhoff, M., et al., *ATPS-33 Evidence of absence for cytomegalovirus in patients with glioblastomas and other high-grade gliomas by real-time PCR for DNA, immunohistochemistry for protein and in-situ hybridization for mRNA*. Neuro-oncology, 2015. **17**(suppl_5): p. v25-v25.
12. Woodworth, M.B., K.M. Girsakis, and C.A. Walsh, *Building a lineage from single cells: genetic techniques for cell lineage tracking*. Nature Reviews Genetics, 2017. **18**(4): p. 230-244.
13. Megyola, C.M., et al., *Dynamic Migration and Cell-Cell Interactions of Early Reprogramming Revealed by High-Resolution Time-Lapse Imaging*. Stem Cells, 2013. **31**(5): p. 895-905.
14. Quintanilla Jr, R.H., et al., *Kinetic Measurement and Real Time Visualization of Somatic Reprogramming*. JoVE (Journal of Visualized Experiments), 2016(113): p. e54190-e54190.
15. Jo, C.H., et al., *Intra-articular injection of mesenchymal stem cells for the treatment of osteoarthritis of the knee: a proof-of-concept clinical trial*. Stem cells, 2014. **32**(5): p. 1254-1266.
16. Sohni, A. and C.M. Verfaillie, *Mesenchymal stem cells migration homing and tracking*. Stem cells international, 2013. **2013**.

17. Kim, S.J., et al., *Superparamagnetic iron oxide nanoparticles for direct labeling of stem cells and in vivo MRI tracking*. Contrast media & molecular imaging, 2016. **11**(1): p. 55-64.
18. Aswendt, M., et al., *Novel bimodal iron oxide particles for efficient tracking of human neural stem cells in vivo*. Nanomedicine, 2015. **10**(16): p. 2499-2512.
19. Hitchens, T.K., et al., *Combining perfluorocarbon and superparamagnetic iron-oxide cell labeling for improved and expanded applications of cellular MRI*. Magnetic resonance in medicine, 2015. **73**(1): p. 367-375.
20. Tan, W., K. Wang, and T.J. Drake, *Molecular beacons*. Current opinion in chemical biology, 2004. **8**(5): p. 547-553.
21. Tan, W., et al., *Molecular beacons: a novel DNA probe for nucleic acid and protein studies*. Chemistry-A European Journal, 2000. **6**(7): p. 1107-1111.
22. Desai, H.V., et al., *Live-cell, temporal gene expression analysis of osteogenic differentiation in adipose-derived stem cells*. Tissue Engineering Part A, 2014. **20**(5-6): p. 899-907.
23. Boutorine, A.S., et al., *Fluorescent probes for nucleic acid visualization in fixed and live cells*. Molecules, 2013. **18**(12): p. 15357-15397.
24. Li, N., et al., *A tumour mRNA-triggered nanocarrier for multimodal cancer cell imaging and therapy*. Chemical Communications, 2014. **50**(56): p. 7473-7476.
25. Ke, G., et al., *L-DNA molecular beacon: a safe, stable, and accurate intracellular nano-thermometer for temperature sensing in living cells*. Journal of the American Chemical Society, 2012. **134**(46): p. 18908-18911.
26. Yannas, I.V., *Similarities and differences between induced organ regeneration in adults and early foetal regeneration*. Journal of The Royal Society Interface, 2005. **2**(5): p. 403-417.
27. Gurtner, G.C., et al., *Wound repair and regeneration*. Nature, 2008. **453**(7193): p. 314.
28. Conboy, I.M. and T.A. Rando, *Aging, stem cells and tissue regeneration: lessons from muscle*. Cell cycle, 2005. **4**(3): p. 407-410.
29. Beausejour, C.M. and J. Campisi, *Ageing: balancing regeneration and cancer*. Nature, 2006. **443**(7110): p. 404-406.
30. Gomes, M.E., et al., *Tissue Engineering and Regenerative Medicine: New Trends and Directions—A Year in Review*. Tissue Engineering Part B: Reviews, 2017. **23**(3): p. 211-224.
31. Xu, Y., Y. Shi, and S. Ding, *A chemical approach to stem-cell biology and regenerative medicine*. Nature, 2008. **453**(7193): p. 338.
32. Yagi, H., et al., *Human-scale whole-organ bioengineering for liver transplantation: a regenerative medicine approach*. Cell transplantation, 2013. **22**(2): p. 231-242.
33. Horch, R.E., L.M. Popescu, and E. Polykandriotis, *History of Regenerative Medicine, in Regenerative Medicine-from Protocol to Patient*. 2016, Springer. p. 1-19.
34. Kaiser, L., *The future of multihospital systems*. Topics in health care financing, 1991. **18**(4): p. 32-45.
35. Kealey, T., *Liberation biology: The scientific and moral case for the biotech revolution by ronald bailey*. Economic Affairs, 2007. **27**(2): p. 104-105.

36. Haseltine, W.A., *The emergence of regenerative medicine: a new field and a new society*. e-biomed: the journal of regenerative medicine, 2001. **2**(4): p. 17-23.
37. Gonfiotti, A., et al., *The first tissue-engineered airway transplantation: 5-year follow-up results*. The Lancet, 2014. **383**(9913): p. 238-244.
38. Kamao, H., et al., *Characterization of human induced pluripotent stem cell-derived retinal pigment epithelium cell sheets aiming for clinical application*. Stem cell reports, 2014. **2**(2): p. 205-218.
39. Assawachananont, J., et al., *Transplantation of embryonic and induced pluripotent stem cell-derived 3D retinal sheets into retinal degenerative mice*. Stem cell reports, 2014. **2**(5): p. 662-674.
40. Zakrzewski, J.L., M.R. Van Den Brink, and J.A. Hubbell, *Overcoming immunological barriers in regenerative medicine*. Nature biotechnology, 2014. **32**(8): p. 786-794.
