

**EFFECTS OF CRYOPRESERVATION CONDITIONS ON
HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM
CELLS AND ITS POTENTIAL APPLICATION
IN CARDIAC FIBROSIS**

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**THESIS SUBMITTED IN FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY**

**DEPARTMENT OF BIOMEDICAL ENGINEERING
FACULTY OF ENGINEERING
UNIVERSITY OF MALAYA
KUALA LUMPUR**

2016

UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

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Registration/Matric No: **KHA130037**

Name of Degree: **Doctor of Philosophy**

Title of Thesis:

**EFFECTS OF CRYOPRESERVATION CONDITIONS ON HUMAN
ADIPOSE-DERIVED MESENCHYMAL STEM CELLS AND ITS
POTENTIAL APPLICATION IN CARDIAC FIBROSIS**

Field of Study: **Biomedical Engineering**

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ABSTRACT

Human adipose-derived mesenchymal stem cells (hASCs) hold great potential for clinical application (*e.g.*, regenerative medicine and cell-based therapies) due to their multilineage differentiation ability and paracrine function. To achieve sufficient numbers of hASCs for off-the-shelf-use in an intensive clinical setting (*e.g.*, cardiac fibrosis therapy), hASCs at early passage should be capable of being cryopreserved in the long-term with cell functionality maintained without raising biosafety concerns (*e.g.*, tumourigenesis). The objectives of this study are to evaluate the effects of cryopreservation conditions on hASCs and the potential application of cryopreserved hASCs in cardiac fibrosis. In this study, hASCs were cryopreserved for 3 months using a slow freezing method in various combinations of 3 general used cryoprotective agents (CPAs), including dimethylsulfoxide (DMSO), trehalose, and fetal bovine serum (FBS). Following rapid thawing, hASCs cryopreserved in a cryopreservation medium containing DMSO at a reduced concentration without FBS (5% DMSO) were found to maintain high viability and functional properties in terms of differentiation potential (including adipogenic, osteogenic and chondrogenic), proliferation potential, and stemness. Moreover, hASCs cryopreserved in 5% DMSO have a low risk of tumourigenesis, as indicated by normal expression levels of tumour suppressor markers and human telomerase reverse transcriptase (hTERT), normal telomere length, and normal telomerase activity without significant DNA damage or p53 mutation. In addition, it was found that fresh (non-cryopreserved) hASCs and hASCs cryopreserved in 5% DMSO both display a similar potential to inhibit cardiac myofibroblast differentiation *in vitro* via paracrine signalling, and thus may decrease the incidence of cardiac fibrosis. In summary, 5% DMSO without FBS may be an ideal CPA for efficient long-term cryopreservation of

hASCs for clinical applications. Further, long-term cryopreserved hASCs demonstrate their significant therapeutic value in cardiac fibrosis therapy.

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ABSTRAK

Sel stem mesenkima dari tisu adipos manusia (hASCs) merupakan sel yang mempunyai keupayaan untuk menjalani pembezaan ke pelbagai sel spesifik dan fungsi parakrin, maka ia mempunyai potensi yang tinggi dalam penggunaan klinikal (contohnya, terapi sel dan perubatan pertumbuhan semula). Demi mendapat sel yang mencukupi untuk tersedia kepada penggunaan dalam rawatan intensif (contohnya, terapi fibrosis jantung), hASCs pada kultur awal sepatutnya mempunyai keupayaan untuk dikriopreservasi dalam jangka masa yang panjang supaya fungsi sel dapat dikekalkan tanpa menyebabkan kebimbangan dalam keselamatan biologi (contohnya, pertumbuhan tumor). Tujuan kajian ini adalah untuk menentukan kesan keadaan kriopreservasi terhadap hASCs dan potensi penggunaan hASCs yang telah dikriopreservasikan dalam fibrosis jantung. Dalam kajian ini, hASCs dikriopreservasi dalam pelbagai kombinasi agen perlindungan penyejukbekuan yang biasa digunakan, iaitu dimethylsulfoxide (DMSO), trehalose, dan serum fetus lembu (FBS), untuk jangka masa selama 3 bulan dengan menggunakan kaedah penyejukbekuan secara perlahan. Berikutan dengan pencairan secara cepat, hASCs yang dikriopreservasi dalam medium penyejukbekuan yang mengandungi DMSO yang berkepekatan yang rendah tanpa FBS (5% DMSO) didapati mengekalkan viabiliti yang tinggi dan fungsinya termasuk keupayaan menjalani pembezaan ke pelbagai sel (termasuk adipos, tulang dan rawan), keupayaan proliferasi dan sifat stem. Tambahan pula, hASCs yang dikriopreservasi dalam 5% DMSO menunjukkan ekspresi penanda penindasan tumor dan *human telomerase reverse transcriptase* (hTERT) yang normal, aktiviti telomerase dan panjang telomere yang normal tanpa mutasi pada p53 atau kerosakan DNA yang signifikan, maka ia mempunyai risiko yang rendah dalam pertumbuhan tumor. Selain itu, didapati bahawa hASCs yang segar (tanpa dikriopreservasikan) dan dikriopreservasi dalam 5% DMSO mempunyai potensi yang

sama dalam perencatan pembezaan miofibroblas jantung secara *in vitro* melalui isyarat parakrin, maka ini mungkin dapat mengurangkan insiden fibrosis jantung. Secara ringkasnya, 5% DMSO tanpa FBS merupakan CPA yang sesuai untuk kriopreservasi hASCs secara berkesan dalam jangka masa yang panjang untuk penggunaan klinikal. Tambahan pula, hASCs yang dikriopreservasi dalam jangka masa yang panjang menunjukkan nilai terapi yang signifikan dalam terapi fibrosis jantung.

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ACKNOWLEDGEMENTS

I would like to express deepest gratitude to my supervisors, Assoc. Prof. Ir. Dr. Belinda Murphy and Dr. Wan Safwani Wan Kamarul Zaman, for their constant support and guidance throughout my studies. I am grateful to them who have spent their valuable time to give me idea and suggestion for solving the problems I had during my research. Despite their busy schedule, they are able to revise my manuscripts and improve their quality to the level required for publication.

Special thanks to Prof. Dr. Xu Feng and Dr. Chua Kien Hui for allowing me to learn and improve my research skills from their post-doctoral researchers and students. I particularly acknowledge Prof. Dr. Xu Feng for inviting me to have attachment in his center (BEBC) at Xi'an Jiaotong University, China. They are always available to give me valuable suggestion and comment which helped me a lot in my manuscript writing.

Many thanks to my family for their endless prayer, encouragement and support all the way. Thanks to my friends and colleagues in Tissue Engineering Lab and BEBC who helped me in completing this research.

Finally, I would like to express my endless appreciation to Ms. Choi Jane Ru for her unconditional love and support. She also worked with me in optimizing the assay for my research. Thank you very much to you.

This research was supported by University of Malaya, High Impact Research Grant (UM.C/HIR/MOHE/ENG/44) and Postgraduate Research Grant (PPP) (PG007-2014A) from Ministry of Education Malaysia.

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LIST OF SYMBOLS AND ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
ACAN	Aggrecan
AFM	Atomic force microscopy
ALPL	Alkaline phosphatase
anti-HGF	HGF antibody
ASCs	Adipose-derived stem cells
AT ₁ R	Angiotensin II type 1 receptor
α -SMA	Alpha smooth muscle actin
Col I	Collagen type I
COL-2	Collagen type II
Col III	Collagen type III
CPAs	Cryoprotective agents
DAPI	4'-6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
ECM	Extracellular matrix
ESCs	Embryonic stem cells
FABP4	Fatty acid binding protein 4
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
hASCs	Human adipose-derived stem cells

HGF	Hepatocyte growth factor
hMSCs	Human mesenchymal stem cells
IGF-1	Insulin-like growth factor 1
LPL	Lipoprotein lipase
M	mol/L
MBA	<i>N,N</i> methylene-bis-acrylamide
Min	minute
MSCs	Mesenchymal stem cells
OSC	Osteocalcin
PA	Polyacrylamide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PPAR- γ	Peroxisome proliferator-activated receptor γ
qPCR	Quantitative Real-Time polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SEM	Scanning electron microscopy
SOX-9	Sry-related HMG box-9
SVF	Stromal vascular fraction
TERT	Telomerase reverse transcriptase
TGF- β 1	Transforming growth factor beta 1
$^{\circ}$ C	Degree celsius

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CHAPTER 1: INTRODUCTION

1.1 Background

Human stem cells, particularly human mesenchymal stem cells (hMSCs), are an ideal candidate for many biomedical applications such as regenerative medicine and cell-based therapies (Arthur et al., 2009; Mimeault et al., 2007). hMSCs obtained from various locations of the human body (including adipose tissue, bone marrow and periosteum) are capable of self-renewing and differentiating into multiple specific types of cell, such as those in bone or cartilage (Callihan et al., 2011; Doulatov & Daley, 2013). Further, hMSCs also have paracrine function and immunosuppressive property (Baraniak & McDevitt, 2010; De Miguel et al., 2012; Nauta & Fibbe, 2007), which support their use in various clinical settings. Implantation of such multi-functional hMSCs may overcome the problems of organ shortage crisis worldwide to treat the fatal diseases, *e.g.*, heart failure and liver failure. Among the hMSCs, human adipose-derived mesenchymal stem cells (hASCs) have gained great attraction due to the abundance and readily accessibility of adipose tissues (Gomillion & Burg, 2006; Kolle et al., 2013; Mizuno et al., 2012).

In general, each clinical therapy may require a huge amount of cells (including hASCs), approximately 200 million cells (Faustini et al., 2010). Therefore, the cells need to be expanded *in vitro* extensively (Pittenger et al., 1999). During the extensive cell expansion, hASCs may be at a risk of tumourigenesis and chromosomal aberration (Froelich et al., 2013; Roemeling-van Rhijn et al., 2013) and thus may raise biosafety concerns in clinical application (Sharpe et al., 2012). For instance, it has been reported that hASCs displayed spontaneous malignant transformation and chromosomal aberration after culture beyond 10 passages (Froelich et al., 2013; Roemeling-van Rhijn et al., 2013). Such adverse changes may be caused by a potential contamination with an external cell

source such as tumour cells and the stressful culture conditions (*e.g.*, frequent enzymatic cell dissociation and long-term culture) (Barkholt et al., 2013; de la Fuente et al., 2010). In addition, long-term culture of hASCs was associated with senescence, reduced differentiation potential, and alteration of stemness and phenotype (Safwani et al., 2012, 2013; Wan Safwani et al., 2011). These concerns limit the wide use of long-term cultured hASCs in clinical applications. In fact, hASCs at an early passage (within passage 4) are safe for clinical use (Barkholt et al., 2013). Therefore, a method for long-term storage of hASCs at an early passage is required for off-the-shelf clinical use. Cryopreservation represents the potential storage method which maintains functional properties of hASCs in long-term (Gonda et al., 2008; Yong et al., 2015c).

Among the cryopreservation methods (*e.g.*, slow freezing and vitrification), slow freezing is the most preferable method for hASCs due to easier processing and low risk of contamination, whereas vitrification requires a good manipulation skill and has a high risk of causing contamination (Güven & Demirci, 2012). To achieve an efficient cryopreservation using a slow freezing method, optimization of the use of cryoprotective agents (CPAs) is essential to protect hASCs from freezing injury (*e.g.*, cell death and compromised functional properties). Among the CPAs, 10% dimethylsulfoxide (DMSO) and fetal bovine serum (FBS) is the most widely used for cryopreservation of hASCs. However, clinical uses of cells preserved in such CPA formulations have raised the issues of adverse reactions (*e.g.*, respiratory depression and neurotoxicity and xenogeneic immune response) in recipients after implantation of cryopreserved cells (Benekli et al., 2000; Tuschong et al., 2002; Windrum & Morris, 2003). This has resulted the need for development of an alternative CPA or a CPA formulation consisting of DMSO in a reduced concentration and without FBS. To date, a standardized cryopreservation protocol of hASCs for clinical applications has not been fully established. As the

biosafety profile of cryopreserved hASCs has not been established, it is also essential to evaluate the biosafety (*e.g.*, tumourigenic potential and chromosomal abnormality) of cryopreserved hASCs prior to clinical applications (Yong et al., 2015b). Understanding the storage conditions and characteristics of cryopreserved hASCs following thawing is essential prior to clinical application to ensure a good and safe product.

Cardiac fibrosis, an initial healing process essential for heart repair after damage due to heart diseases such as myocardial infarction, is often dysregulated once it has begun, causing adverse remodeling of cardiac tissues that may ultimately lead to cardiac failure (Wynn & Ramalingam, 2012; Zeisberg et al., 2007). To date, cardiac fibrosis cannot be effectively halted or reversed by surgery or drug therapies (*e.g.*, anti-fibrotic agents) (Daskalopoulos et al., 2012). Recently, undifferentiated MSCs with an ability to secrete soluble factors (*e.g.*, hepatocyte growth factor (HGF) and insulin growth factor-1 (IGF-1)), have been found to be capable of altering the behaviors of neighboring cells (*e.g.*, myofibroblast differentiation (a hallmark of fibrosis) and cardiomyocyte regeneration) through their paracrine functions (Ramkisoensing et al., 2014), indicating their great potential for treating cardiac fibrosis. The existing studies showed that MSCs could suppress cardiac fibrosis by many mechanisms, *e.g.*, ECM degradation and suppression of alpha-smooth muscle actin (α -SMA) expression (a defining marker of cardiac myofibroblast differentiation) (Li et al., 2015; Mao et al., 2013; Mias et al., 2009; Ohnishi et al., 2007; Wang et al., 2011). However, these studies were performed on cardiac fibroblasts cultured in non-physiological or mechanically irrelevant conditions (*e.g.*, cell culture plastic plates and glass slides). To date, the paracrine effects of MSCs on cardiac myofibroblast differentiation in conditions mimicking the stiffness of *in vivo* normal and fibrotic cardiac tissues has not been explored, which would provide a better understanding for the therapeutic use of MSCs in cardiac fibrosis. In addition,

cryopreserved hASCs might hold great potential for cardiac fibrosis therapy as such an intensive clinical setting might require a large number of cells for off-the-shelf use.

In the present study, the types and concentrations of CPAs used to cryopreserve hASCs for 3 months using a slow freezing method were optimized by evaluating the effect of cryopreservation on the phenotype, viability, functional properties (*e.g.*, proliferation and differentiation potential) and biosafety (*e.g.*, tumourigenic potential) of hASCs. Based on these evaluation, the ideal CPA used to cryopreserve hASCs was determined. Further, an *in vitro* cardiac fibrosis model was developed based on evaluation of cardiac myofibroblast differentiation markers in cardiac fibroblasts cultured on hydrogels mimicking the stiffness of native fibrotic cardiac tissues. Then, conditioned medium of fresh (non-cryopreserved) and cryopreserved hASCs was applied to the model to investigate the paracrine effects of hASCs on cardiac fibrosis. The findings of this study would impact the establishment of a standardized cryopreservation protocol for hASCs and therapeutic use of long-term cryopreserved hASCs in cardiac fibrosis in future.

1.2 Research question

- i. Does cryopreservation have any effect on hASCs in terms of cell phenotype, viability, proliferation, and differentiation?
- ii. Do cryopreserved hASCs raise any biosafety concerns (*e.g.*, tumourigenesis)?
- iii. Do cryopreserved hASCs have the potential to be used in therapeutic application of cardiac fibrosis?

1.3 Research hypotheses

- i. Cryopreservation maintains cell phenotype, high viability, proliferation potential, multilineage differentiation potential of hASCs.
- ii. Cryopreserved hASCs do not undergo tumourigenesis.
- iii. Cryopreserved hASCs have a high potential to be used in therapeutic application of cardiac fibrosis.

1.4 Research objectives

The objectives of this study include:

- i. To determine the cryoprotective effect of various CPAs *e.g.*, on cell phenotype, cell viability, cell proliferation and differentiation (including adipogenic, osteogenic and chondrogenic) of hASCs.
- ii. To evaluate the biosafety of cryopreserved hASCs through tumourigenic potential assessment.
- iii. To develop an *in vitro* cardiac fibrosis model.
- iv. To evaluate the paracrine effects of cryopreserved hASCs on cardiac fibrosis.

1.5 General methodology

In general, this study was approved by the Medical Ethics Committee of University Malaya Medical Centre (UMMC) (reference no. 996.46) (**Appendix A**), and carried out in accordance with the approved guidelines and experimental protocols which conform the declaration of Helsinki. The scopes of the ethical clearance in this study are as follows:

- i. A written informed consent can be obtained from the donor of adipose tissues in the presence of a physician. If the donor is physically or mentally incapable of

giving consent, one of his/her relative is allowed to give consent on the behalf of him/her.

- ii. Isolation of adipose tissues must be performed by a physician.
- iii. Adipose tissues of the donor can only be used for research purpose.
- iv. The personal information of donor are confidential. The results of research can only be presented and published without including the personal information of donor.

After collection of adipose tissues, the tissues were processed to obtain hASCs. hASCs were cryopreserved using various combinations of general used CPAs (*e.g.*, DMSO, trehalose and FBS) for 3 months. After 3 months of cryopreservation, hASCs were subjected to the evaluation of cell phenotype, viability, proliferation, differentiation and tumourigenic potential. Fresh or non-cryopreserved hASCs were used as control. Based on these evaluations, an ideal CPA used to cryopreserve hASCs was determined.

On the other hand, an *in vitro* cardiac fibrosis model made by rat cardiac fibroblasts cultured on a hydrogel which mimics the stiffness of native fibrotic cardiac tissues was developed. Cardiac fibroblasts were isolated from the hearts of neonatal rats (1-3 day old), which conform the National Institutes of Health (NIH) guidelines (Guide for the care and use of laboratory animals). Further, hASCs which was cryopreserved in the ideal CPA, was applied to the model to evaluate the potential therapeutic application of cryopreserved hASCs in cardiac fibrosis. Flow chart of the general methodology was shown in the **Fig. 1.1**. The research methodology will be elaborated in detail in the section of materials and methods in chapter 3, 4 and 5.

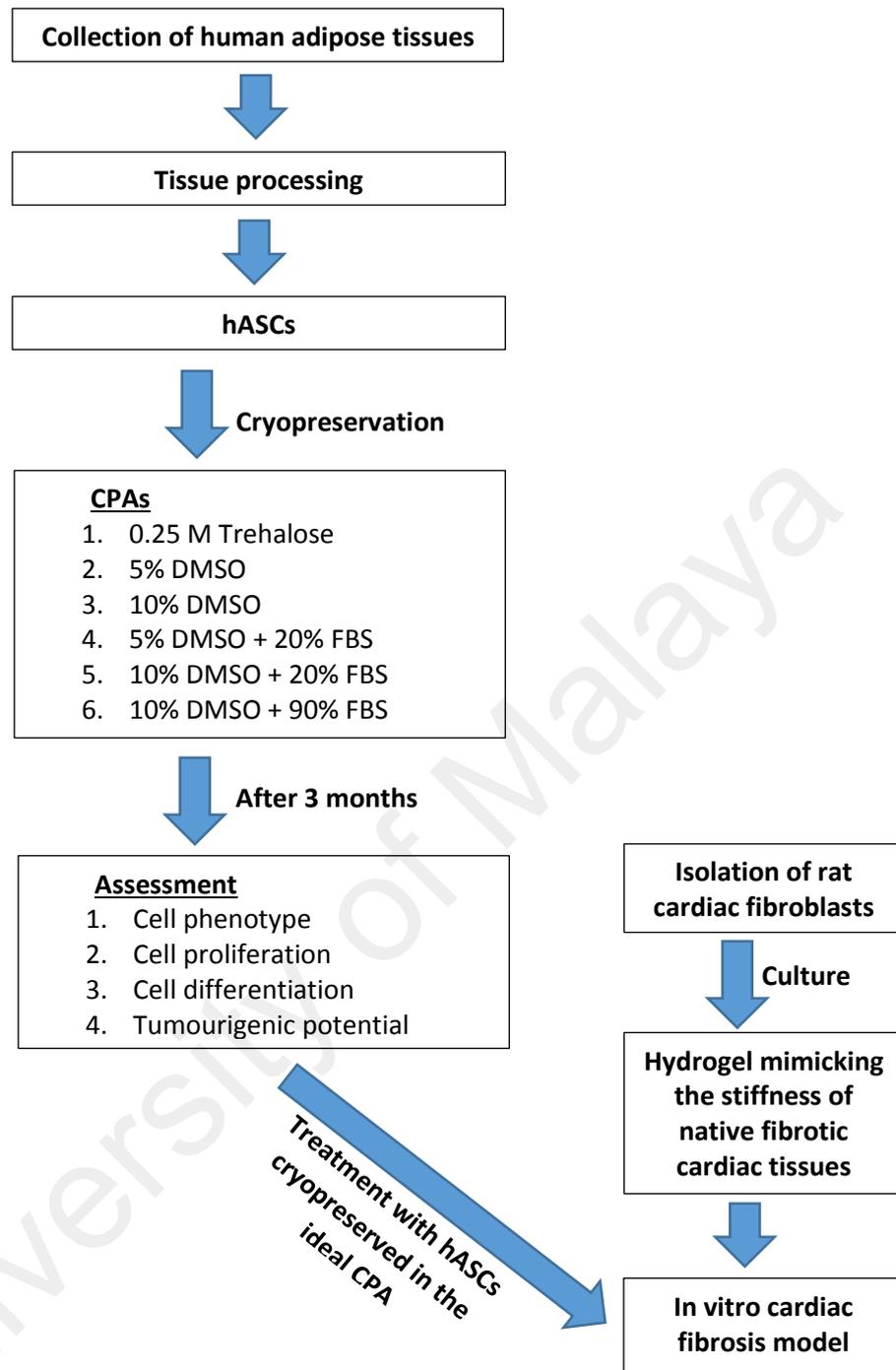


Figure 1.1. Flow chart of general methodology

1.6 Thesis framework

The thesis is written in accordance with the article style format. It is comprised of six chapters as described as follows:

- i. Chapter 1 gives a brief overview of the research background, research question, research hypotheses, research objectives, general methodology and the content of each chapter.
- ii. Chapter 2 provides a critical review on the research background, including stem cells, mesenchymal stem cells, ASCs, cryopreservation, the existing studies of cryopreservation of hASCs, biosafety assessments of mesenchymal stem cells, and cardiac fibrosis. This chapter contains selected figures and table reprinted in part with permission from my review articles as follows:
 - **Yong, K. W.**, Wan Safwani, W. K., Xu, F., Wan Abas, W. A., Choi, J. R., & Pinguan-Murphy, B. (2015). Cryopreservation of Human Mesenchymal Stem Cells for Clinical Applications: Current Methods and Challenges. *Biopreserv Biobank*, 13(4), 231-239. Copyright (2016) Mary Ann Liebert.
 - **Yong, K. W.**, Li, Y., Huang, G., Lu, T. J., Safwani, W. K., Pinguan-Murphy, B., & Xu, F. (2015). Mechanoregulation of Cardiac Myofibroblast Differentiation: Implications for Cardiac Fibrosis and Therapy. *Am J Physiol Heart Circ Physiol*, 309(4), 19. Copyright (2016) American Physiological Society.
- iii. Chapter 3 presents the characterization of hASCs isolated from human adipose tissues.
- iv. Chapter 4 presents the effect of cryopreservation on hASCs in terms of cell phenotype, viability, proliferation, differentiation (adipogenesis, osteogenesis and chondrogenesis), stemness and biosafety (tumourigenic potential). Chapter 3 and

4 contains selected figures and table reprinted in part with permission from my research articles as follows:

- **Yong, K. W.**, Pingguan-Murphy, B., Xu, F., Abas, W. A., Choi, J. R., Omar, S. Z., . . . Wan Safwani, W. K. (2015). Phenotypic and Functional Characterization of Long-Term Cryopreserved Human Adipose-Derived Stem Cells. *Sci Rep*, 5(9596). Copyright (2016) Nature Publishing Group.
 - **Yong, K. W.**, Safwani, W. K., Xu, F., Zhang, X., Choi, J. R., Abas, W. A., . . . Pingguan-Murphy, B. (2016). Assessment of tumourigenic potential in long-term cryopreserved human adipose-derived stem cells. *J Tissue Eng Regen Med*, 12(10). Copyright (2016) John Wiley & Sons.
- v. Chapter 5 presents the development of an *in vitro* cardiac fibrosis model and the evaluation of paracrine effects of cryopreserved hASCs on cardiac fibrosis.
- vi. Chapter 6 includes thesis contribution, conclusion, limitation of the study and future work.

CHAPTER 2: LITERATURE REVIEW

2.1 Stem cells

Stem cells are unspecialized and undifferentiated cells that have the ability to undergo self-renewal and multilineage differentiation potential (Conrad & Huss, 2005). Due to these characteristics, stem cells have become an ideal candidate for regenerative medicine and tissue engineering, as well as cell-based and gene therapies (Mimeault et al., 2007). Ideally, to meet the requirement of regenerative medicinal applications, stem cells should fulfill the following set of criteria: First, stem cells should be harvested by a minimally invasive procedure and cultivated to obtain millions to billions of cells. Second, stem cells can be differentiated into multiple specific types of cells in a reproducible manner. Last but not least, stem cells should be effectively and safely implanted into either an allogeneic or autologous host (Gimble, 2003).

In general, stem cells can be divided into four categories based on their potency. These categories are totipotent, pluripotent, multipotent and unipotent (Callihan et al., 2011; Eisenberg & Eisenberg, 2003). Zygotes, an example of totipotent stem cells, are created by egg fertilization by sperm, have the greatest differentiation potential, thus capable of producing all kinds of cells including embryonic stem cells (ESCs) (Callihan et al., 2011). Pluripotent stem cells can give rise to almost all the specialized cells of the three germ layers, including endoderm (gastrointestinal tract and lungs), mesoderm (urogenital, bone, blood and muscle) and ectoderm (nervous system and epidermal tissues) (Binder et al., 2009). Multipotent stem cells have the ability to differentiate into multiple but limited cell types, *e.g.*, mesenchymal stem cells (MSCs) can differentiate into specialized cells such as those in bones and cartilages but cannot differentiate into blood cells such as lymphocytes and monocytes (**Fig. 2.1**). Stem cells that can only

differentiate into one type of cells are termed unipotent stem cells (Eisenberg & Eisenberg, 2003).

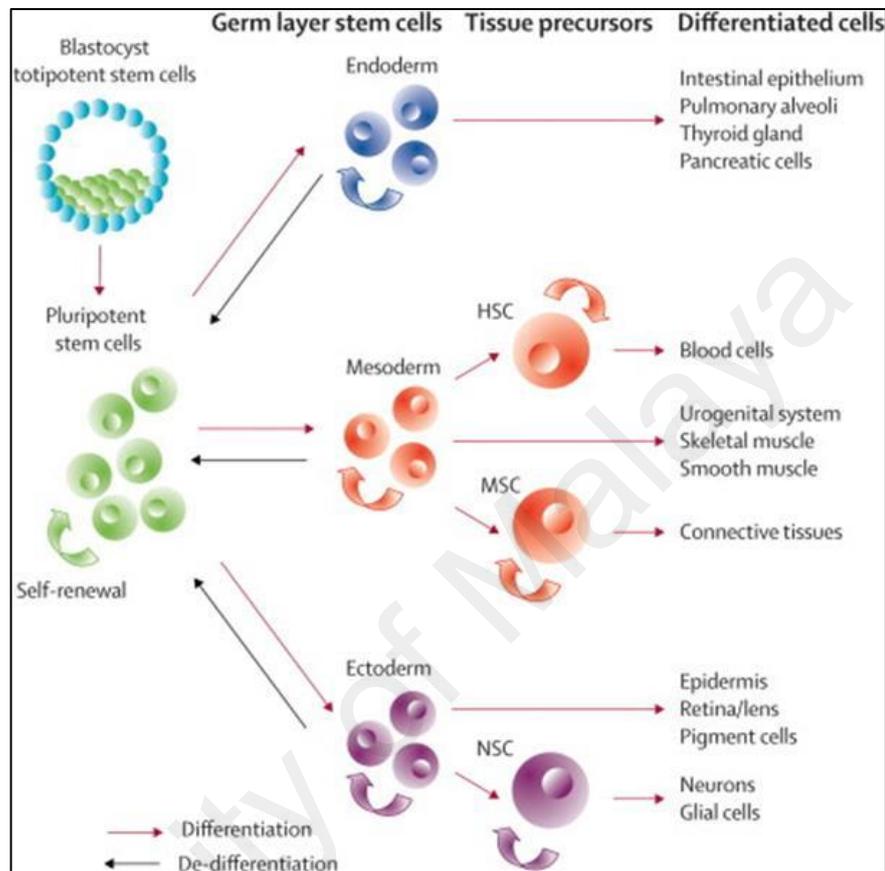


Figure 2.1. Classification of stem cells based on potency. Blastocyst totipotent stem cells produce pluripotent stem cells (also namely embryonic stem cells) that give rise to adult stem cells (including hematopoietic stem cells (HSC), neural stem cells (NSC) and mesenchymal stem cell (MSCs)) which can differentiate into various types of specialized cells. Reproduced from Corsten and Shah (2008).

Stem cells can also be divided into three broad types, including ESCs which are isolated from the inner cell mass of blastocysts, adult stem cells which are found in adult tissues, and induced-pluripotent stem cells (iPSCs) (Estrov, 2009). iPSCs are derived from somatic cells that are genetically reprogrammed into cells mimicking ESCs by possessing the pluripotency of ESCs. These cells require pluripotency-associated genes or transcription factors (*e.g.*, OCT-4, SOX-2, cMyc and Klf4) to maintain their ESCs-like properties (Power & Rasko, 2011).

2.1.1 Embryonic stem cells

ESCs can be induced *in vitro* and *in vivo* in a simple culture condition to differentiate into specific progenitor cells or specialized cells (Mimeault & Batra, 2006), *e.g.*, hematopoietic cell lineages, neuron-like cells, hepatocytes, pancreatic islet-like cells, osteocytes, chondrocytes, adipocytes, and cardiomyocytes (Trounson, 2006; Wu et al., 2007). Three markers, particularly SOX-2, NANOG, and OCT-4, are expressed in human ESCs to maintain their pluripotency (Callihan et al., 2011). However, the implantation of differentiated cells generated from ESCs into recipients may cause adverse effects such as, teratoma formation and immune rejection, due to the presence of residual undifferentiated and pluripotent ESCs (Andrews et al., 2005; Mimeault & Batra, 2006). Further, the destruction of embryos to obtain ESCs is ethically unacceptable (Young, 2000).

2.1.2 Adult stem cells

The use of stem cells derived from adult tissues avoids many ethical concerns related to the use of ESCs (Gomillion & Burg, 2006). Adult stem cells are capable of self-renewing and differentiating into the major specialized cells of the tissue in which they reside (Vats et al., 2002). Adult stem cells typically are divided into hematopoietic stem cells, neural stem cells and MSCs (Gomillion & Burg, 2006). Hematopoietic stem cells are primitive and undifferentiated cells that are capable of self-renewing and differentiating into all types of blood cells such as white blood cells and red blood cells (Mayani, 2003). Neural stem cells can differentiate into almost all the cells in the adult central nervous system such as, neurons and glia (Price & Williams, 2001; Temple, 2001).

2.2 Mesenchymal stem cells

Among the adult stem cells, MSCs hold great promise for cell therapy (Chagastelles et al., 2010). In addition to multipotent differentiation potential, MSCs have paracrine functions and immunosuppressive effects, which support their use in various clinical settings (Baraniak & McDevitt, 2010; Caplan, 2007; Caplan & Bruder, 2001). Morphologically, MSCs resemble fibroblasts in their spindle shape morphology (Pittenger et al., 1999). MSCs should possess the following criteria as proposed by Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy such as adherence to plastic, positively express specific surface antigens for MSCs (*e.g.*, CD90, CD105, CD73 and CD44), negatively express specific antigens for hematopoietic cells (*e.g.*, CD14, CD19, CD34 and CD45), and multipotent differentiation (including adipogenic, osteogenic and chondrogenic) (Dominici et al., 2006). MSCs can be isolated from various locations in the human body, such as bone marrow (Ginis et al., 2012; Pravdyuk et al., 2013), adipose tissue (De Rosa et al., 2009; Minonzio et al., 2014), periosteum (Ferretti et al., 2012), amniotic fluid (Angelo et al., 2012) and umbilical cord blood (Liu et al., 2011).

Bone marrow is the most recognized source of MSCs (Ringden & Le Blanc, 2005), which can be found in the bone marrow stroma. These cells are able to differentiate into multiple lineages of cells such as adipocytes, osteocytes, myocytes and neural cells (Barry & Murphy, 2004; Jiang et al., 2002). During bone marrow biopsy, about 10-40 mL bone marrow is aspirated from the iliac crest, and MSCs are then isolated in culture by their adherence properties (Caplan, 2000; Pittenger et al., 1999). However, isolation of bone marrow often causes morbidity donor site pain, and yields low numbers of MSCs (0.01% of the total nucleated cells in marrow) that thus requires extensive *in vitro* cultivation (Pittenger et al., 1999; Tholpady et al., 2006). Therefore, adipose tissue, which harbours

a high yield of MSCs, has been identified as a potential source of MSCs to replace bone marrow. Adipose tissue is particularly attractive because of its abundance and readily accessibility (Gomillion & Burg, 2006).