41. Cools, P., et al., *Non-thermal Plasma Technology for the Improvement of Scaffolds for Tissue Engineering and Regenerative Medicine-A Review*. 2016.
42. Hipp, J. and A. Atala, *Sources of stem cells for regenerative medicine*. Stem Cell Reviews, 2008. **4**(1): p. 3-11.
43. Stoltz, J.-F., et al., *Stem cells and regenerative medicine: myth or reality of the 21st century*. Stem cells international, 2015. **2015**.
44. Mason, C. and P. Dunnill, *A brief definition of regenerative medicine*. 2008.
45. Richardson, S.M., et al., *Mesenchymal stem cells in regenerative medicine: focus on articular cartilage and intervertebral disc regeneration*. Methods, 2016. **99**: p. 69-80.
46. Lee, W.C., et al., *Multivariate biophysical markers predictive of mesenchymal stromal cell multipotency*. Proceedings of the National Academy of Sciences, 2014. **111**(42): p. E4409-E4418.
47. Pittenger, M.F., et al., *Multilineage potential of adult human mesenchymal stem cells*. science, 1999. **284**(5411): p. 143-147.
48. Prockop, D.J., et al., *Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs)*. Journal of cellular and molecular medicine, 2010. **14**(9): p. 2190-2199.
49. Von Bahr, L., et al., *Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation*. Stem cells, 2012. **30**(7): p. 1575-1578.
50. Lee, R.H., et al., *Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6*. Cell stem cell, 2009. **5**(1): p. 54-63.
51. Majumdar, M.K., et al., *Human marrow-derived mesenchymal stem cells (MSCs) express hematopoietic cytokines and support long-term hematopoiesis when differentiated toward stromal and osteogenic lineages*. Journal of hematotherapy & stem cell research, 2000. **9**(6): p. 841-848.
52. English, K., *Mechanisms of mesenchymal stromal cell immunomodulation*. Immunology and cell biology, 2013. **91**(1): p. 19.
53. Kode, J.A., et al., *Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration*. Cytotherapy, 2009. **11**(4): p. 377-391.

54. Ren, G., et al., *Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide*. Cell stem cell, 2008. **2**(2): p. 141-150.
55. Sukmawati, D. and R. Tanaka, *Introduction to next generation of endothelial progenitor cell therapy: a promise in vascular medicine*. American journal of translational research, 2015. **7**(3): p. 411.
56. Kalka, C., et al., *Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization*. Proceedings of the National Academy of Sciences, 2000. **97**(7): p. 3422-3427.
57. Losordo, D.W., et al., *Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina*. Circulation, 2007. **115**(25): p. 3165-3172.
58. Yu, J., et al., *Induced pluripotent stem cell lines derived from human somatic cells*. science, 2007. **318**(5858): p. 1917-1920.
59. Warren, L., et al., *Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA*. Cell stem cell, 2010. **7**(5): p. 618-630.
60. Zhang, Y., et al., *Small molecules, big roles—the chemical manipulation of stem cell fate and somatic cell reprogramming*. J Cell Sci, 2012. **125**(23): p. 5609-5620.
61. Tashiro, K., et al., *Efficient adipocyte and osteoblast differentiation from mouse induced pluripotent stem cells by adenoviral transduction*. Stem cells, 2009. **27**(8): p. 1802-1811.
62. Vierbuchen, T., et al., *Direct conversion of fibroblasts to functional neurons by defined factors*. Nature, 2010. **463**(7284): p. 1035.
63. Schwartz, M., et al., *The remedy may lie in ourselves: prospects for immune cell therapy in central nervous system protection and repair*. Journal of molecular medicine, 1999. **77**(10): p. 713-717.
64. Anguille, S., et al., *Clinical use of dendritic cells for cancer therapy*. The lancet oncology, 2014. **15**(7): p. e257-e267.
65. Hinrichs, C.S. and S.A. Rosenberg, *Exploiting the curative potential of adoptive T-cell therapy for cancer*. Immunological reviews, 2014. **257**(1): p. 56-71.
66. Mcconville, H., et al., *Car T-cell Therapy Effects: Review of procedures and patient education*. Clinical Journal of Oncology Nursing, 2017. **21**(3): p. E79-E86.
67. Chan, B. and K. Leong, *Scaffolding in tissue engineering: general approaches and tissue-specific considerations*. European spine journal, 2008. **17**(4): p. 467-479.
68. Martin, I., D. Wendt, and M. Heberer, *The role of bioreactors in tissue engineering*. TRENDS in Biotechnology, 2004. **22**(2): p. 80-86.
69. O'brien, F.J., *Biomaterials & scaffolds for tissue engineering*. Materials today, 2011. **14**(3): p. 88-95.
70. Rehfeldt, F., et al., *Cell responses to the mechanochemical microenvironment—implications for regenerative medicine and drug delivery*. Advanced drug delivery reviews, 2007. **59**(13): p. 1329-1339.
71. Babensee, J.E., et al., *Host response to tissue engineered devices*. Advanced drug delivery reviews, 1998. **33**(1): p. 111-139.
72. Hutmacher, D.W., *Scaffolds in tissue engineering bone and cartilage*. Biomaterials, 2000. **21**(24): p. 2529-2543.

73. Yoshimoto, H., et al., *A biodegradable nanofiber scaffold by electrospinning and its potential for bone tissue engineering*. Biomaterials, 2003. **24**(12): p. 2077-2082.
74. Ko, H., B.K. Milthorpe, and C.D. McFarland, *Engineering thick tissues-the vascularisation problem*. European Cells and Materials, 2007.
75. Phelps, E.A. and A.J. Garcia, *Update on therapeutic vascularization strategies*. Regenerative medicine, 2009. **4**(1): p. 65-80.