2.3 Adipose-derived mesenchymal stem cells

Adipose tissue is abundant, easily accessible, and harvested following minimally invasive procedures. ASCs have been identified within the stromal vascular fraction (SVF) of white adipose tissue (Tang et al., 2008). The SVF of adipose tissue harbours up to 2% of MSCs compared to 0.01% for those isolated from bone marrow (Strem & Hedrick, 2005), which makes adipose tissue to be the favourite source of MSCs. Adipose tissue can be harvested by two types of procedures, namely lipectomy (direct excision) or lipoaspiration (liposuction surgery) (Vermette et al., 2007). The initial methods to isolate ASCs were developed by Rodbell and his group members. Adipose tissue can be digested with collagenase type 1 to obtain SVF which comprised of various types of cells, including ASCs, endothelial cells, pre-adipocytes, macrophages or monocytes, lymphocytes, and pericytes (Rodbell, 1966) (**Fig. 2.2**). When SVF cells are cultured, a subset of spindle shaped cells adhere to the plastic cell culture plate. These adherent cells, namely ASCs, can be purified by culture expansion with stem cell culture media to deplete the cells from hematopoietic, endothelial, and pericyte lineage (Bourin et al., 2013).

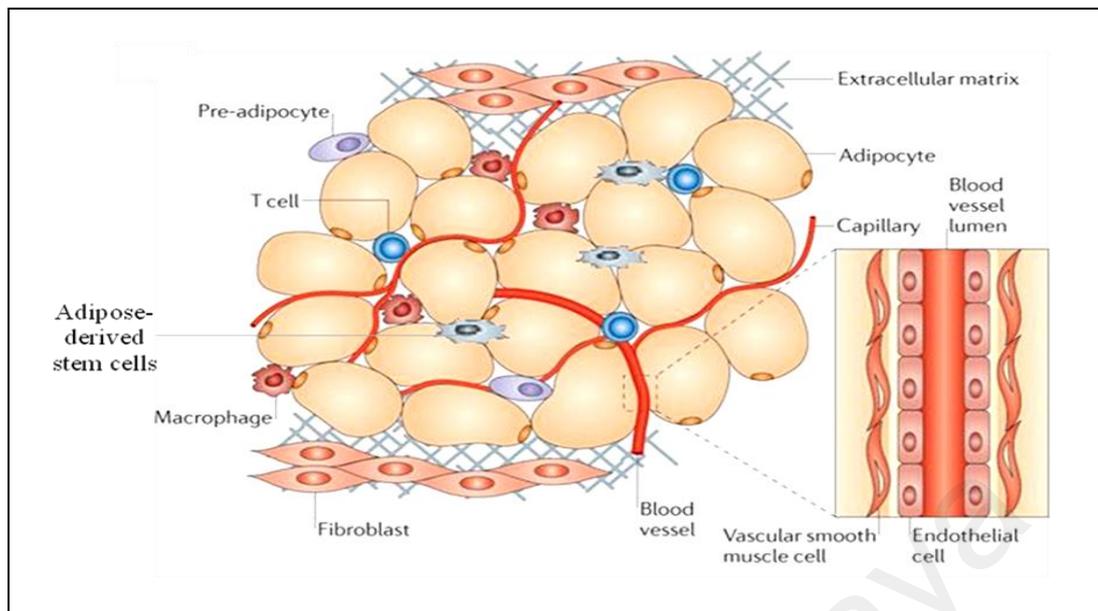


Figure 2.2: The composition of adipose tissue. Adapted from Cao (2010).

ASCs have spindle shaped morphology (Wan Safwani et al., 2011) (**Fig. 2.3**), which is similar in appearance with bone marrow-derived MSCs. Further, ASCs are similar with bone marrow-derived MSCs in terms of cell surface phenotype and differentiation potential (De Ugarte et al., 2003). As a type of MSCs, ASCs highly express surface markers such as CD90, CD73, CD105 and CD44 (Zaman et al., 2012). In addition, ASCs are capable of *in vitro* differentiation into adipocytes (Ogawa et al., 2004; Rodriguez et al., 2004), osteocytes (Hattori et al., 2004; Hicok et al., 2004), chondrocytes (Estes & Guilak, 2011), cardiomyocytes (Lee et al., 2009; van Dijk et al., 2008), neuron-like cells (Dhar et al., 2007; Ning et al., 2006) and pancreatic endocrine-like insulin-producing cells (Silva et al., 2012; Timper et al., 2006). They also possess an ability to maintain their stemness and multipotency by expressing stemness markers, such as OCT-4, REX-1, SOX-2 and NANOG (Izadpanah et al., 2006; Zuk et al., 2002).

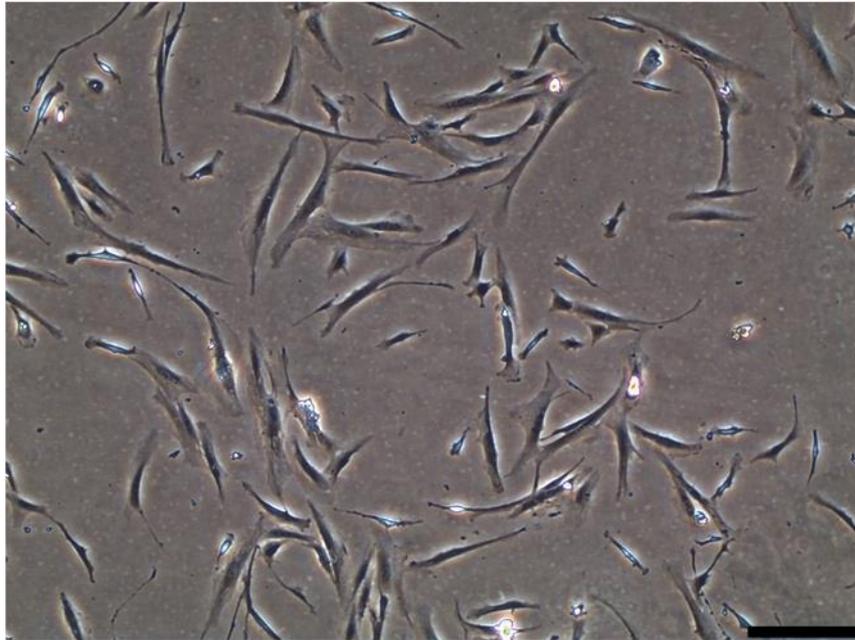


Figure 2.3. Morphology of ASCs. ASCs present spindle shaped morphology (Magnification: 40 ×; Scale bar: 200 μm).

2.4 Cryopreservation

Cryopreservation is a process of maintaining biological function and viability of cells by freezing and storing them below -80°C , *e.g.*, at the temperature of liquid nitrogen (-196°C) (Karlsson & Toner, 1996). In general, the first step in cryopreservation is harvesting the cells followed by the addition of cryopreservation medium containing cryoprotective agents (CPAs). Then, ice crystal induction is performed with an optimum cooling rate. Finally, the cells are stored in liquid nitrogen (Hubel, 1997). Long-term cryopreservation of living tissues and cells offers a great potential for clinical applications, including blood transfusion (Wagner et al., 2002), bone marrow transplantation (Rowley et al., 2003), *in vitro* fertilization (Anger et al., 2003), vascular grafts and bone grafts (Kofron et al., 2003). In the case of MSCs, preclinical trials demonstrated that implantation of cryopreserved MSCs was capable of treating intestinal inflammation (Castelo-Branco et al., 2012) and restoring myocardial function (Chin et al., 2010), but their therapeutic use is still not well established yet.

2.4.1 Benefits of cryopreservation

Cryopreservation allows transportation and pooling of cells to reach cell numbers required for cell therapy while maintaining their functional properties. It also allows the completion of quality control and safety testing of cells prior to clinical applications (Hubel, 1997). Without cryopreservation, the cells are forced to be continuously subcultured, which may accumulate genetic changes and result in tumorigenicity or heterogeneity (Stubban et al., 2007). On the other hand, at $-196\text{ }^{\circ}\text{C}$, the cells have no metabolic demands, thus avoiding biological variation due to genetic drift for years (Rowley, 1992). Therefore, cryopreservation produces a bank of cells at specific passages with intact functional properties and genetic characteristics. These validated cells can be used to initiate new experiments, maximize the long-term use of cells, and minimize experimental variation (Stubban et al., 2007).

2.4.2 Methods of cryopreservation

2.4.2.1 Slow freezing

The slow freezing method is the preferred method for cryopreservation of high volume cells such as ASCs and cell lines. With a freezing rate of $1\text{ }^{\circ}\text{C}/\text{min}$ using a non-programmable time freezing protocol in a commercially available freezing container “Mr. Frosty” (temperature is lowered at such rate due to the slow freezing property of isopropanol), a large number of cells can be frozen in one cryovial at a low concentration ($<1.5\text{ M}$) of CPAs (Zhang et al., 2011c). To achieve such a low freezing rate, direct contact of cells with non-sterile liquid nitrogen is not required during freezing, thus avoiding potential contamination with other microorganism or pathogens. By using a slow freezing method, a large amount of cryopreserved cells which are contamination-free can be pooled to provide sufficient cells for off-the-shelf clinical use. In order to further improve the efficiency of cryopreservation, a high-cost controlled

freezing/warming rate freezer which adopts a programmable freezing time protocol was developed. However, it has been reported that both protocols which have been used to freeze MSCs display similar potential to maintain phenotype, viability, and functional properties of MSCs (Janz Fde et al., 2012). With the advance of technology, “Cell Alive System” (CAS), a programmed freezer which adopts an approach of vibrating the water molecules and cells during freezing to prevent intra- and extra-cellular ice formation using alternating magnetic field and electric field, has been developed (Wowk, 2012). It has been reported that the risk of freeze injury to MSCs can be further reduced by using this system (Lee et al., 2012). Among the slow freezing protocols, a nonprogrammable freezing time protocol is favorable for cryopreservation of ASCs due to its low cost and high cryopreservation efficiency. To preserve ASCs efficiently using a slow freezing method, it is essential to optimize and determine the ideal CPAs used to preserve ASCs.

2.4.2.2 Rapid freezing/vitrification

Vitrification is a process which requires a very high freezing rate to convert a cell-laden CPA suspension directly from its aqueous phase to a glass state upon in contact with liquid nitrogen (Rall & Fahy, 1985). Vitrification is usually applied to small volume cells such as ESCs, embryo and oocyte because it requires low volume of cell suspension to achieve high freezing rate (Song et al., 2010). Therefore, it is ill-suited to cryopreservation of ASCs in large volumes. Further, it requires a high concentration of CPAs (6-8 M) which can cause osmotic shock and chemical toxicity to cells (Karlsson & Toner, 1996; Zhang et al., 2011c). Vitrification systems can be divided into carrier-based systems and carrier-free systems (**Fig. 2.4**). Cryoloops (700,000 °C/min), quartz microcapillaries (250,000 °C/min) and plastic straws (2500 °C/min) are among the carriers that have been developed for vitrification with each of them offering a different freezing rate (Zhang et al., 2011c). The use of higher freezing rate allows vitrification using CPA at a lower concentration, thereby reducing the risk of osmotic damage and chemical toxicity to cells.

On the other hand, carrier-free systems which adopt an approach of generating cell-laden CPAs in microdroplets followed by ejection to liquid nitrogen, were developed to further increase freezing rate for vitrification. (Zhang et al., 2011c).

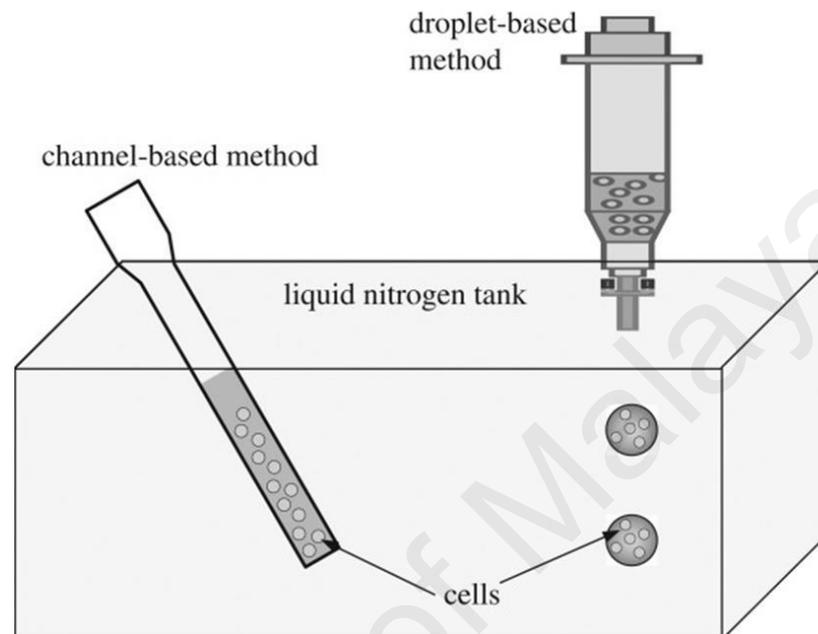


Figure 2.4. Carrier/channel-based and carrier-free (microdroplet-based) vitrification system. Adapted from Xu et al. (2010).

Post-thaw viability of cells was reported to be higher using vitrification compared to slow freezing method as the high freezing rate reduces the time of intra- and extracellular ice formation (Li et al., 2010). However, vitrification which adopts an approach of direct cell-to-liquid nitrogen contact, might lead to potential pathogenic contamination (Tedder et al., 1995). The Hepatitis B virus and *Aspergillus sp.* are among the pathogens which have been reported to contaminate liquid nitrogen (Fountain et al., 1997; Tedder et al., 1995). The contamination may come from non-sterile liquid nitrogen itself or cross-contamination from infected samples in the liquid nitrogen storage tank (AbdelHafez et al., 2011). Moreover, good manipulation skill is required for vitrification and it is impractical on a large scale of cell cryopreservation. The recycling of cells is labor-consuming as it needs manual picking up of individual cell colonies (Li et al., 2010),

potentially causing cell loss. The advantages and disadvantages of slow freezing and vitrification are described in brief in **Table 2.1**.

Table 2.1 Comparison between slow freezing and vitrification method

No	Aspect	Slow freezing	Rapid freezing
1	Concentration of CPAs required	Low	High
2	Risk of freeze injury	High	Low
3	Post-thaw viability	High	Higher
4	Risk of toxicity of cryoprotective agents	Low	High
5	Potential contamination with pathogenic agents	Low	High
6	Manipulation Skill	Easy	Good manipulation skill is needed.

Reproduced from Yong *et al.* (2015b)

Overall, the slow freezing method is preferable because cryopreservation of ASCs can be done on a large scale, the post-thaw viability of cells is high, and it avoids the risk of pathogenic contamination, allowing cryopreserved ASCs to be used for clinical applications.

2.4.3 Cryoprotective agents

The roles of CPAs in cell cryopreservation are to stabilize the cell membrane, minimize osmotic stress to the cells, and protect cells against intracellular and extracellular ice crystal formation which are both harmful to cells (McGann, 1978; Rudolph & Crowe, 1985) (**Fig. 2.5**). CPAs are divided into two broad classes: 1) permeating CPAs that freely permeate cell membranes and 2) nonpermeating CPAs (McGann, 1978).