76. Hench, L.L. and J.M. Polak, *Third-generation biomedical materials*. Science, 2002. **295**(5557): p. 1014-1017.
77. Ambrosio, A., et al., *A novel amorphous calcium phosphate polymer ceramic for bone repair: I. Synthesis and characterization*. Journal of Biomedical Materials Research Part A, 2001. **58**(3): p. 295-301.
78. Frenkel, S.R. and P.E. Di Cesare, *Scaffolds for articular cartilage repair*. Annals of biomedical engineering, 2004. **32**(1): p. 26-34.
79. Hussain, A., et al., *Functional 3-D cardiac co-culture model using bioactive chitosan nanofiber scaffolds*. Biotechnology and bioengineering, 2013. **110**(2): p. 637-647.
80. Oh, S.H., et al., *Fabrication and characterization of hydrophilic poly (lactic-co-glycolic acid)/poly (vinyl alcohol) blend cell scaffolds by melt-molding particulate-leaching method*. Biomaterials, 2003. **24**(22): p. 4011-4021.
81. Rowlands, A., et al., *Polyurethane/poly (lactic-co-glycolic) acid composite scaffolds fabricated by thermally induced phase separation*. Biomaterials, 2007. **28**(12): p. 2109-2121.
82. Friess, W. and M. Schlapp, *Sterilization of gentamicin containing collagen/PLGA microparticle composites*. European journal of pharmaceutics and biopharmaceutics, 2006. **63**(2): p. 176-187.
83. Pek, Y.S., A.C. Wan, and J.Y. Ying, *The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel*. Biomaterials, 2010. **31**(3): p. 385-391.
84. Engler, A.J., et al., *Matrix elasticity directs stem cell lineage specification*. Cell, 2006. **126**(4): p. 677-689.
85. Engler, A.J., et al., *Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating*. Journal of cell science, 2008. **121**(22): p. 3794-3802.
86. Ji, J., et al., *Patient-Derived Human Induced Pluripotent Stem Cells From Gingival Fibroblasts Compositated With Defined Nanohydroxyapatite/Chitosan/Gelatin Porous Scaffolds as Potential Bone Graft Substitutes*. Stem cells translational medicine, 2016. **5**(1): p. 95-105.
87. Yoon, J.J., et al., *Heparin-immobilized biodegradable scaffolds for local and sustained release of angiogenic growth factor*. Journal of Biomedical Materials Research Part A, 2006. **79**(4): p. 934-942.
88. Bianco, P. and P.G. Robey, *Stem cells in tissue engineering*. Nature, 2001. **414**(6859): p. 118.
89. Chiu, L.L. and M. Radisic, *Scaffolds with covalently immobilized VEGF and Angiopoietin-1 for vascularization of engineered tissues*. Biomaterials, 2010. **31**(2): p. 226-241.

90. Hadjizadeh, A., F. Ghasemkhah, and N. Ghasemzaie, *Polymeric Scaffold Based Gene Delivery Strategies to Improve Angiogenesis in Tissue Engineering: A Review*. Polymer Reviews, 2017. **57**(3): p. 505-556.
91. Berthiaume, F., T.J. Maguire, and M.L. Yarmush, *Tissue engineering and regenerative medicine: history, progress, and challenges*. Annual review of chemical and biomolecular engineering, 2011. **2**: p. 403-430.
92. Lysaght, M.J., A. Jaklenec, and E. Deweerdt, *Great expectations: private sector activity in tissue engineering, regenerative medicine, and stem cell therapeutics*. Tissue Engineering Part A, 2008. **14**(2): p. 305-315.
93. Aboody, K., et al., *Translating stem cell studies to the clinic for CNS repair: current state of the art and the need for a Rosetta stone*. Neuron, 2011. **70**(4): p. 597-613.
94. Tabar, V. and L. Studer, *Pluripotent stem cells in regenerative medicine: challenges and recent progress*. Nature reviews. Genetics, 2014. **15**(2): p. 82.
95. Juhászová, J., et al., *Osteogenic differentiation of miniature pig mesenchymal stem cells in 2D and 3D environment*. Physiological research, 2011. **60**(3): p. 559.
96. Nussbaum, J., et al., *Transplantation of undifferentiated murine embryonic stem cells in the heart: teratoma formation and immune response*. The FASEB Journal, 2007. **21**(7): p. 1345-1357.
97. Nguyen, P.K., J. Riegler, and J.C. Wu, *Stem cell imaging: from bench to bedside*. Cell stem cell, 2014. **14**(4): p. 431-444.
98. Salmikangas, P., et al., *Marketing regulatory oversight of advanced therapy medicinal products (ATMPs) in Europe: the EMA/CAT perspective*, in *Regulatory Aspects of Gene Therapy and Cell Therapy Products*. 2015, Springer. p. 103-130.
99. Emmert, M.Y., E.S. Fioretta, and S.P. Hoerstrup, *Translational challenges in cardiovascular tissue engineering*. Journal of Cardiovascular Translational Research, 2017. **10**(2): p. 139-149.
100. Goldring, C.E., et al., *Assessing the safety of stem cell therapeutics*. Cell stem cell, 2011. **8**(6): p. 618-628.
101. Bara, J.J., et al., *Concise review: Bone marrow-derived mesenchymal stem cells change phenotype following in vitro culture: implications for basic research and the clinic*. Stem cells, 2014. **32**(7): p. 1713-1723.
102. Vacanti, V., et al., *Phenotypic changes of adult porcine mesenchymal stem cells induced by prolonged passaging in culture*. Journal of cellular physiology, 2005. **205**(2): p. 194-201.
103. Dominici, M., et al., *Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement*. Cytotherapy, 2006. **8**(4): p. 315-317.
104. Das, K.P., T.M. Freudenrich, and W.R. Mundy, *Assessment of PC12 cell differentiation and neurite growth: a comparison of morphological and neurochemical measures*. Neurotoxicology and teratology, 2004. **26**(3): p. 397-406.