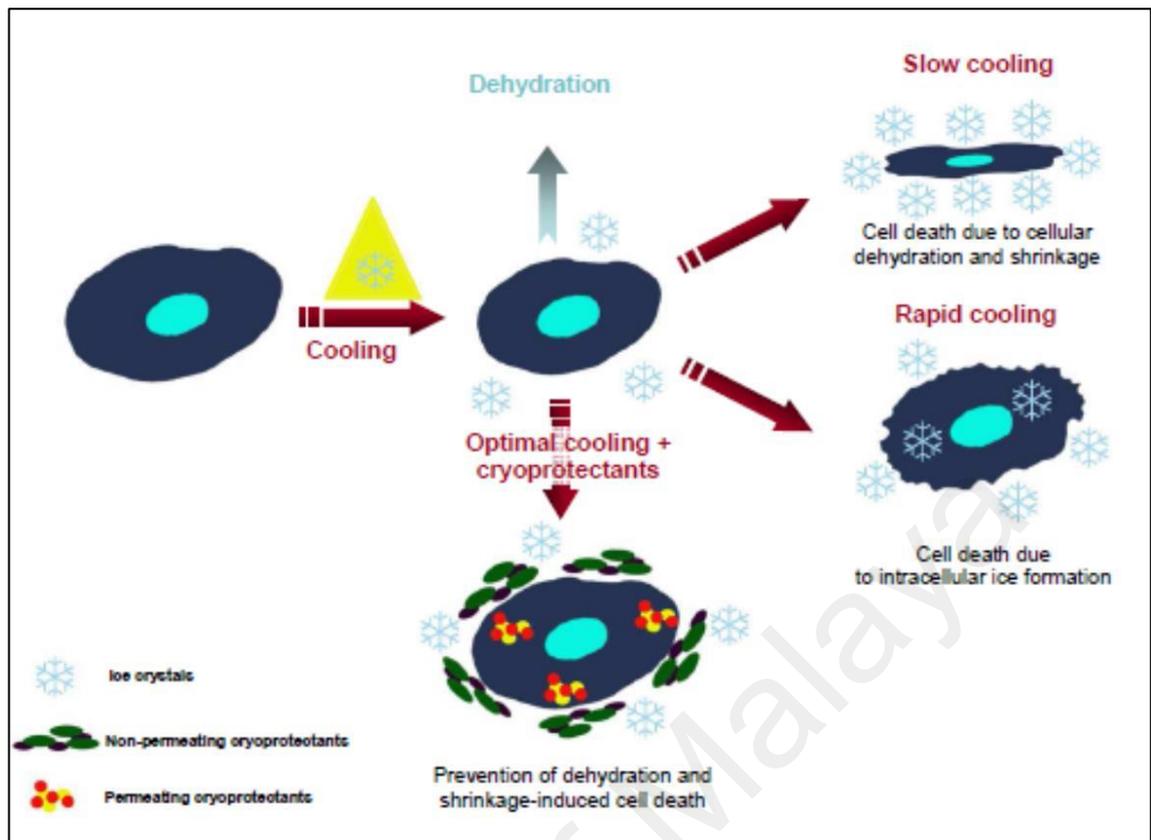


Figure 2.5. The role of CPAs in cell cryopreservation. Cryopreservation requires CPAs (either permeating or non-permeating) to protect cells from freeze injury (*e.g.*, cell death) by avoiding the formation of intra- and extra-cellular ice crystals which are harmful to cells during freezing. Reproduced from Martin-Ibanez et al. (2012).

2.4.3.1 Permeating CPAs

The most commonly used permeating CPAs are DMSO (dimethylsulfoxide) and glycerol (Buchanan et al., 2004). Permeating CPAs can penetrate the cell membrane due to their low molecular weight (Karlsson, 2002; Karlsson & Toner, 1996). Permeating CPAs prevent cell dehydration and physical injury induced by intracellular ice formation by reducing the incidence of increasing concentrations of electrolytes in the extracellular space during freezing (Rowley, 1992). DMSO is the most widely used CPA for large numbers of tissue and cell cryopreservation. DMSO can penetrate the cell membrane by removing the water within the cells to prevent the formation of intracellular ice that can cause cell rupture (Berz et al., 2007). Further, DMSO is able to stabilize the plasma membrane by electrostatic interaction (Anchordoguy et al., 1991). Generally, DMSO with

a concentration of 10% (v/v) combined with FBS (20% - 90%) (v/v) is used to preserve MSCs (Liu et al., 2008; Miranda-Sayago et al., 2012; Zhang et al., 2011b).

However, DMSO is toxic at temperatures beyond 4 °C (Zambelli et al., 1998). The clinical uses of cells preserved in 10% DMSO have caused many adverse effects in recipients, such as neurotoxicity and respiratory depression (Benekli et al., 2000; Windrum & Morris, 2003). Nevertheless, DMSO is not only cytotoxic but it also induces undesired differentiation of stem cells to neuronal-like cells (Woodbury et al., 2000). Therefore, the transplantation protocol has included the step of post-thaw washing of the cells to remove DMSO prior to transplantation (Fraser et al., 1998; Rubinstein et al., 1995). However, washing cells by centrifugation prior to removal of DMSO can cause significant cell loss. Further, total removal of DMSO from cryopreserved cells is time-consuming and complex (Thirumala et al., 2009). Therefore, the development of an alternative cryopreservation medium or a cryopreservation medium consisting of a reduced concentration of DMSO is required to avoid such adverse effects (Son et al., 2010).

2.4.3.2 Non-permeating CPAs

The commonly used non-permeating CPAs are polymers (*e.g.*, polyvinylpyrrolidone / PVP) and sugars (*e.g.*, trehalose and sucrose), which cannot enter the cells (Buchanan et al., 2004). Non-permeating CPAs cannot move across the cell membrane but protect the cell membrane by forming a viscous glassy shell around the outer surface of cells (Karlsson, 2002; Karlsson & Toner, 1996). Sucrose and PVP have been used for cryopreservation of MSCs to replace DMSO, but the results demonstrated that they are inferior to DMSO in terms of maintaining viability of MSCs (Janz Fde et al., 2012; Thirumala et al., 2010a).

In general, FBS is usually added to the cryopreservation media as a source of nutrients (Jochems et al., 2002). FBS stabilizes cell membranes and adjusts osmotic pressure inside and outside the cells to maintain viability of cells. It has been reported that cryopreservation medium containing FBS contributes to the prevention of viability loss of ASCs throughout the cryopreservation process (Gonda et al., 2008). However, FBS contains a number of proteins and peptides which can initiate xenogeneic immune responses (Mackensen et al., 2000; Tuschong et al., 2002). In addition, serum could possibly transfer pathogens (Zambelli et al., 1998). Therefore, it is recommended to minimize or exclude the use of FBS as part of the cryopreservation medium (Balci & Can, 2013). Overall, an ideal CPA should maintain functional properties and high survival rate of cells after thawing, and allow for cell transplantation without raising biosafety issues (*e.g.*, xenogeneic immune response, cytotoxicity and tumourigenesis).

2.4.4 Methods of thawing

Besides freezing, it is essential to select the best method to thaw frozen cells. The standard thawing method is to warm cells at a rapid rate (> 100 °C/min) at 37 °C in a water bath until all ice disappear (Thirumala et al., 2009), resulting in recovery of a high numbers of viable cells following thawing without the need for high-cost equipment. A dry warming procedure has been proposed to thaw frozen cells to avoid possible contamination of cells with microorganism presence in a water bath (Rollig et al., 2002). Therefore, a controlled-rate freezing/thawing chamber was developed to thaw frozen cells at a freezing rate of 10 °C/min, resulting in high viability of cells following thawing which is comparable to those thawed with the standard method (Thirumala et al., 2005). After thawing, cells are washed by centrifugation to remove CPAs, which are particularly toxic (*e.g.*, DMSO) prior to clinical applications, but resulting in cell loss that might affect the clinical

outcome. In the future, it is essential to develop a controlled-rate dry warming device which is low cost, reliable and portable for thawing frozen cells, and a method which can remove CPAs while minimizing cell loss (Fleming & Hubel, 2006) for efficient clinical applications.

2.4.5 Challenges in cryopreservation

The issue of adverse effects in recipients of cryopreserved cells is a challenge to be addressed to cryopreserve cells using a slow freezing method for clinical applications (Li et al., 2010; Martin-Ibanez et al., 2012). To date, the introduction of non-toxic polymers, such as PVP, and disaccharides, such as sucrose, as a CPA (Janz Fde et al., 2012; Thirumala et al., 2010b), did not achieve expectation in replacing DMSO for cryopreservation of MSCs because they are less efficient than DMSO in maintaining the viability of MSCs. Further investigation is required to explore alternative CPAs to replace DMSO completely. On the other hand, cryopreservation of cells using FBS-free cryopreservation medium seems to be possible, for instance, 5% human albumin solution and sericin have been proposed to replace FBS for cryopreservation of MSCs (Minonzio et al., 2014; Verdanova et al., 2014). Taken together, an effective and safe cell cryopreservation protocol with an optimal concentration of CPAs is still under investigation.

To vitrify cells for clinical applications, many issues need to be addressed, including small scale cryopreservation, high risk of contamination and cell loss due to inefficient collection of frozen cells. With the advance of technology, an ejection-based micro-droplet generation system which permits efficient vitrification of micro-droplets in a continuous manner has been developed, improving throughput capability of vitrification required for cryopreservation of MSCs. A closed, sterile, and fully-automated

vitrification system (*e.g.*, ejection-based micro-droplet generation system) with a high throughput capability which allows vitrification of cells via non-contact with liquid nitrogen should be developed to avoid potential contamination and reduce cell loss (Güven & Demirci, 2012; Shi *et al.*, 2015). On the other hand, a sterile polytetrafluoroethylene cartridge with filtration and ultraviolet radiation can be used to sterilize liquid nitrogen prior to vitrification of cells (Parmegiani *et al.*, 2009).

2.5 The effect of cryopreservation on hASCs

2.5.1 Cell phenotype

The cell phenotype of hASCs can be determined through assessment of their morphological appearance and expression of specific surface markers. Morphologically, hASCs have spindle-like or fibroblast-like shapes (Wan Safwani *et al.*, 2011). It has been reported that cryopreservation does not cause any morphological changes to hASCs (De Rosa *et al.*, 2009). Gonda *et al.* (2008) and Liu *et al.* (2008) reported that cryopreserved hASCs maintain a similar expression level of negative (CD14 and CD45) and positive (CD44, CD90 and CD105) CD markers as fresh (non-cryopreserved) hASCs. However, the effect of cryopreservation on full panels of specific surface markers suggested by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has not been evaluated elsewhere.

2.5.2 Cell proliferation

The proliferative potential of hASCs can be determined through the Resazurin reduction assay and their growth kinetics (Choi *et al.*, 2015). The degree of enzymatic reduction of Resazurin corresponds to the numbers of viable cells is a valuable parameter to assess cell proliferation (Czekanska, 2011). Growth kinetics can be analyzed to calculate population doubling time of cells (Martinello *et al.*, 2011; Seo *et al.*, 2011). It has been

reported that cryopreserved and fresh hASCs have a similar population doubling time (De Rosa et al., 2009; Gonda et al., 2008). These results show that the proliferative potential of hASCs is maintained following the cryopreservation.

2.5.3 Cell differentiation

The differentiation of hASCs into chondrocytes, osteocytes and adipocytes can be evaluated by histochemical staining methods. Cryopreserved hASCs were found to display formation of round lipid droplets which are positively stained by Oil red O upon adipogenic induction, indicating adipogenic differentiation (Minonzio et al., 2014; Thirumala et al., 2010b). Upon osteogenic induction, cryopreserved hASCs showed formation of calcium deposits which are positively stained by Alizarin red. (Liu et al., 2008; Minonzio et al., 2014). Upon chondrogenic induction, the presence of proteoglycan in cryopreserved hASCs was confirmed by Safranin O or Alcian blue staining, indicating chondrogenic differentiation (Gonda et al., 2008; Minonzio et al., 2014). Besides histochemical staining, gene expression analysis of specific differentiation markers through polymerase chain reaction (PCR) and gel agarose electrophoresis also can be performed to evaluate differentiation potential of cryopreserved hASCs. For instance, osteogenic-like cells differentiated from cryopreserved hASCs expressed osteogenic markers such as alkaline phosphatase, osteocalcin and osteopontin (Liu et al., 2008). However, most of the data were analyzed in a qualitative manner, which cannot be accurately compared with those of fresh hASCs and also among hASCs preserved in various CPAs. For instance, quantitative Real-Time PCR analysis revealed that hASCs preserved in 10% DMSO and 90% FBS show low gene expression levels of osteogenic and adipogenic markers when compared to fresh hASCs (James et al., 2011). Therefore, it is also essential to assess differentiation ability of cryopreserved hASCs in a

quantitative manner, *e.g.*, through Real-Time PCR assay to observe molecular changes that might occur following cell freezing (Davies et al., 2014).

Stemness markers such as OCT-4, SOX-2, REX-1 and NANOG can be used to analyze the multipotent differentiation potential of stem cells (Choi et al., 2014; Chua et al., 2014). It has been reported that the reduction in differentiation potential of hASCs was associated with reduced expression of such markers (Chua et al., 2014; Wan Safwani et al., 2011). Therefore, stemness markers in cryopreserved hASCs should be assessed as cryopreservation might compromise differentiation potential of hASCs by downregulating those markers. To date, analysis of stemness markers in hASCs following the cryopreservation has not been evaluated yet.

2.5.4 Cell viability

Viability assays such as live-dead cell staining (acetomethoxy derivate of calcein (calcein-AM)/ethidium bromide), annexin V-propidium iodide (annexin V-PI) and trypan blue exclusion assays can be used to assess cell viability following cryopreservation. These assays have consistently demonstrated that DMSO (at a concentration of $\leq 10\%$) gives a high viability of MSCs ($> 75\%$) following cryopreservation (Ginis et al., 2012; Janz Fde et al., 2012; Pravdyuk et al., 2013; Thirumala et al., 2010a). Among the assays, trypan blue exclusion assay is more preferable due to its high reliability, low cost and ease of processing (without the need of sophisticated external analyzing systems such as flow cytometry system and fluorescence imaging system). In general, types and concentration of CPAs are the main factors which affect cell viability. For instance, amnion-derived MSCs preserved in DMSO display a higher cell viability than those preserved in sucrose (Janz Fde et al., 2012), indicating a relatively high efficiency of DMSO in sustaining the viability of amnion-derived MSCs throughout the cryopreservation. Methylcellulose and

PVP which were used to replace DMSO in cryopreservation of hASCs, were found to display lower viability than those preserved in 10% DMSO. On the other hand, Pravdyuk et al. (2013) showed that bone marrow derived-MSCs preserved in 10% DMSO have a higher post-thaw cell viability than those preserved in 5% DMSO. Therefore, it is essential to assess the cell viability of hASCs following cryopreservation to ensure that a sufficient therapeutic effect from instantly applied cryopreserved hASCs can be achieved.

2.6 Biosafety assessments of MSCs

Knowledge of the biosafety of stem cells including MSCs is still limited, therefore it is essential to assess their biosafety in addition to their therapeutic efficacy (Goldring et al., 2011). Potential risks in terms of chromosomal abnormalities and tumorigenicity in stem cells should be evaluated prior to clinical application (Fink 2009). Adult stem cells may become tumorigenic cells due to mutations acquired during long term expansion (Goldring et al., 2011). For instance, hMSCs have been shown to undergo spontaneous malignant transformation and chromosomal aberration after culture beyond 10 passages (Froelich et al., 2013; Roemeling-van Rhijn et al., 2013; Rosland et al., 2009). As genetic aberrations are strongly associated with formation of tumours, stem cells for clinical use must be free from tumour-associated genomic alteration (Goldring et al., 2011; Laurent et al., 2011). To this end, genetic characterization and culture conditions of stem cells should be clearly defined. Further, parameters that can be used to assess biosafety of cells from the aspect of tumorigenicity are telomerase activity, telomere length, tumour suppressor gene expression level, and detection of p53 mutation (Zaman et al., 2012).

Recently, it has been reported that cells (*e.g.*, ESCs and lymphocytes) cryopreserved in DMSO may display chromosomal abnormality and changes in telomere length, which in turn might lead to formation of tumour (Diaferia et al., 2008; Jenkins et

al., 2012; Yong et al., 2015b). These concerns have established the need for assessment of tumourigenic potential and chromosomal abnormality in cryopreserved hMSCs including hASCs prior to clinical applications. Chromosomal abnormality can be detected through karyotyping which allows detection of abnormal structures (*e.g.*, duplication, translocation, depletions or inversion) and numbers (*e.g.*, gain or loss) of chromosomes (Dittmar et al., 2010). It has been reported that cryopreservation does not alter chromosomes' structures and numbers in hMSCs (Angelo et al., 2012; de Lima Prata et al., 2012; Luetzkendorf et al., 2015; Miranda-Sayago et al., 2012) Angelo et al., 2012. However, the tumourigenic potential of cryopreserved hMSCs has not been evaluated elsewhere.

2.7 Cardiac fibrosis

Heart injury from many causes, *e.g.*, hypertension and myocardial infarction, can trigger cardiac fibrosis, a healing process for heart repair. During cardiac fibrosis, extracellular matrix (ECM) (*e.g.*, collagen) is actively secreted by cardiac fibroblasts and myofibroblasts to replace damaged heart tissues. However, this process is often dysregulated once it has begun, causing adverse remodeling of cardiac tissues which leads to permanent scarring, impair cardiac function and congestive heart failure (Davis & Molkenin, 2014; Weber, 1997; Wynn & Ramalingam, 2012; Zeisberg et al., 2007). Heart failure is a major public health issue with a prevalence of over 23 million worldwide and approximately 300 thousand deaths per year are directly attributable to it (Lloyd-Jones et al., 2010).

In general, cardiac fibrosis is characterized by increased α -SMA expression and collagen production which indicate cardiac myofibroblast differentiation (differentiation of cardiac fibroblasts to myofibroblasts), a hallmark of cardiac fibrosis (Thompson et al.,

2011; van den Borne et al., 2010). Besides resident cardiac fibroblasts, cardiac myofibroblasts can be derived from cells from other lineages, *e.g.*, endothelial cells and bone marrow-derived precursors in response to profibrotic growth factors and cytokines released by immune cells and cardiac cells (Kis et al., 2011; Lajiness & Conway, 2014).