105. Leferink, A.M., C.A. van Blitterswijk, and L. Moroni, *Methods of monitoring cell fate and tissue growth in three-dimensional scaffold-based strategies for in vitro tissue engineering*. Tissue Engineering Part B: Reviews, 2016. **22**(4): p. 265-283.
106. Mather, M.L., S.P. Morgan, and J.A. Crowe, *Meeting the needs of monitoring in tissue engineering*. Regenerative medicine, 2007. **2**(2): p. 145-160.

107. Holmes, C., M. Tabrizian, and P.O. Bagnaninchi, *Motility imaging via optical coherence phase microscopy enables label-free monitoring of tissue growth and viability in 3D tissue-engineering scaffolds*. Journal of tissue engineering and regenerative medicine, 2015. **9**(5): p. 641-645.
108. Kang, J.H. and J.-K. Chung, *Molecular-genetic imaging based on reporter gene expression*. Journal of Nuclear Medicine, 2008. **49**(Suppl 2): p. 164S-179S.
109. Swenson, E.S., et al., *Limitations of green fluorescent protein as a cell lineage marker*. Stem Cells, 2007. **25**(10): p. 2593-2600.
110. Paul, A., et al., *Microencapsulated stem cells for tissue repairing: implications in cell-based myocardial therapy*. Regenerative Medicine, 2009. **4**(5): p. 733-745.
111. Li, S.-H., et al., *Tracking cardiac engraftment and distribution of implanted bone marrow cells: comparing intra-aortic, intravenous, and intramyocardial delivery*. The Journal of thoracic and cardiovascular surgery, 2009. **137**(5): p. 1225-1233.e1.
112. Zheng, M.-H., et al., *Matrix-induced autologous chondrocyte implantation (MACI®): biological and histological assessment*. Tissue engineering, 2007. **13**(4): p. 737-746.
113. Hoehn, M., et al., *Monitoring of implanted stem cell migration in vivo: a highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat*. Proceedings of the National Academy of Sciences, 2002. **99**(25): p. 16267-16272.
114. Moore, X., et al., *Endothelial progenitor cells''homing'specificity to brain tumors*. Gene therapy, 2004. **11**(10): p. 811.
115. Vilalta, M., et al., *Biodistribution, long-term survival, and safety of human adipose tissue-derived mesenchymal stem cells transplanted in nude mice by high sensitivity non-invasive bioluminescence imaging*. Stem cells and development, 2008. **17**(5): p. 993-1004.
116. Chen, I.Y., et al., *Comparison of optical bioluminescence reporter gene and superparamagnetic iron oxide MR contrast agent as cell markers for noninvasive imaging of cardiac cell transplantation*. Molecular Imaging and Biology, 2009. **11**(3): p. 178-187.
117. O'Keefe, R.J. and J. Mao, *Bone tissue engineering and regeneration: from discovery to the clinic—an overview*. Tissue Engineering Part B: Reviews, 2011. **17**(6): p. 389-392.
118. Ratcliffe, E., R.J. Thomas, and D.J. Williams, *Current understanding and challenges in bioprocessing of stem cell-based therapies for regenerative medicine*. British medical bulletin, 2011: p. ldr037.
119. Keith, B. and M.C. Simon, *Hypoxia-inducible factors, stem cells, and cancer*. Cell, 2007. **129**(3): p. 465-472.
120. Mohyeldin, A., T. Garzón-Muvdi, and A. Quiñones-Hinojosa, *Oxygen in stem cell biology: a critical component of the stem cell niche*. Cell stem cell, 2010. **7**(2): p. 150-161.
121. Diepart, C., et al., *Arsenic trioxide treatment decreases the oxygen consumption rate of tumor cells and radiosensitizes solid tumors*. Cancer research, 2012. **72**(2): p. 482-490.

122. Dmitriev, R.I. and D.B. Papkovsky, *Optical probes and techniques for O₂ measurement in live cells and tissue*. Cellular and molecular life sciences, 2012. **69**(12): p. 2025-2039.
123. Wang, X.-d., et al., *Ultra-small, highly stable, and membrane-impermeable fluorescent nanosensors for oxygen*. Methods and Applications in Fluorescence, 2013. **1**(3): p. 035002.
124. Wilson, D., *Measuring oxygen using oxygen dependent quenching of phosphorescence: a status report*, in *Optical Imaging of Brain Function and Metabolism*. 1993, Springer. p. 225-232.
125. Koo Lee, Y.-E., et al., *Near infrared luminescent oxygen nanosensors with nanoparticle matrix tailored sensitivity*. Analytical chemistry, 2010. **82**(20): p. 8446-8455.
126. Wang, X.-d., et al., *Self-referenced RGB colour imaging of intracellular oxygen*. Chemical Science, 2011. **2**(5): p. 901-906.
127. Martin, C.H., D. Wu, and K.L.J. Prather, *Integrated bioprocessing for the pH-dependent production of 4-valerolactone from levulinate in Pseudomonas putida KT2440*. Applied and environmental microbiology, 2010. **76**(2): p. 417-424.
128. Chaudhry, M.A., B.D. Bowen, and J.M. Piret, *Culture pH and osmolality influence proliferation and embryoid body yields of murine embryonic stem cells*. Biochemical Engineering Journal, 2009. **45**(2): p. 126-135.
129. Chan, K.W., et al., *MRI-detectable pH nanosensors incorporated into hydrogels for in vivo sensing of transplanted cell viability*. Nature materials, 2013. **12**(3): p. 268.
130. Sharma, P., et al., *Gadolinium-Doped Silica Nanoparticles Encapsulating Indocyanine Green for Near Infrared and Magnetic Resonance Imaging*. Small, 2012. **8**(18): p. 2856-2868.
131. Mehta, M., et al., *Biomaterial delivery of morphogens to mimic the natural healing cascade in bone*. Advanced drug delivery reviews, 2012. **64**(12): p. 1257-1276.