To date, cardiac fibrosis is a substantial problem that is difficult to manage, as it cannot be effectively halted or reversed by surgery or drug therapies (*e.g.*, anti-fibrotic agents) once it has begun (Daskalopoulos et al., 2012). Recently it has been realized that activities (*e.g.*, collagen secretion) and differentiation of cardiac myofibroblasts should be controlled in order to treat cardiac fibrosis. Therefore, it is essential to understand cardiac myofibroblast differentiation to explore several possible targets for intervention as effective cardiac fibrosis therapy (Yong et al., 2015a).

2.7.1 Cardiac myofibroblast differentiation

In general, myofibroblasts present spindle-shaped and extensive areas of endoplasmic reticulum with protruding dendrite-like processes (Eyden, 2008; Hinz et al., 2007). The defining marker of myofibroblasts is a relatively high expression of α -SMA that incorporates into stress fibers which contribute to their contractile function (Dobaczewski et al., 2012). The α -SMA expression can be determined using protein based (*e.g.*, Western Blotting or immunofluorescence staining) or molecular-based (*e.g.*, quantitative Real-Time PCR) assays (Galie et al., 2012; Watson et al., 2014; Watson et al., 2012). Further, soluble factors (*e.g.*, transforming growth factor-beta 1 (TGF- β 1)) and ECM proteins such as collagen I (Col I), collagen III (Col III) and fibronectin-extra domain A (EDA) are actively secreted by myofibroblasts (Daskalopoulos et al., 2012; van den Borne et al., 2010). The function of Col I and III (major components of ECM in heart) is to replace the damaged heart tissue (Ma et al., 2014), whereas fibronectin EDA connects integrins and

stress fibers in myofibroblasts to ECM, allowing myofibroblasts to contract and generate traction force on the ECM (Santiago et al., 2010). Cell traction forces (tensile forces exerted by the cells to ECM through focal adhesions such as integrins) are important for migration, maintenance of shaped and mechanotransduction (Balaban et al., 2001). The criteria for assessment of cardiac myofibroblast differentiation are described in brief in **Fig. 2.6**.

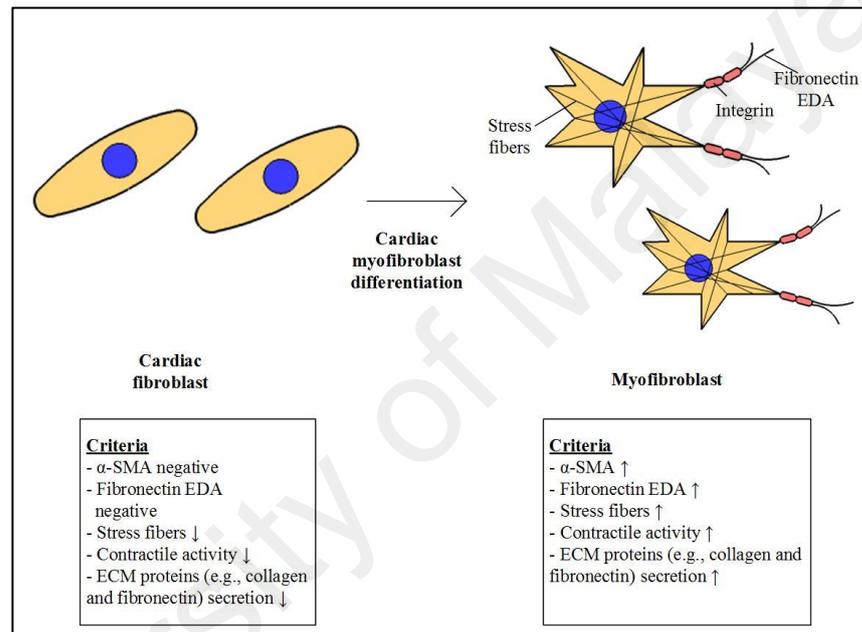


Figure 2.6. Criteria for assessment of cardiac myofibroblast differentiation. Adapted from Yong et al. (2015a).

Initially, cardiac myofibroblasts are known to be derived from cardiac fibroblasts in response to biochemical cues. Soluble factors (*e.g.*, angiotensin II (AngII) and TGF- β 1), extracellular proteins (*e.g.*, fibronectin-EDA), matricellular proteins (*e.g.*, connective tissue growth factor), and cytokines (*e.g.*, interleukin-6), are found to induce cardiac myofibroblast differentiation (Daniels et al., 2009; Leask, 2010). Among the biochemical cues, TGF- β 1, a major inducer of cardiac myofibroblast differentiation (Ma et al., 2014), binds to TGF- β 1 receptor on cardiac fibroblasts to enhance α -SMA expression and synthesis of ECM molecules (*e.g.*, collagen and fibronectin EDA) which further mediate myofibroblast differentiation in a positive feedback loops manner (Serini et al., 1998).

Recently, it has been realized that biochemical cues are not the only inducer for cardiac myofibroblast differentiation.

Over the past decade, cardiac myofibroblast differentiation is also found to be regulated by the mechanical properties of ECM (*e.g.*, matrix stiffness) (Hinz, 2010). Matrix stiffness is defined as a measure of matrix resistance to mechanical deformation (Hinz, 2009). Cardiac fibroblasts have been found to differentiate into cardiac myofibroblasts in response to mechanical stimuli (*e.g.*, ECM stiffness) (Galie et al., 2011; Zhao et al., 2014). Stiff ECM generates mechanical stress to the cells which in turn activates TGF- β 1 to induce myofibroblast differentiation (Galie et al., 2012). The prolonged existence of fibrosis is due to increasing secretion of TGF- β 1 in response to persistence of stiff injured and fibrotic tissues (Wells, 2013). These demonstrate that matrix stiffness and biochemical cues are actually interdependent in myofibroblast differentiation (MacKenna et al., 2000). Generally, TGF- β 1 produced by the cells (*e.g.*, cardiac fibroblasts) is stored in the ECM as part of a latent complex. One of the components of latent complex called latency-associated peptide (LAP) which connects with integrins (*e.g.*, $\alpha_v\beta_5$) controls the release of TGF- β 1 from the latent complex (Wells, 2013). TGF- β 1 is released when the latent complex undergoes conformational changes in response to traction forces generated by the cells to ECM (Wipff et al., 2007). When cells apply traction to soft ECM, soft ECM can deform easily, leaving no conformational changes to the latent complex, thus disables the release of TGF- β 1. However, when cells apply traction to stiff ECM, stiff ECM can resist deformation, causing distortion of the latent complex which in turn releases the active TGF- β 1. TGF- β 1 binds to its receptor at the cell membrane and phosphorylates or activates Smad2 to increase expression of α -SMA protein or myofibroblast differentiation (Wells & Discher, 2008). These proteins interact with myosin to generate increased traction to stiff ECM, which in turn activates

more and more TGF- β 1 to maintain myofibroblast phenotype and promote myofibroblast differentiation (**Fig. 2.7**). The whole process is a positive feedback loop that incorporates both mechanical and biochemical signals (Wipff et al., 2007). In short, myofibroblast differentiation requires stiff ECM, cell traction force, and activation of TGF- β 1. In addition to extracellular release of TGF- β 1 from the latent complex, cardiac fibroblasts can secrete TGF- β 1 in a paracrine or autocrine manner through mechanical stress to induce myofibroblast differentiation (Dalla Costa et al., 2010; Galie et al., 2012).

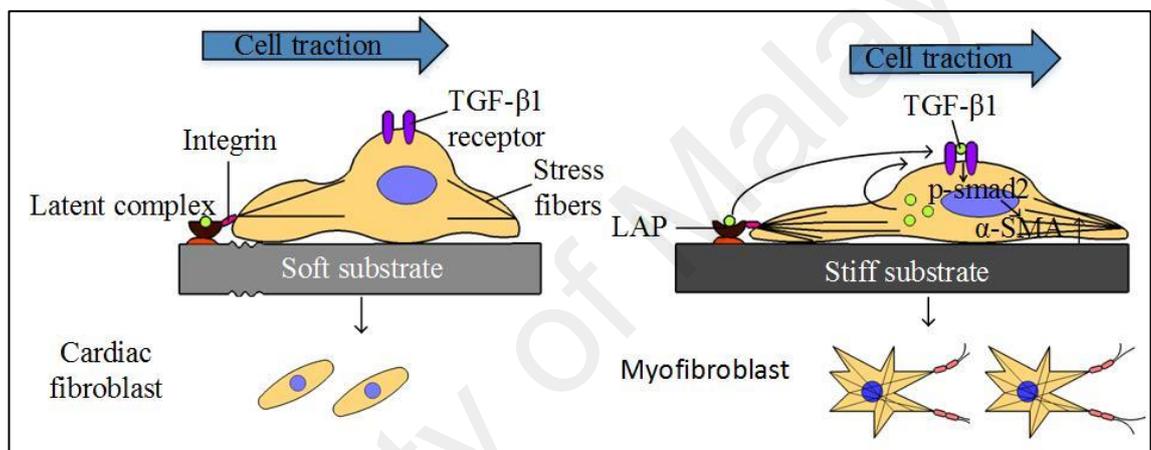


Figure 2.7. The existing mechanism of matrix-stiffness induced cardiac myofibroblast differentiation. LAP: latency-associated peptide. p-Smad2: phosphorylated Smad2. Adapted from Yong et al. (2015a).

2.7.2 Matrix stiffness-induced cardiac fibrosis model

In normal hearts, a stable cross-linked ECM network at healthy myocardium protects cardiac fibroblasts from mechanical stress. Upon myocardial infarction, prolonged cardiac remodeling disrupts the structural integrity of ECM network at the infarcted areas. The surrounding cardiac fibroblasts were exposed to increased mechanical stress due to stiff and disorganized matrix at the infarcted areas, thus leading to cardiac fibrosis and myofibroblast differentiation (Dobaczewski et al., 2012). Therefore, animal models of myocardial infarction were used to develop matrix stiffness-induced *in vivo* cardiac fibrosis models as they showed increased expression of Col I, Col III and TGF- β 1, which can be seen in cardiac fibrosis (Xu et al., 2005). Though such models, it is possible to

explore signaling pathways, integrins and inducers involved in cardiac fibrosis and myofibroblast differentiation. For instance, it was found that cardiac myofibroblast differentiation is associated with elevation of collagen IV in rat myocardium 20 weeks after myocardial infarction (Naugle et al., 2006). Further, cardiac function was shown to be improved while cardiac fibrosis was shown to be decreased in animal models of myocardial infarction following the intervention on collagen IV (Bryant et al., 2009; Luther et al., 2012). It seems that cardiac myofibroblast differentiation might be mediated by interaction of collagen IV with α_3 integrin acquired by cardiac fibroblasts (Bryant et al., 2009; Shamhart & Meszaros, 2010). Although animal models may give deep insights into the cell response *in vivo*, however, to avoid ethical issues of discomfort or pain to animals, *in vitro* models which are more conducive to repetitive and systematic investigation of cell pathophysiology and allow high-throughput testing with less time-consuming, are advantageous and preferable (Elliott & Yuan, 2011).

In the past decade, cardiac fibroblasts which spontaneously differentiate into cardiac myofibroblasts on stiff plastic cell culture plate or glass slide were used as an *in vitro* cardiac fibrosis model (Li et al., 2015; Mao et al., 2013; Mias et al., 2009; Ohnishi et al., 2007; Wang et al., 2011). However, it was realized that the stiffness of such plastic and glass substrates (around 1 GPa) does not represent the stiffness of the native fibrotic cardiac tissues, which could not accurately reflect the cell response *in vivo*. To study cardiac fibrosis and cardiac myofibroblast differentiation in an accurate manner, it is essential to develop a cardiac fibrosis model mimicking the stiffness of native fibrotic cardiac tissues. For instance, a two dimensional (2D) *in vitro* cardiac fibrosis model which is made of rat cardiac fibroblasts cultured on PEGDA hydrogels with patterned stiffness (10 kPa and 40 kPa) which correspond to the stiffness of native normal and fibrotic cardiac tissues respectively, was created. The stiffness of normal rat cardiac tissues is 10-20 kPa,

whereas the stiffness of fibrotic rat cardiac tissues is 30-70 kPa (Berry et al., 2006; Engler et al., 2008). This model revealed that cardiac fibroblasts migrate across the border from the soft hydrogel substrate (stiffness of 10 kPa) to the stiff hydrogel substrates (stiffness of 40 kPa) which in turn differentiate into myofibroblasts on the stiff substrate, as indicated by a relatively high expression of α -SMA and fibronectin. Cardiac fibroblasts were found to maintain their inactivated phenotype on soft substrates (Zhao et al., 2014).

Recently, it has been suggested that cell signaling and differentiation which result from responses to mechanical stress induced by ECM in 2D culture, might arise from matrix protein (*e.g.*, collagen) tethering besides matrix stiffness (Chaudhuri & Mooney, 2012; Trappmann et al., 2012). For instance, varying collagen tethering degree has been found to induce different cell behaviors (*e.g.*, differentiation and spreading) in human epidermal stem cells cultured on substrates with various stiffness (Trappmann et al., 2012). Collagen tethering can be controlled through varying the distance between two adjacent collagen anchoring points, by adjusting concentration of protein-substrate linker or substrate pore size (Trappmann et al., 2012; Wen et al., 2014). Therefore, it is essential to develop a 2D *in vitro* cardiac fibrosis model which incorporates substrate pore size and substrate stiffness mimicking native fibrotic cardiac tissues which may regulate cardiac myofibroblast differentiation. For instance, Engler and his group members have developed a mechanical tunable cell culture system which decouples substrate stiffness and pore size to investigate the independent effect of each factor on adipogenic and osteogenic differentiation of hASCs (Wen et al., 2014).

To date, certain challenges in engineering a three-dimensional (3D) *in vitro* matrix stiffness-induced cardiac fibrosis still remain. For instance, the properties of hydrogels such as stiffness and pore size are still coupled to each other, disabling the investigation

on the independent effect of each factor on cardiac myofibroblast differentiation. In future, new fabrication techniques of 3D cell-laden hydrogels are needed to decouple such factors.

2.7.3 The effect of MSCs on matrix stiffness-induced cardiac fibrosis

The beneficial effects of MSCs on cardiac fibrosis have been evaluated mostly in animal models of myocardial infarction for preclinical trials. Most of the studies demonstrated that MSCs derived either from bone marrow or adipose tissue were found to attenuate cardiac fibrosis following the transplantation of MSCs near infarcted or myofibroblast-rich areas (Badimon et al., 2015; Ramkisoensing et al., 2014). The inhibitory effects of MSCs on cardiac fibrosis in the infarcted heart were indicated by improved left ventricular heart function, decreased ECM production and decreased scar size (Ishikane et al., 2013; Miyahara et al., 2006; Song et al., 2013). It seems that MSCs decrease ECM (*e.g.*, collagen) production by attenuating the secretion of TGF- β 1 to reduce the size of scar formed post-myocardial infarction, thus restoring heart function (Xu et al., 2005). Taken together, the existing evidence found in the preclinical trials demonstrates the potential of MSCs to treat cardiac fibrosis post-myocardial infarction by restoring cardiac function and reducing scar size, which encourages more clinical trials in this area. To date, the use of MSCs in such clinical trials is still in phase I-II. The results seem to be promising as they are in accordance with the findings of preclinical trials, which could prompt the clinical trial into the next phase (Badimon et al., 2015). Although such *in vivo* studies have been conducted extensively, these studies restrict mechanistic investigations of anti-fibrotic effects of MSCs, which can help to optimize therapeutic benefit of MSCs in cardiac fibrosis therapy. The effects could be mediated by either paracrine signaling, direct cell-to-cell contact or differentiation to cardiomyocytes (Ramkisoensing et al.,

2014). Therefore, *in vitro* studies are required to further investigate the exact and comprehensive inhibitory mechanism of MSCs on cardiac fibrosis.

The therapeutic effect based on cardiomyogenic differentiation potential of MSCs on cardiac fibrosis has been shown to be greatly reduced under stiff ECM. For instance, when undifferentiated MSCs were implanted into the heart of a rat model of cardiac fibrosis post myocardial infarction, they showed bone tissue formation instead of cardiomyogenic differentiation (Breitbach et al., 2007). MSCs were shown to present low expressions of early cardiac transcription factors (*e.g.*, GATA4 and Nkx2.5) in response to stiff ECM upon cardiomyogenic induction (Sullivan et al., 2014). These suggest that the therapeutic effect of MSCs on cardiac fibrosis might not be mediated by differentiation to cardiomyocytes.

On the other hand, a conditioned medium of bone marrow-derived MSCs has been applied to *in vitro* cardiac fibrosis models made of rat cardiac fibroblasts cultured on plastic cell culture plate (Mao et al., 2013; Mias et al., 2009; Ohnishi et al., 2007; Wang et al., 2011). Bone marrow-derived MSCs were found to attenuate production of Col I and Col III (Ohnishi et al., 2007). It was also observed that MSCs decrease viability of cardiac fibroblasts and α -SMA expression, suggesting the loss or phenotypic change of myofibroblasts. Further, ECM degradation was activated in response to upregulation of matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9) and downregulation of MMP endogenous inhibitor (TIMP) upon treatment with conditioned medium of MSCs (Mias et al., 2009). On the other hand, bone marrow derived-MSCs transfected by integrin-linked kinase were found to reduce expression of Col I and Col III, TIMP, α -SMA and connective tissue growth factor through paracrine signaling (Mao et al., 2013). Several soluble factors with anti-fibrotic effects secreted by MSCs were determined, such as HGF, IGF-1, basic fibroblast growth factor (bFGF) and adrenomedullin (ADM)

(Ramkisoensing et al., 2014). HGF and IGF-1 may suppress pro-fibrotic signaling by miR-155 and miR-21 to reduce cardiac fibrosis (Iekushi et al., 2012; Kishore et al., 2013). bFGF may enhance the secretion of HGF which in turn increases the anti-fibrotic effects of MSCs (Tang et al., 2015). ADM was found to inhibit the synthesis of Col I and Col III (Li et al., 2009). Among the soluble factors, HGF is well-known as major contributor for MSCs anti-fibrotic function to reduce fibrosis in multiple organs (Gorji et al., 2012; Semedo et al., 2009; Tsai et al., 2009; Zhang et al., 2011a). However, to date, the comprehensive inhibitory mechanism of soluble factors, particularly HGF, secreted by MSCs on cardiac fibrosis still remain elusive. Investigation of the paracrine effects of MSCs toward an *in vitro* cardiac fibrosis model mimicking the stiffness of native fibrotic cardiac tissues should be performed to elucidate such mechanism.

Besides paracrine signaling, MSCs may interact with cardiac myofibroblasts to reduce cardiac fibrosis through direct cell-to-cell contact. To study such a mechanism, an engineered microfluidic-based co-culture platform was developed to co-culture hASCs with rat cardiac myofibroblasts using a gap co-culture method. Tube-like structures which connect hASCs with cardiac myofibroblasts were observed, suggesting the formation of tunnels for transporting vesicles containing anti-fibrotic factor from hASCs to myofibroblasts. It was showed that hASCs decrease viability and α -SMA expression of myofibroblasts via cell-to-cell contact. By using the same platform, conditioned medium of hASCs was used to culture cardiac myofibroblasts, resulting in low viability and α -SMA expression of myofibroblasts, but this method seems to be less potent than the gap co-culture method (Li et al., 2015). Notably, the existing *in vitro* studies focused on the reduction of interstitial fibrosis (development of myofibroblasts) rather than scar elimination which can only be observed in animal-based cardiac fibrosis model upon treatment with MSCs.

2.8 Literature review summary

hASCs gain special attention due to the abundance and readily accessibility of adipose tissues in human body. To date, cryopreservation is the existing method used to preserve and store hASCs for clinical applications. However, a standardized protocol of cryopreservation of hASCs is not well established yet. Optimization of type and concentration of CPA used in the cryopreservation of hASCs still remains challenging. In addition, the biosafety (*e.g.*, risk of tumourigenesis) of cryopreserved hASCs is also not well established. Taken together, this has strengthened the need to fully evaluate the effects of cryopreservation conditions (*e.g.*, CPA) on hASCs in term of their cell viability, functional properties and tumourigenic potential.

Cardiac fibrosis is a pathological condition that cannot be completely stopped by any treatment to date, resulting in heart failure which is a leading cause of mortality worldwide. It has been suggested that MSCs which can secrete soluble factors to alter behavior of neighbouring cells (*e.g.*, cardiac myofibroblast differentiation and cardiomyocyte regeneration), holding great potential in cardiac fibrosis therapy. Since cardiac fibrosis may require intensive cell therapies, hASCs with a high capability of being cryopreserved in long-term for off-the-shelf clinical use, would be an ideal cell source for such therapy. To elucidate the therapeutic potential and mechanism of hASCs in cardiac fibrosis therapy, it is essential to investigate the paracrine effects of hASCs on an *in vitro* cardiac fibrosis model which mimics the stiffness of the native fibrotic cardiac tissues.

CHAPTER 3: ISOLATION AND CHARACTERIZATION OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

3.1 Introduction

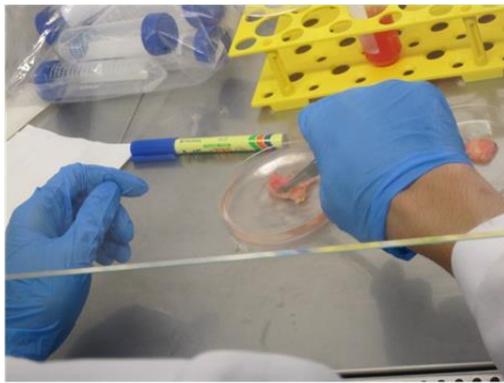
In the past, investigators used to report studies of hMSCs obtained from various sources of the human body and using differing cell isolation and characterization approaches (Zuk et al., 2001). In order to promote the trade of data among investigators for comparison of biological properties and experimental outcomes in hMSCs, characterization of hMSCs should be perform in accordance to the standard criteria of hMSCs defined by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (Dominici et al., 2006). Assessment of surface antigen/marker expression, which allows identification of cell population, can be performed using immunophenotyping through a flow cytometry assessment (Diaz-Romero et al., 2005). Multipotent differentiation can be induced by culturing cells in various cell differentiation induction culture media, and the outcome can be assessed through a histochemical staining method (*e.g.*, Oil red O, Alizarin red and Alcian blue) (Choi et al., 2015). In the present study, the cells isolated from human adipose tissues were characterized based on the proposed criteria of hMSCs prior to further experiments. In addition to histochemical staining, quantitative Real-Time PCR was performed to evaluate multipotent differentiation potential of the cells.

3.2 Material and methods

3.2.1 Isolation and culture of hASCs

Human adipose tissue samples were obtained from 6 distinct female donors undergoing caesarean section, aged between 25 and 35 years old, with prior informed written consent (**Appendix B**). The size for each sample was within 5 – 15 mL. Procedures for isolation and culture of hASCs were described briefly in **Fig. 3.1**. First, adipose tissues were

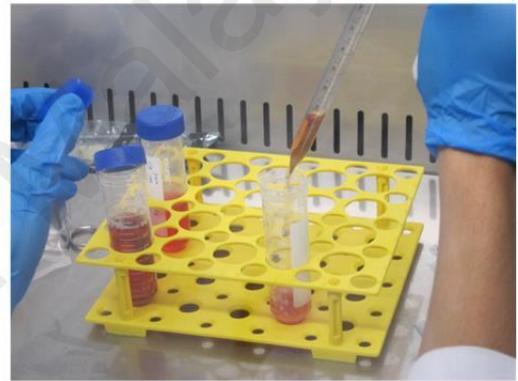
cleaned using phosphate buffer saline (PBS) (Sigma Aldrich, St. Louis, USA) to remove the blood and oil. Blood vessels were removed and adipose tissues were cut into small pieces. Digestion of 5 mL of adipose tissue was conducted using 5 mL of 0.3% (w/v) collagenase type I enzyme (Worthington, New Jersey, USA) solution with 250 rpm agitation at 37°C for 10 mins in an incubator shaker (LSI-3016A, Daihan Labtech, India). Following centrifugation with 1000 rpm at room temperature, 3 fractions of digested tissues (oil, collagenase solution and pellet) were seen. Oil and collagenase solution were removed followed by washing to obtain the pellets. Finally, the pellets were resuspended with complete culture medium composed of Dulbecco's Modified Eagle's medium (DMEM)/Ham F-12, 10% FBS, 1% glutamax, 1% antibiotic/antimycotic (Gibco, New York, USA) and 1% vitamin C solution (5 mg/mL) (Sigma-Aldrich, USA), and seeded in a cell culture flask. Cells were cultured in a CO₂-controlled incubator (Galaxy® 170 R, New Brunswick Scientific, USA) at 37°C and 5% CO₂. The number of mononuclear cells for each isolation was approximately 2×10^5 cells per mL of adipose tissue. Cells were subcultured to passage 3 prior to being used for all assays for characterization of hMSCs, including cell phenotyping and differentiation (adipogenic, osteogenic, and chondrogenic) assessment.



Washing adipose tissues



Mincing adipose tissues



Tissue digestion with agitation at 37 °C

Adding 0.3% collagenase type 1 solution



Cell culture

Incubation at 37 °C and 5% CO₂

Figure 3.1. A flow chart demonstrates the procedures for isolation and culture of hASCs.

3.2.2 Trypsinization

Culture medium from culture vessel was removed and the monolayer of cells was washed with PBS to remove all traces of FBS. PBS was then removed and trypsinization solution (Accutase cell detachment solution (Innovative Cell Technologies, Inc., San Diego, USA) or 0.25% trypsin solution (Gibco)) was added into culture vessel to completely cover the monolayer of adherent cells (hASCs or cardiac fibroblasts). Culture vessel was placed into a cell incubator (37 °C, 5% CO₂) for 5 mins. Progress was checked by microscopic examination. The cells in suspension appeared rounded when trypsinization process is complete. Culture medium containing FBS was added to the cell suspension to inhibit further trypsinization activity which is harmful to cells. Cell suspension was then centrifuged with 1000 rpm at room temperature. All supernatant was removed and the pellet was resuspended with fresh culture medium for cell count, assays, subculturing or further experiment.

3.2.3 Cell phenotyping

Cell phenotyping of hASCs was performed through microscopic observation and immunophenotyping. Microscopic examination was conducted to assess the morphological appearance and adherent properties of hASCs, whereas surface markers expressed by hASCs were determined by immunophenotyping. To conduct immunophenotyping, hASCs were subjected to trypsinization using Accutase cell detachment solution (Innovative Cell Technologies, Inc.) following method as described in the section 3.2.2, and then washed and centrifuged (1000 rpm, room temperature) with 0.5% (w/v) bovine serum albumin solution (BSA) (Amresco, Ohio, USA) in PBS. Cell suspension in BSA was filtered using a cell strainer with a pore size of 70 µm (Becton Dickinson, San Jose, USA) to eliminate the cell clumps followed by cell counting. About 5×10^5 cells were transferred into a 5 mL falcon tube (Becton Dickinson) and centrifuged.

The cell pellets were resuspended in 100 μ L sheath fluid (Becton Dickinson). Cells were then incubated with antibodies labeled with fluorochrome (Becton Dickinson) as follows: CD 105-FITC, CD 14-PE, CD 90-FITC, CD 34-FITC, HLA DRDPDQ-FITC, CD 73-PE, CD19-PE, CD 44-FITC, CD 45-FITC and HLA ABC-FITC, each for 30 mins on ice in the dark. Cells also were incubated with negative control antibodies (Becton Dickinson) as follows: FITC-conjugated mouse IgG1 and IgG2 α isotypes, and PE-conjugated mouse IgG1 and IgG2 α isotypes. After 30 mins, cells were washed and centrifuged with sheath fluid to remove unbound and residual antibody. Finally, cells were resuspended in 500 μ L sheath fluid, and the data were immediately acquired using a flow cytometry system (BD FACSCanto II, Becton Dickinson). For each sample, a minimum of 10,000 events were acquired. The data were then analyzed using FlowJo software (Treestar, OR, USA).

3.2.4 Tri-lineage differentiation (adipogenic, osteogenic and chondrogenic) assessment

The tri-lineage differentiation potential (adipogenesis, osteogenesis and chondrogenesis) of hASCs was determined using histochemical staining and Real-Time PCR. To induce adipogenesis, hASCs were cultured in adipogenic induction medium composed of complete culture medium, 1 μ M dexamethasone (Sigma), 0.5 μ M isobutyl-1-methylxanthine (Sigma), insulin-transferrin-selenium (ITS) (Becton Dickinson) and 200 μ M indomethacin (Sigma) for 21 days. Adipogenic induction medium was changed alternately with complete culture medium every 2 days, as cells incubated in the adipogenic induction medium for more than 3 consecutive days may die due to the toxic effect of adipogenic induction medium. After 21 days, the cells were fixed in 4% neutral buffered formalin (Sigma) at room temperature for 10 mins. After the removal of formalin, the cells were incubated in 60% isopropanol at room temperature for 5 mins. A fresh 60% Oil red O (Sigma) working solution was prepared by diluting a stock solution (0.3% (w/v)

Oil Red O in 60% isopropanol (Sigma)) using deionized water followed by filtration using syringe filter with a pore size of 20 μm . After discarding the isopropanol, the cells were stained with Oil Red O working solution at room temperature for 5 mins. Finally, the cells were washed with deionized water to remove the excess stain, and then observed using a light microscope (Nikon Eclipse TS100, Tokyo, Japan). Differentiation of hASCs into adipogenic-like cells was indicated by the formation of lipid droplets stained by Oil Red O. Further, the adipogenic potential of hASCs before and after adipogenic induction was compared in terms of adipogenic markers expression determined using Real-Time PCR. Lipoprotein lipase (LPL) (Hs00173425_m1), fatty acid binding protein (FABP4) (Hs01086177_m1) and peroxisome proliferator-activated receptor- γ (PPARG) (Hs01115513_m1) serve as markers for adipogenic differentiation.

For inducing osteogenic differentiation, hASCs were cultured in osteogenic induction medium composed of complete culture medium, 0.5 mM ascorbic acid-2-phosphate (Sigma), 10 mM β -glycerophosphate (Sigma) and 100 nM dexamethasone (Sigma) for 21 days. Osteogenic induction medium was changed every 3 days. After 21 days, the cells were fixed in 4% neutral buffered formalin at room temperature for 10 mins. After the removal of formalin, the cells were incubated in deionized water at room temperature for 5 mins. After discarding deionized water, the cells were stained with 2% (w/v) Alizarin Red S (Sigma) solution at room temperature for 5 mins. Finally, the cells were washed with deionized water to remove the excess stain, and then observed using a light microscope. Differentiation of hASCs into osteogenic-like cells was indicated by the presence of calcium deposits stained by Alizarin Red (Sigma). Further, osteogenic potential of hASCs before and after osteogenic induction was compared in terms of osteogenic markers expression. Runt-related transcription factor 2 (RUNX2)

(Hs00231692_m1), Osteocalcin (OSC) (Hs015878914_m1) and Alkaline phosphatase (ALPL) (Hs01029144_m1) serve as markers for osteogenic differentiation.

For inducing chondrogenesis, hASCs in pellet forms were cultured in chondrogenic induction medium composed of complete culture medium with 50 ng/ml IGF-1 (Peprotech, New Jersey, USA), 100 nM dexamethasone (Sigma), 10 ng/ml TGF- β 1 (Peprotech), 40 μ g/ml L-proline (Sigma), 50 μ g/ml ascorbate-2-phosphate (Sigma) and 1% ITS (Becton Dickinson) for 21 days. Chondrogenic induction medium was changed every 3 days. After 21 days, the pellets were fixed in 4% neutral buffered formalin overnight at room temperature. Then, the pellets were dehydrated in the series of ascending concentrations of ethanol, cleared with xylene, infiltrated with paraffin and moulded into tissue block. Then, the tissue block were cut into 5 mm thick section and placed on a glass slide. Tissue section on the slide were deparaffinized with xylene and rehydrated through series of descending concentrations of ethanol. The tissue section was stained with 1% (w/v) Alcian blue (Sigma) in 3% acetic acid with pH 2.5. Nuclear fast red solution at 0.1% (Sigma) was used as a counterstain to stain cytoplasm of the cells. Tissue sections were then dehydrated through series of ascending concentrations of ethanol. Finally, tissue section was mounted with DPX (Sigma) and then observed using a light microscope. Further, the chondrogenic potential of hASCs before and after chondrogenic induction was compared in terms of chondrogenic markers expression. Sry-related HMG box-9 (SOX-9) (Hs00165814_m1), Collagen type II (COL-2) (Hs00264051_m1) and Aggrecan (ACAN) (Hs00153936_m1) serve as markers for chondrogenic differentiation. Cells before differentiation induction were used as negative control in staining and Real-Time PCR assays.