132. Wang, X.d., R.J. Meier, and O.S. Wolfbeis, *Fluorescent pH-Sensitive Nanoparticles in an Agarose Matrix for Imaging of Bacterial Growth and Metabolism*. Angewandte Chemie International Edition, 2013. **52**(1): p. 406-409.
133. Schambach, S.J., et al., *Application of micro-CT in small animal imaging*. Methods, 2010. **50**(1): p. 2-13.
134. Massoud, T.F. and S.S. Gambhir, *Molecular imaging in living subjects: seeing fundamental biological processes in a new light*. Genes & development, 2003. **17**(5): p. 545-580.
135. Kazakova, L.I., et al., *Chemosensors and biosensors based on polyelectrolyte microcapsules containing fluorescent dyes and enzymes*. Analytical and bioanalytical chemistry, 2013. **405**(5): p. 1559-1568.
136. Sandros, M.G., V. Shete, and D.E. Benson, *Selective, reversible, reagentless maltose biosensing with core-shell semiconducting nanoparticles*. Analyst, 2006. **131**(2): p. 229-235.
137. Quinti, L., R. Weissleder, and C.-H. Tung, *A fluorescent nanosensor for apoptotic cells*. Nano letters, 2006. **6**(3): p. 488-490.

138. Kim, K., et al., *Cell-permeable and biocompatible polymeric nanoparticles for apoptosis imaging*. Journal of the American Chemical Society, 2006. **128**(11): p. 3490-3491.
139. Kasili, P.M., J.M. Song, and T. Vo-Dinh, *Optical sensor for the detection of caspase-9 activity in a single cell*. Journal of the American Chemical Society, 2004. **126**(9): p. 2799-2806.
140. Neves, A.A. and K.M. Brindle, *Imaging cell death*. Journal of Nuclear Medicine, 2014. **55**(1): p. 1-4.
141. Crabtree, J., et al., *Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis*. Gut, 1991. **32**(12): p. 1473-1477.
142. Cha, T.-G., et al., *Optical nanosensor architecture for cell-signaling molecules using DNA aptamer-coated carbon nanotubes*. ACS nano, 2011. **5**(5): p. 4236-4244.
143. Seferos, D.S., et al., *Nano-flares: probes for transfection and mRNA detection in living cells*. Journal of the American Chemical Society, 2007. **129**(50): p. 15477-15479.
144. Prigodich, A.E., et al., *Multiplexed nanoflares: mRNA detection in live cells*. Analytical chemistry, 2012. **84**(4): p. 2062-2066.
145. Halo, T.L., et al., *NanoFlares for the detection, isolation, and culture of live tumor cells from human blood*. Proceedings of the National Academy of Sciences, 2014. **111**(48): p. 17104-17109.
146. He, L., et al., *Fluorescence Resonance Energy Transfer-Based DNA Tetrahedron Nanotweezer for Highly Reliable Detection of Tumor-Related mRNA in Living Cells*. ACS nano, 2017. **11**(4): p. 4060-4066.
147. Li, N., et al., *A Multicolor Nanoprobe for Detection and Imaging of Tumor-Related mRNAs in Living Cells*. Angewandte Chemie International Edition, 2012. **51**(30): p. 7426-7430.
148. Taylor, A., et al., *Long-term tracking of cells using inorganic nanoparticles as contrast agents: are we there yet?* Chemical Society Reviews, 2012. **41**(7): p. 2707-2717.
149. Bulte, J.W., *In vivo MRI cell tracking: clinical studies*. American Journal of Roentgenology, 2009. **193**(2): p. 314-325.
150. Rosen, A.B., et al., *Finding Fluorescent Needles in the Cardiac Haystack: Tracking Human Mesenchymal Stem Cells Labeled with Quantum Dots for Quantitative In Vivo Three-Dimensional Fluorescence Analysis*. Stem Cells, 2007. **25**(8): p. 2128-2138.
151. Wu, T.-J., et al., *Tracking the engraftment and regenerative capabilities of transplanted lung stem cells using fluorescent nanodiamonds*. Nature nanotechnology, 2013. **8**(9): p. 682-689.
152. Wang, H.H., et al., *Durable mesenchymal stem cell labelling by using polyhedral superparamagnetic iron oxide nanoparticles*. Chemistry-A European Journal, 2009. **15**(45): p. 12417-12425.
153. Barnett, B.P., et al., *Magnetic resonance-guided, real-time targeted delivery and imaging of magnetocapsules immunoprotecting pancreatic islet cells*. Nature medicine, 2007. **13**(8): p. 986.

154. Lewin, M., et al., *Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells*. Nature biotechnology, 2000. **18**(4): p. 410.
155. Terrovitis, J., et al., *Magnetic resonance imaging overestimates ferumoxide-labeled stem cell survival after transplantation in the heart*. Circulation, 2008. **117**(12): p. 1555-1562.
156. Winter, E., et al., *Cell tracking using iron oxide fails to distinguish dead from living transplanted cells in the infarcted heart*. Magnetic resonance in medicine, 2010. **63**(3): p. 817-821.
157. Zhang, H.F., et al., *Functional photoacoustic microscopy for high-resolution and noninvasive in vivo imaging*. Nature biotechnology, 2006. **24**(7): p. 848.
158. Helmchen, F. and W. Denk, *Deep tissue two-photon microscopy*. Nature methods, 2005. **2**(12): p. 932-940.
159. Golub, T.R., et al., *Molecular classification of cancer: class discovery and class prediction by gene expression monitoring*. science, 1999. **286**(5439): p. 531-537.
160. Chang, H.Y., et al., *Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds*. PLoS biology, 2004. **2**(2): p. e7.
161. Kulterer, B., et al., *Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation*. BMC genomics, 2007. **8**(1): p. 70.
162. Hung, S.-C., et al., *Gene expression profiles of early adipogenesis in human mesenchymal stem cells*. Gene, 2004. **340**(1): p. 141-150.