3.2.5 RNA extraction, cDNA synthesis and quantitative Real-Time polymerase chain reaction (PCR)

RNA of cells was extracted using TRI reagent (Ambion, Austin, USA) according to the manufacturer's recommendations. Briefly, cells were trypsinized using trypsin following method as described in the section 3.2.2, and then resuspended in 1 mL TRI reagent followed by grinding using a pestle to lyse the cells. Phase separation was performed using 200 μ L chloroform (Fisher Scientific, Pittsburg, USA), with 15 mins incubation at room temperature followed by centrifugation ($12,000 \times g$, 15 mins, 4°C). Three solution phases were seen: clear aqueous (contained RNA), white cloudy (contained DNA) and red organic (contained protein) phase. Clear aqueous solution was collected followed by RNA precipitation with 500 μ L isopropanol (Sigma) and 5 μ L polyacryl carrier (Molecular Research Center, Ohio, USA). After centrifugation ($12,000 \times g$, 10 mins, 4°C), RNA pellets were washed with 1 mL 75% absolute ethanol (Fisher Scientific) and centrifuged ($12,000 \times g$, 5 mins, 4°C). Finally, RNA pellets were dried at room temperature for 3 mins and then resuspended in 15 μ L ultrapure nuclease-free water (Invitrogen, Carlsbad, USA). The purity and concentration of the total RNA were determined using a Nanodrop (Thermo Scientific, Waltham, USA). The RNA concentration of each total RNA of cells is within 500 – 1000 ng/ μ L. On the other hand, the ratio of absorbance at 260 nm/280 nm was used to assess the purity of RNA. The ratio of absorbance at 260 nm/280 nm for each total RNA of cells is within 1.9 – 2.0. A ratio of ~ 2.0 is generally accepted as “pure” for RNA, indicating only RNA was isolated. The representative data were shown in the **Table 3.1**.

Table 3.1: Concentration and absorbance at 260 nm/280 nm of total RNA of cells

	Concentration (ng/μL)	Absorbance at 260 nm/280 nm
Fresh hASCs before differentiation induction	632.5	1.93
hASCs preserved in 5% DMSO before differentiation induction	939.3	1.97
Fresh hASCs after adipogenic induction	935.1	1.96
hASCs preserved in 5% DMSO after adipogenic induction	949.3	1.93
Fresh hASCs after osteogenic induction	667.0	1.94
hASCs preserved in 5% DMSO after osteogenic induction	984.3	1.98
Fresh hASCs after chondrogenic induction	543.6	1.94
hASCs preserved in 5% DMSO after chondrogenic induction	570.9	1.92

The high capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, USA) was used to convert RNAs to cDNAs. Samples for RNA-to-cDNA conversion were prepared in a 0.2 ml microcentrifuge tube, as shown in **Table 3.2**. The tubes were then placed into a thermal cycler (MyGene TM Series Peltier, Hangzhou, China) for RNA-to-cDNA conversion using the setting recommended by manufacturer (**Table 3.3**).

Table 3.2: Components for cDNA synthesis

Component	Volume (μL)
RNA sample (2 μ g)	Up to 9 μ L
20 \times reverse transcriptase enzyme	1
2 \times reaction buffer	10
Nuclease-free water	Top up to a final reaction volume of 20 μ L
Total	20

Table 3.3: RNA-to-cDNA conversion setting

Process	Condition (temperature, duration)
cDNA synthesis	37 °C, 60 mins
Enzyme denaturation	95 °C, 5 mins
Cooling	4 °C, 30 mins

Real-Time PCR was conducted for gene expression analysis using the *TaqMan* gene expression assay (Applied Biosystems) and the Real-Time PCR system (StepOnePlus, Applied Biosystems). Briefly, samples for Real-Time PCR were prepared and loaded into an optical 96-wells reaction plate (Applied Biosystems), as shown in **Table 3.4**. Once all the samples were loaded, the reaction plate was covered with an optical adhesive film (Applied Biosystems) and then placed into the Real-Time PCR system for gene expression analysis. The thermal cycling profile of Real-Time PCR is shown in **Table 3.5**, following the manufacturer's recommendation. The genes to be evaluated include differentiation markers as mentioned earlier. The housekeeping gene used for normalization was GAPDH (Hs99999905_m1). The gene expression level of the control group (hASCs before differentiation) was normalized to 1. Data were expressed as fold change in relative to the control.

Table 3.4: Sample preparation for Real-Time PCR

Component	Volume (µL)
cDNA	1
Taqman gene expression assay (target marker)	1
Taqman fast advanced master mix	10
Nuclease-free water	8
Total per well	20

Table 3.5: Thermal cycling profile for Real-Time PCR

Process	Conditions (temperature, duration)
Polymerase activation	95 °C, 20 secs
Denaturation	95 °C, 1 secs
+ Primer annealing/extending	+ 60 °C, 20 secs
} 40 cycles	} 40 cycles

3.2.6 Statistical analysis

Statistical analysis was performed using a paired *t*-test to compare data before and after the differentiation induction in the gene expression. Each datum was expressed as mean \pm standard error of mean of six different donors ($n = 6$). Each experiment was conducted in triplicates per donor. Statistical significance was accepted at $p < 0.05$. All data analyses were performed using SPSS 18.0 software.

3.3 Results

3.3.1 Phenotype of hASCs

Through microscopic examination, it was observed that cells at passage 3 presented fibroblast-like or spindle shaped and adherent property (**Fig. 3.2**). Flow cytometry analysis showed that cells express high levels of CD90 ($99.60 \pm 0.23\%$), CD73 ($98.88 \pm 0.24\%$), CD105 ($84.67 \pm 0.90\%$), CD44 ($98.15 \pm 0.76\%$) and HLA ABC ($97.83 \pm 0.27\%$), and expressed low level of CD14 ($0.117 \pm 0.040\%$), CD19 ($0.083 \pm 0.031\%$), CD34 ($0.183 \pm 0.054\%$), CD45 ($0.317 \pm 0.142\%$) and HLA DQDPDR ($0.233 \pm 0.072\%$) (**Fig. 3.3**).

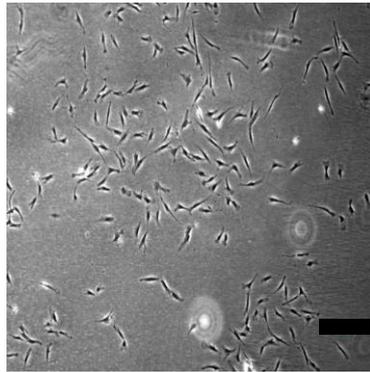


Figure 3.2. Morphology of hASCs. Spindle-shaped cells were observed (magnification: 40×). Scale bar: 200 μ m.

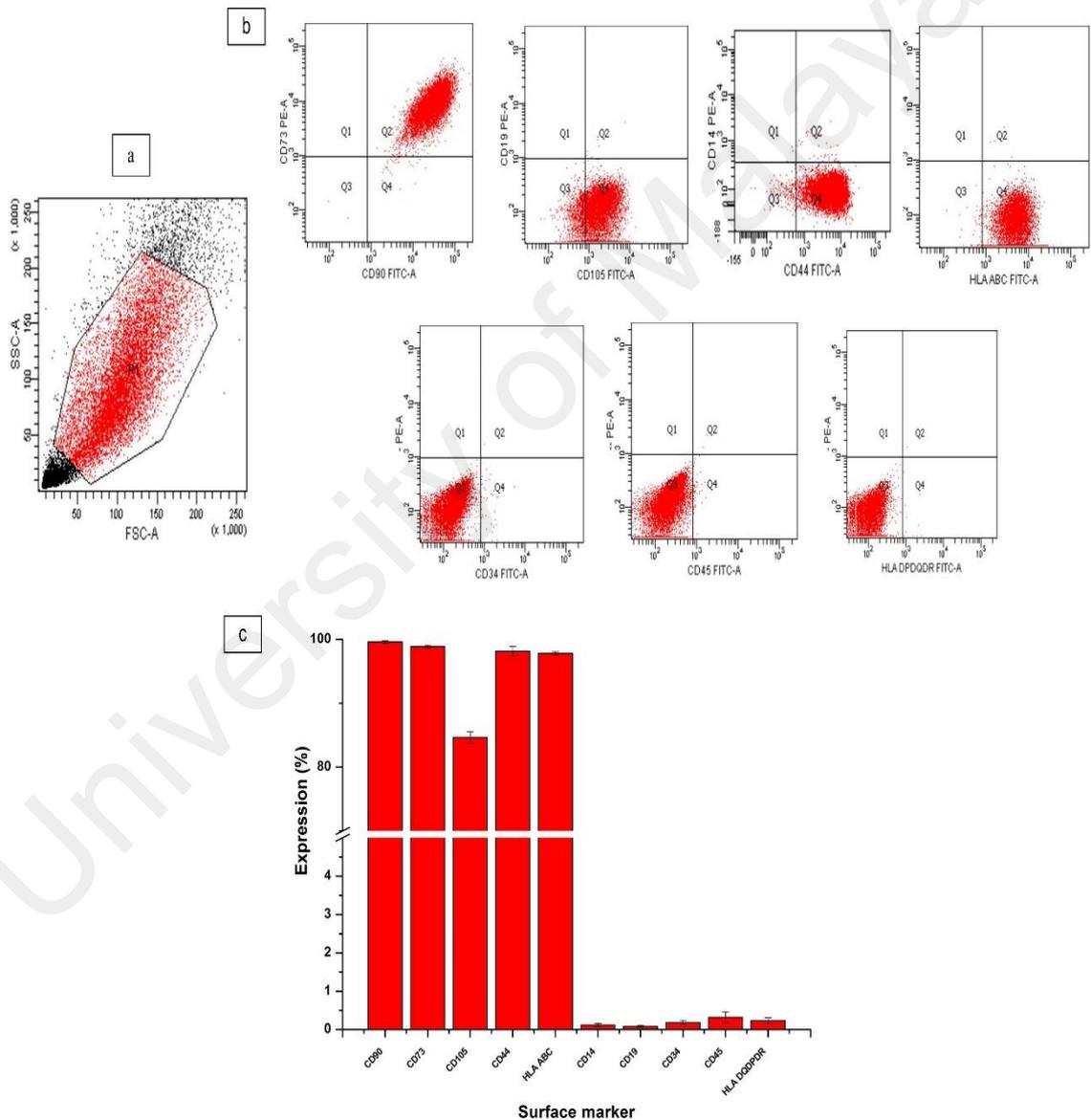


Figure 3.3. Surface marker expression of hASCs. Cells were positive for surface markers such as CD90, CD73, CD105, CD44 and HLA ABC, while negative for CD14, CD19, CD34, CD45 and HLA DQDPDR. **a)** 10,000 events within the gated area P1 were acquired. **b)** The representative dot plot for the expression of each surface marker of hASCs. **c)** Mean expression of each surface marker of hASCs (n = 6).

3.3.2 Tri-lineage differentiation of hASCs

Upon adipogenic induction, cells presented the formation of lipid droplets which are positively stained by Oil red O. Real-Time PCR analysis revealed that cells after adipogenic induction expressed significantly ($p < 0.05$) higher gene expression levels of adipogenic markers (PPARG, FABP4 and LPL) than those before adipogenic induction (**Fig. 3.4a**). Upon osteogenic induction, many dark red regions stained by Alizarin red, indicating the presence of calcium deposits, were observed. In addition, cells after osteogenic induction expressed significantly ($p < 0.05$) higher gene expression levels of osteogenic markers (ALPL, RUNX2 and OSC) than those before osteogenic induction (**Fig. 3.4b**). Upon chondrogenic induction, histological section of cells showed the formation of proteoglycans which are stained positively by Alcian blue. Further, cells after chondrogenic induction expressed significantly ($p < 0.05$) higher gene expression levels of the chondrogenic markers ACAN, COL-2 and SOX-9 than those before chondrogenic induction (**Fig. 3.4c**). As cells before chondrogenic induction were incapable to form pellet in the absence of chondrogenic induction medium, cells in monolayer were used as negative control for Alcian Blue staining. As a result, the cells were not stained by Alcian Blue and only positively stained by nuclear fast red.

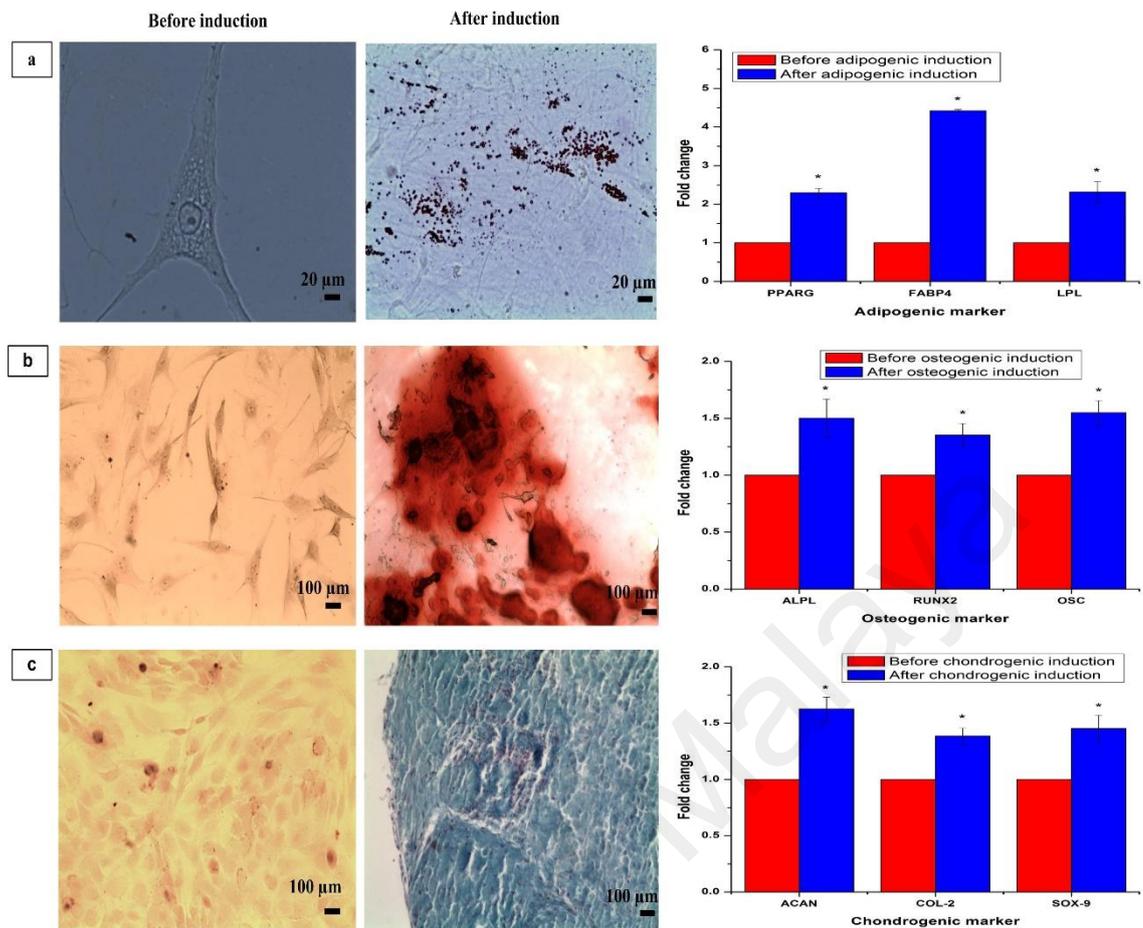


Figure 3.4. Tri-lineage differentiation of hASCs. **a)** Cells differentiated to adipogenic-like cells as indicated by lipid droplets formation (magnification: 400×) and a significant ($p < 0.05$) high gene expression level of adipogenic markers. No sign of lipid droplet was observed in the cells before adipogenic induction. **b)** Cells differentiated to osteogenic-like cells as indicated by calcium deposition (magnification: 100×) and a significantly ($p < 0.05$) higher gene expression level of osteogenic markers. No sign of calcium deposits was observed in the cells before osteogenic induction. **c)** Cells differentiated to chondrogenic-like cells as indicated by proteoglycan formation (magnification: 100×) and a significantly ($p < 0.05$) higher gene expression level of chondrogenic markers. No sign of proteoglycan was seen in the cells before chondrogenic induction.