163. Bustin, S.A., *Real-time, fluorescence-based quantitative PCR: a snapshot of current procedures and preferences*. Expert review of molecular diagnostics, 2005. **5**(4): p. 493-498.
164. Nolan, T., R.E. Hands, and S.A. Bustin, *Quantification of mRNA using real-time RT-PCR*. Nature protocols, 2006. **1**(3): p. 1559.
165. Tyagi, S., *Imaging intracellular RNA distribution and dynamics in living cells*. natuRe methods, 2009. **6**(5): p. 331-338.
166. Yang, C.J., C.D. Medley, and W. Tan, *Monitoring nucleic acids using molecular beacons*. Current pharmaceutical biotechnology, 2005. **6**(6): p. 445-452.
167. Chen, A.K., M.A. Behlke, and A. Tsourkas, *Efficient cytosolic delivery of molecular beacon conjugates and flow cytometric analysis of target RNA*. Nucleic acids research, 2008. **36**(12): p. e69-e69.
168. Chen, A.K., M.A. Behlke, and A. Tsourkas, *Avoiding false-positive signals with nuclease-vulnerable molecular beacons in single living cells*. Nucleic acids research, 2007. **35**(16): p. e105.
169. Li, J.J., R. Geyer, and W. Tan, *Using molecular beacons as a sensitive fluorescence assay for enzymatic cleavage of single-stranded DNA*. Nucleic acids research, 2000. **28**(11): p. e52-e52.
170. Song, F., et al., *Nanocomposite hydrogels and their applications in drug delivery and tissue engineering*. Journal of biomedical nanotechnology, 2015. **11**(1): p. 40-52.
171. Li, M., et al., *Functional nanoparticles in targeting glioma diagnosis and therapies*. Journal of nanoscience and nanotechnology, 2014. **14**(1): p. 415-432.

172. Chen, T., et al., *DNA micelle flares for intracellular mRNA imaging and gene therapy*. *Angewandte Chemie*, 2013. **125**(7): p. 2066-2070.
173. Pan, W., et al., *Simultaneous visualization of multiple mRNAs and matrix metalloproteinases in living cells using a fluorescence nanoprobe*. *Chemistry-A European Journal*, 2015. **21**(16): p. 6070-6073.
174. Xu, C., et al., *Tracking mesenchymal stem cells with iron oxide nanoparticle loaded poly (lactide-co-glycolide) microparticles*. *Nano letters*, 2012. **12**(8): p. 4131-4139.
175. Rosi, N.L., et al., *Oligonucleotide-modified gold nanoparticles for intracellular gene regulation*. *Science*, 2006. **312**(5776): p. 1027-1030.
176. Yue, H., et al., *Particle size affects the cellular response in macrophages*. *European Journal of Pharmaceutical Sciences*, 2010. **41**(5): p. 650-657.
177. Wang, B., et al., *Cellular uptake of covalent poly (allylamine hydrochloride) microcapsules and its influences on cell functions*. *Macromolecular bioscience*, 2012. **12**(11): p. 1534-1545.
178. Selvi, R.B., et al., *ATP driven clathrin dependent entry of carbon nanospheres prefer cells with glucose receptors*. *Journal of nanobiotechnology*, 2012. **10**(1): p. 35.
179. Gao, Y., et al., *Tracking mesenchymal stem cell tumor-homing using fluorescent silica nanoparticles*. *Journal of Materials Chemistry B*, 2015. **3**(7): p. 1245-1253.
180. Zolnik, B.S. and D.J. Burgess, *Effect of acidic pH on PLGA microsphere degradation and release*. *Journal of Controlled Release*, 2007. **122**(3): p. 338-344.
181. Zhang, Z.-Y., et al., *The potential of human fetal mesenchymal stem cells for off-the-shelf bone tissue engineering application*. *Biomaterials*, 2012. **33**(9): p. 2656-2672.
182. Zhang, Z.Y., et al., *Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells*. *Stem cells*, 2009. **27**(1): p. 126-137.
183. Lee, Z., et al., *Imaging stem cell differentiation for cell-based tissue repair*. *Methods Enzymol*, 2012. **506**(2): p. 247-263.
184. Nakamura, A., et al., *Osteocalcin secretion as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells*. *Tissue Engineering Part C: Methods*, 2009. **15**(2): p. 169-180.
185. Song, I.H., A.I. Caplan, and J.E. Dennis, *In vitro dexamethasone pretreatment enhances bone formation of human mesenchymal stem cells in vivo*. *Journal of orthopaedic research*, 2009. **27**(7): p. 916-921.
186. Corn, D.J., et al., *Imaging early stage osteogenic differentiation of mesenchymal stem cells*. *Journal of Orthopaedic Research*, 2013. **31**(6): p. 871-879.
187. Progatzy, F., M.J. Dallman, and C.L. Celso, *From seeing to believing: labelling strategies for in vivo cell-tracking experiments*. *Interface focus*, 2013. **3**(3): p. 20130001.
188. Newman, R.H., M.D. Fosbrink, and J. Zhang, *Genetically encodable fluorescent biosensors for tracking signaling dynamics in living cells*. *Chemical reviews*, 2011. **111**(5): p. 3614-3666.

189. Qiu, L., et al., *A targeted, self-delivered, and photocontrolled molecular beacon for mRNA detection in living cells*. Journal of the American Chemical Society, 2013. **135**(35): p. 12952-12955.
190. Maurisse, R., et al., *Comparative transfection of DNA into primary and transformed mammalian cells from different lineages*. BMC biotechnology, 2010. **10**(1): p. 9.
191. Zhou, J., et al., *Octa-functional PLGA nanoparticles for targeted and efficient siRNA delivery to tumors*. Biomaterials, 2012. **33**(2): p. 583-591.
192. Wiraja, C., et al., *Molecular beacon-loaded polymeric nanoparticles for non-invasive imaging of mRNA expression*. Journal of Materials Chemistry B, 2015. **3**(30): p. 6148-6156.
193. Lim, J., et al., *Polymer Powder Processing of Cryomilled Polycaprolactone for Solvent-Free Generation of Homogeneous Bioactive Tissue Engineering Scaffolds*. Small, 2014. **10**(12): p. 2495-2502.
194. Lu, Z. and H. Zreiqat, *Beta-tricalcium phosphate exerts osteoconductivity through $\alpha 2\beta 1$ integrin and down-stream MAPK/ERK signaling pathway*. Biochemical and biophysical research communications, 2010. **394**(2): p. 323-329.
195. Kim, Y.-J., et al., *Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2*. Journal of Biological Chemistry, 2004. **279**(49): p. 50773-50780.
196. Okano, H., et al., *Steps toward safe cell therapy using induced pluripotent stem cells*. Circulation research, 2013. **112**(3): p. 523-533.
197. Lee, A.S., et al., *Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies*. Nature medicine, 2013. **19**(8): p. 998-1004.
198. Dacic, S., et al., *Col1a1-Driven Transgenic Markers of Osteoblast Lineage Progression*. Journal of bone and mineral research, 2001. **16**(7): p. 1228-1236.
199. Lakshmipathy, U., et al., *Efficient transfection of embryonic and adult stem cells*. Stem cells, 2004. **22**(4): p. 531-543.
200. Braam, S.R., et al., *Improved genetic manipulation of human embryonic stem cells*. Nature Methods, 2008. **5**(5): p. 389-392.
201. Kim, T.-H., K.-B. Lee, and J.-W. Choi, *3D graphene oxide-encapsulated gold nanoparticles to detect neural stem cell differentiation*. Biomaterials, 2013. **34**(34): p. 8660-8670.
202. Yeo, D.C., et al., *Nanosensors for regenerative medicine*. Journal of biomedical nanotechnology, 2014. **10**(10): p. 2722-2746.
203. Luu, H.H., et al., *Distinct roles of bone morphogenetic proteins in osteogenic differentiation of mesenchymal stem cells*. Journal of Orthopaedic Research, 2007. **25**(5): p. 665-677.
204. Vinatier, C., et al., *Cartilage engineering: a crucial combination of cells, biomaterials and biofactors*. Trends in biotechnology, 2009. **27**(5): p. 307-314.
205. Nejadnik, H., et al., *Autologous bone marrow-derived mesenchymal stem cells versus autologous chondrocyte implantation: an observational cohort study*. The American journal of sports medicine, 2010. **38**(6): p. 1110-1116.
206. Homicz, M.R., et al., *Effects of serial expansion of septal chondrocytes on tissue-engineered neocartilage composition*. Otolaryngology—Head and Neck Surgery, 2002. **127**(5): p. 398-408.

207. Stewart, M.C., et al., *Phenotypic stability of articular chondrocytes in vitro: the effects of culture models, bone morphogenetic protein 2, and serum supplementation*. Journal of bone and mineral research, 2000. **15**(1): p. 166-174.
208. Ahmed, T. and M.T. Hincke, *Mesenchymal stem cell-based tissue engineering strategies for repair of articular cartilage*. Histol Histopathol, 2014. **29**(6): p. 669-89.
209. Bosnakovski, D., et al., *Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells (MSCs) in different hydrogels: influence of collagen type II extracellular matrix on MSC chondrogenesis*. Biotechnology and bioengineering, 2006. **93**(6): p. 1152-1163.
210. Johnstone, B., et al., *Tissue engineering for articular cartilage repair—the state of the art*. Eur Cell Mater, 2013. **25**(248): p. e67.
211. Goldring, M.B., K. Tsuchimochi, and K. Ijiri, *The control of chondrogenesis*. Journal of cellular biochemistry, 2006. **97**(1): p. 33-44.
212. Nagesha, D., et al., *In vitro imaging of embryonic stem cells using multiphoton luminescence of gold nanoparticles*. International journal of nanomedicine, 2007. **2**(4): p. 813.
213. Villa, C., et al., *Stem cell tracking by nanotechnologies*. International journal of molecular sciences, 2010. **11**(3): p. 1070-1081.
214. Kan, A., et al., *Screening of chondrogenic factors with a real-time fluorescence-monitoring cell line ATDC5-C2ER: Identification of sorting nexin 19 as a novel factor*. Arthritis & Rheumatology, 2009. **60**(11): p. 3314-3323.
215. Woods, N.-B., et al., *Lentiviral vector transduction of NOD/SCID repopulating cells results in multiple vector integrations per transduced cell: risk of insertional mutagenesis*. Blood, 2003. **101**(4): p. 1284-1289.
216. Bhirde, A., et al., *Nanoparticles for cell labeling*. Nanoscale, 2011. **3**(1): p. 142-153.
217. Hachani, R., et al., *Tracking stem cells in tissue-engineered organs using magnetic nanoparticles*. Nanoscale, 2013. **5**(23): p. 11362-11373.
218. Xu, C., et al., *Nanoparticle-based monitoring of cell therapy*. Nanotechnology, 2011. **22**(49): p. 494001.
219. Yeo, D., et al., *A nanoparticle-based sensor platform for cell tracking and status/function assessment*. Scientific reports, 2015. **5**: p. srep14768.
220. Santangelo, P.J., et al., *Dual FRET molecular beacons for mRNA detection in living cells*. Nucleic acids research, 2004. **32**(6): p. e57-e57.
221. Sarkar, D., et al., *Cellular and extracellular programming of cell fate through engineered intracrine-, paracrine-, and endocrine-like mechanisms*. Biomaterials, 2011. **32**(11): p. 3053-3061.
222. Wiraja, C., et al., *Nanosensors for continuous and noninvasive monitoring of mesenchymal stem cell osteogenic differentiation*. Small, 2016. **12**(10): p. 1342-1350.
223. Barry, F., et al., *Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation-dependent gene expression of matrix components*. Experimental cell research, 2001. **268**(2): p. 189-200.

224. Yang, Z., et al., *Improved mesenchymal stem cells attachment and in vitro cartilage tissue formation on chitosan-modified poly (L-lactide-co-epsilon-caprolactone) scaffold*. Tissue Engineering Part A, 2011. **18**(3-4): p. 242-251.
225. Karp, J.M. and W. Zhao, *Micro-and Nanoengineering of the Cell Surface*. 2014: William Andrew.
226. Bian, L., et al., *Enhanced MSC chondrogenesis following delivery of TGF-β3 from alginate microspheres within hyaluronic acid hydrogels in vitro and in vivo*. Biomaterials, 2011. **32**(27): p. 6425-6434.
227. Xu, J., et al., *Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels*. Tissue Engineering Part A, 2008. **14**(5): p. 667-680.
228. Feichtinger, G.A., et al., *Enhanced reporter gene assay for the detection of osteogenic differentiation*. Tissue Engineering Part C: Methods, 2010. **17**(4): p. 401-410.
229. Gil, J.S., H.B. Machado, and H.R. Herschman, *A method to rapidly and accurately compare the relative efficacies of non-invasive imaging reporter genes in a mouse model and its application to luciferase reporters*. Molecular Imaging and Biology, 2012. **14**(4): p. 462-471.
230. Bagó, J.R., et al., *In vivo bioluminescence imaging of cell differentiation in biomaterials: a platform for scaffold development*. Tissue Engineering Part A, 2012. **19**(5-6): p. 593-603.
231. Ilieva, M. and M. Dufva, *SOX2 and OCT4 mRNA-expressing cells, detected by molecular beacons, localize to the center of neurospheres during differentiation*. PloS one, 2013. **8**(8): p. e73669.
232. Lin, X., et al., *Backbone-modified molecular beacons for highly sensitive and selective detection of microRNAs based on duplex specific nuclease signal amplification*. Chemical Communications, 2013. **49**(65): p. 7243-7245.
233. Brouwer, M., H. Zhou, and N.N. Kasri, *Choices for induction of pluripotency: recent developments in human induced pluripotent stem cell reprogramming strategies*. Stem Cell Reviews and Reports, 2016. **12**(1): p. 54-72.
234. Robinton, D.A. and G.Q. Daley, *The promise of induced pluripotent stem cells in research and therapy*. Nature, 2012. **481**(7381): p. 295.
235. Hou, P., et al., *Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds*. Science, 2013. **341**(6146): p. 651-654.
236. Wang, A., et al., *Induced pluripotent stem cells for neural tissue engineering*. Biomaterials, 2011. **32**(22): p. 5023-5032.
237. Merrell, A.J. and B.Z. Stanger, *Adult cell plasticity in vivo: de-differentiation and transdifferentiation are back in style*. Nature Reviews Molecular Cell Biology, 2016. **17**(7): p. 413-425.
238. Wu, S.M. and K. Hochedlinger, *Harnessing the potential of induced pluripotent stem cells for regenerative medicine*. Nature cell biology, 2011. **13**(5): p. 497-505.
239. Buganim, Y., et al., *The developmental potential of iPSCs is greatly influenced by reprogramming factor selection*. Cell stem cell, 2014. **15**(3): p. 295-309.
240. Schlaeger, T.M., et al., *A comparison of non-integrating reprogramming methods*. Nature biotechnology, 2015. **33**(1): p. 58-63.

241. Silva, M., et al., *Generating iPSCs: translating cell reprogramming science into scalable and robust biomanufacturing strategies*. Cell Stem Cell, 2015. **16**(1): p. 13-17.
242. Hanna, J., et al., *Direct cell reprogramming is a stochastic process amenable to acceleration*. Nature, 2009. **462**(7273): p. 595.
243. Ruiz, S., et al., *Generation of a drug-inducible reporter system to study cell reprogramming in human cells*. Journal of Biological Chemistry, 2012. **287**(48): p. 40767-40778.
244. Lahm, H., et al., *Live Fluorescent RNA-Based Detection of Pluripotency Gene Expression in Embryonic and Induced Pluripotent Stem Cells of Different Species*. Stem Cells, 2015. **33**(2): p. 392-402.
245. Tay, L.M., et al., *Noninvasive Monitoring of Three-Dimensional Chondrogenic Constructs Using Molecular Beacon Nanosensors*. Tissue Engineering Part C: Methods, 2017. **23**(1): p. 12-20.
246. Tyagi, S. and O. Alsmadi, *Imaging native β -actin mRNA in motile fibroblasts*. Biophysical journal, 2004. **87**(6): p. 4153-4162.
247. Wu, H., et al., *Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs*. Journal of Biological Chemistry, 2004. **279**(17): p. 17181-17189.
248. Esteban, M.A., et al., *The mesenchymal-to-epithelial transition in somatic cell reprogramming*. Current opinion in genetics & development, 2012. **22**(5): p. 423-428.
249. Birchmeier, W. and C. Birchmeier, *Mesenchymal-epithelial transitions*. Bioessays, 1994. **16**(5): p. 305-307.
250. Shi, Y., et al., *Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds*. Cell stem cell, 2008. **3**(5): p. 568-574.
251. Anokye-Danso, F., et al., *Highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency*. Cell stem cell, 2011. **8**(4): p. 376-388.
252. Barth, A.L., *Visualizing circuits and systems using transgenic reporters of neural activity*. Current opinion in neurobiology, 2007. **17**(5): p. 567-571.
253. Golding, I., et al., *Real-time kinetics of gene activity in individual bacteria*. Cell, 2005. **123**(6): p. 1025-1036.
254. Zychlinski, D., et al., *Physiological promoters reduce the genotoxic risk of integrating gene vectors*. Molecular Therapy, 2008. **16**(4): p. 718-725.