3.4 Discussion

A minimal criteria has been proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy to define hMSCs (Dominici et al., 2006). First, hMSCs must be plastic-adherent when cultured in tissue culture flasks. In the present study, it was observed that cells isolated from human adipose tissues possess spindle shaped and adherent properties when cultured in plastic cell culture flasks. Second, hMSCs should highly express CD105, CD73, CD90 and CD44 while lacking expression of CD45, CD34, CD14, CD19 and HLA DQDPDR. CD105, CD73, CD90 and CD44 represent panels of surface marker which are usually expressed on the cells possessing mesenchymal phenotype, *e.g.*, hMSCs (Dominici et al., 2006). CD34 represents marker of endothelial cells and primitive hematopoietic cells (Lin et al., 1995), whereas CD45 is a leukocyte marker (Nakano et al., 1990). CD14 is usually expressed on monocytes and macrophages (Jersmann, 2005), whereas CD19 is a marker of B lymphocytes (Wang et al., 2012). HLA DQDPDR is not expressed on hMSCs unless they are stimulated by interferon- γ (Dominici et al., 2006). The results of immunophenotyping were in accordance with the second criteria of hMSCs, suggesting that most of the cells at passage 3 possess mesenchymal phenotype while lack of hematopoietic phenotype. In addition, cells expressed a high level of HLA ABC (only expressed by human nucleated cells including hMSCs) (Jones et al., 1988), excluding the presence of non-nucleated cells (*e.g.*, red blood cells) in culture. These findings indicate the presence of MSCs in culture.

Third, hMSCs have the ability to undergo trilineage differentiation (adipogenic, osteogenic and chondrogenic). Formation of lipid droplets is a main feature of adipogenic differentiation as lipid droplets are lipid-rich organelles which are found mostly in adipose tissue to regulate storage and hydrolysis of lipid (Ducharme & Bickel, 2008). In addition to histochemical staining, we also performed Real-Time PCR to verify the

differentiation by analyzing the gene expression of differentiation markers after differentiation induction. PPAR γ is an adipogenic marker which regulates adipogenic differentiation and maintains adipocyte phenotype (Kawai & Rosen, 2010), whereas LPL which acts as a catalyzer for hydrolyzing triglyceride, is an early marker that indicates the formation of adipocytes (Gonzales & Orlando, 2007). Further, FABP4 which is mainly expressed in adipocytes to facilitate metabolism and transport of fatty acids, acts as an intermediate marker of adipocyte differentiation (Scifres et al., 2011). Upon adipogenic induction, it was observed that cells display formation of lipid droplets and expressed high levels of adipogenic markers, suggesting cells possess adipogenic differentiation ability.

Calcium, which is produced and deposited to form a strong and dense bone tissue (Clarke, 2008), is a typical feature to indicate osteogenic differentiation. ALPL, an early indicator of osteogenic differentiation, is highly expressed upon active formation of bone to allow calcium and phosphate deposition for bone mineralization (Ding et al., 2009). RUNX2 is a transcription factor which regulates skeletal morphogenesis and osteoblastic differentiation (Caetano-Lopes et al., 2007), whereas OSC acts as a late marker of osteogenic differentiation which plays essential role in bone mineralization and matrix synthesis (Caetano-Lopes et al., 2007). Upon osteogenic induction, it was observed that cells display calcium deposition and expressed high levels of osteogenic markers, suggesting cells are capable to differentiate into osteogenic-like cells.

ACAN, a chondroitin sulfate proteoglycan, is a structural component of cartilage (Kiani et al., 2002) which can be stained by Alcian blue to indicate formation of chondrocytes. Further, ACAN represents a late chondrogenic marker which provides osmotic resistance to the cartilage to resist compressive forces (Chandran & Horkay,

2012), whereas SOX-9 is a transcription factor which regulates cartilage formation and maintain chondrocyte phenotype, acts as an early chondrogenic marker (Amarilio et al., 2007). COL-2, another late indicator of chondrocyte formation, provides tensile strength to the cartilage for resisting shearing force (Martel-Pelletier et al., 2008). Upon chondrogenic induction, it was observed that cells display formation of proteoglycan and expressed high levels of chondrogenic markers, indicating the chondrogenic differentiation potential of cells.

3.5 Conclusion

In conclusion, cultured passage 3 cells isolated from human adipose tissues showed a plastic-adherent property, expression of specific surface markers and trilineage differentiation, suggesting that these cells are hASCs. Therefore, hASCs at passage 3 can be used for further experiments.

CHAPTER 4: FUNCTIONALITY AND BIOSAFETY ASSESSMENT OF CRYOPRESERVED HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS

4.1 Introduction

Cryopreservation using a slow freezing method involves issue of freeze injury (*e.g.*, cell death) induced by extra- and intra-cellular ice crystal formation during the freezing process (Gao & Critser, 2000; Li et al., 2010; Samot et al., 2011). Therefore, it is essential to optimize the types and concentrations of CPAs used to preserve hASCs to avoid ice formation. In the present study, hASCs were cryopreserved for 3 months in various combinations of 3 generally used CPAs, including DMSO, trehalose and FBS.

Among the CPAs, 10% DMSO and FBS is the most widely used for cryopreservation of hASCs (De Rosa et al., 2009; Gonda et al., 2008; James et al., 2011; Liu et al., 2008). However, many adverse effects, including respiratory depression and neurotoxicity, have been caused by the clinical use of cells preserved in 10% DMSO (Benekli et al., 2000; Windrum & Morris, 2003), resulting a need to reduce the concentration of DMSO in cryopreservation medium to decrease the risk of such adverse effects. Moreover, FBS should be reduced or excluded in cryopreservation medium due to issues such as transmission of pathogens or xenogeneic immune response in recipient following the transplantation of cryopreserved cells (Tuschong et al., 2002; Zambelli et al., 1998). Although significant efforts have been put to explore new CPAs for preserving hASCs to replace DMSO, but they appeared to be less effective than DMSO. For instance, CPAs such as methylcellulose and PVP, were found to be less effective than DMSO in terms of sustaining the viability of hASCs (Thirumala et al., 2010a; Thirumala et al., 2010b). To date, there is still an unmet need for an alternative cryopreservation medium to completely replace FBS and DMSO. Therefore, trehalose at a concentration of 0.25 M

has been suggested, as it was found to be effective in preserving functional properties and viability of adipocytes from human adipose tissue, a source which hASCs are isolated from (Pu et al., 2005).

Besides evaluation of functional properties, it is also essential to assess the biosafety of cryopreserved hASCs. Recently, it has been reported that cells (*e.g.*, embryonic stem cells and lymphocytes) cryopreserved in DMSO may display chromosome abnormality and changes in telomere length, which in turn might lead to tumour formation (Diaferia et al., 2008; Jenkins et al., 2012; Yong et al., 2015b). These concerns have established the need for assessment of tumourigenic potential and chromosomal abnormality in cryopreserved hMSCs including hASCs prior to clinical applications. Through karyotyping, it has been reported that there are no alteration in chromosome structures and numbers in cryopreserved hMSCs, indicating no chromosomal abnormality in hMSCs after cryopreservation (Angelo et al., 2012; de Lima Prata et al., 2012; Luetzkendorf et al., 2015; Miranda-Sayago et al., 2012). However, the tumourigenic potential of hMSCs cryopreserved in various CPAs has not been evaluated yet.

Herein, quantitative assessments were conducted to accurately compare the effect of various CPAs on hASCs in terms of cell phenotype, viability, proliferation potential, differentiation (including adipogenic, osteogenic and chondrogenic) potential, and stemness. Further, the tumourigenic potential of hASCs preserved in various CPAs were evaluated, in terms of changes in tumour suppressor marker and human telomerase reverse transcriptase (hTERT) expression, telomere length and telomerase activity, as well as the analysis of DNA damage and p53 mutation. Based on the comparison, the ideal CPA to preserve hASCs effectively for clinical applications was determined. The

findings from this study would impact the establishment of a standardized cryopreservation protocol of hASCs and therapeutic use of long-term cryopreserved hASCs in future clinical applications.

4.2 Materials and methods

4.2.1 Isolation and culture of hASCs

Isolation and culture of hASCs were performed using the methods as described in section 3.2.1.

4.2.2 Cryopreservation

About 1×10^6 hASCs at passage 2 were trypsinized using trypsin following method as described in the section 3.2.2, and then suspended with 1 mL DMEM/Ham F-12 containing CPA (which was kept at $-20\text{ }^{\circ}\text{C}$ followed by thawing at room temperature) in a cryovial. The CPAs tested were: 1) 0.25 M trehalose (Sigma); 2) 5% DMSO (Sigma); 3) 10% DMSO; 4) 5% DMSO + 20% FBS; 5) 10% DMSO + 20% FBS and 6) 10% DMSO + 90% FBS. All cryovials were moved into “Mr. Frosty (Nalgene), a freezing container, and kept at $-80\text{ }^{\circ}\text{C}$ overnight. All cryovials were then moved to liquid nitrogen ($-196\text{ }^{\circ}\text{C}$) the next day and stored for 3 months. Procedures of cryopreservation were described in **Fig. 4.1**. After 3 months, the frozen hASCs were thawed rapidly at $37\text{ }^{\circ}\text{C}$ in a water bath, washed with complete cell culture medium and centrifuged to remove the CPAs. Finally, hASCs were resuspended with complete cell culture medium and subcultured to passage 3 prior to be used for all assays.

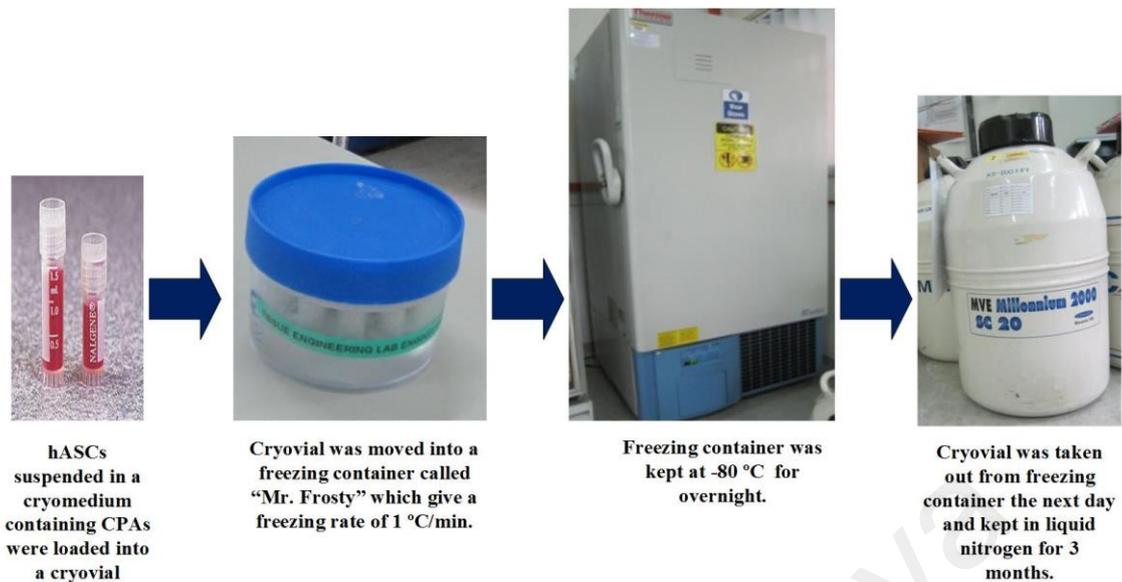


Figure 4.1. A flow chart demonstrates the procedures of cryopreservation of hASCs using a slow freezing method.

4.2.3 Cell phenotyping

Cell phenotyping of cryopreserved hASCs was performed following the method as described in section 3.2.2.

4.2.4 Cell proliferation assay (Resazurin red reduction assay and population doubling time)

hASCs were plated with cell density of 2×10^4 cells / cm^2 into the 24 well plate and left overnight in an incubator for cell attachment. Resazurin red reduction assays were performed after 24 hrs (day 1), and on days 3, 7, 10 and 14. hASCs were incubated with Resazurin (Sigma) working solution (14 mg/L Resazurin in PBS)) for 3 hours prior to absorbance measurement. A microplate reader (Fluostar Optima, BMG Labtech, Germany) was used to measure the absorbance of Resazurin at 570 nm and 595 nm. The percentage of Resazurin reduction of hASCs on each day was calculated and analyzed. In the meantime, a standard curve of percentage of Resazurin reduction versus cell number was generated (**Appendix C**). Briefly, cells were seeded into a 24 wells plate with a series of cell numbers as follows: 1.25×10^4 , 2.5×10^4 , 5.0×10^4 , 7.5×10^4 , $10 \times$

10^4 and 12.5×10^4 cells per well. After overnight incubation for cell attachment, the percentage of Resazurin reduction on each well was calculated. Finally, cell number on days 1, 3, 7, 10 and 14 respectively was determined.

On the other hand, population doubling time (PDT) of hASCs at passage 3 were calculated using the formulas $PD = \log_{10} [(A)/(B)]/\log_{10} 2$ and $PDT = t/PD$, where A represents number of cells harvested; B represents number of cells seeded; and t represents the time between seeding at the previous passage and harvest at the subsequent passage (Luetzkendorf et al., 2015).

4.2.5 Cell differentiation potential assay

The tri-lineage differentiation potential (adipogenesis, osteogenesis and chondrogenesis) of cryopreserved hASCs was performed using methods as described in section 3.2.3.

4.2.6 Cell viability assay

Cell viability of hASCs post-thawing was determined using 0.4% trypan blue solution (Gibco) and haemocytometer (Neubauer, USA). The total number of dead cells (cells stained with trypan blue) and live cells (bright cells without trypan blue stain) was counted under a light microscope (Eclipse TS100, Nikon, USA). Cell viability of hASCs preserved in various CPAs was calculated using the formula as follows.

$$\text{Cell viability} = \frac{\text{Total of live cell}}{\text{Total of live cell} + \text{dead cell}} \times 100\%$$

4.2.7 RNA extraction, cDNA synthesis and Real-Time PCR

RNA extraction, cDNA synthesis and Real-Time PCR were conducted in accordance with the methods as described in section 3.2.4. Genes to be evaluated include differentiation markers (LPL, FABP4, PPARG, Runx2, OSC, ALP, SOX9, COL-2, and ACAN),

stemness markers such as SOX-2 (Hs01053049_s1), REX-1 (Hs01938187_s1), NANOG (Hs01060663_m1) and OCT-4 (Hs04260367_g1), tumour suppressor genes such as RB1/pRb (Hs01078066_ml), CDKN2A/p16 (Hs00923894_ml), CDKN1A/p21 (Hs00355782_m1), TP53/p53 (Hs00153349_m1), and hTERT (Hs00972656_ml). The housekeeping gene used for normalization was GAPDH (Hs99999905_m1). The gene expression level of the control group (fresh hASCs or hASCs before differentiation) was normalized to 1. Data were expressed as fold change in relative to the control.

4.2.8 Telomerase Assay

TeloTAGGG PCR ELISAPLUS kit (Roche Applied Science, Indianapolis, USA) was used to determine the telomerase activity of hASCs, based on telomere repeat amplification protocol (TRAP) recommended by manufacturer. Briefly, 2×10^5 hASCs were suspended in 200 μ L lysis reagent followed by incubation on ice for 30 mins for cell lysis. The lysates obtained were subjected to subsequent PCR using reagents and primers provided in the kit. The thermal cycling profile of PCR is shown in **Table 4.1**. PCR products were denatured at room temperature for 10 mins followed by hybridization with digoxigenin-(DIG)-labelled, telomeric repeat-specific detection probes at 37°C with 300 rpm agitation for 2 hrs. These hybridized products were immobilized to a streptavidin-coated microplate via the biotin conjugated with PCR products. The hybridized products were incubated in a solution containing anti-DIG antibody, which is tagged with horseradish peroxidase (HRP) enzyme at room temperature for 30 mins with agitation followed by incubation in 3', 3', 5', 5'-Tetramethylbenzidine (TMB) substrate solution at room temperature for 20 mins with agitation. The color of solution changed from blue to yellow when stop reagent was added to stop the activity of HRP enzyme. By using a microplate reader (Fluostar Optima, BMG Labtech), the absorbance of the solution at 450

nm was measured. Relative telomerase activity (RTA) of hASCs was calculated using the formula given by the manufacturer.

Table 4.1: Thermal cycling profile for PCR in telomerase assay

Process	Conditions (temperature, duration)
Primer elongation	25 °C, 20 mins
Telomerase inactivation	94 °C, 5 mins
Amplification:	
i. Denaturation	94 °C, 30 secs
ii. Annealing	50 °C, 30 secs
iii. Polymerization	72 °C, 90 secs
	30 cycles
Hold	72 °C, 10 mins

4.2.9 Genomic DNA extraction

PureLink® Genomic DNA Mini Kit (Invitrogen), a spin column-based DNA extraction kit, was used to extract genomic DNA from hASCs according to manufacturer's instruction. Briefly, hASCs were suspended in 200 µL PBS followed by the addition of 20 µL proteinase K (for digestion of protein), 20 µL RNase A (for removal of RNA) and 200 µL lysis/binding buffer. Cell digestion was performed in a water bath at 55 °C for 10 mins. The lysates obtained were mixed with 200 µL absolute ethanol and subjected to DNA binding using a spin column, which contains a silica-based membrane to which only DNA can bind. DNA was washed with washing buffers and eventually eluted in an elution buffer into a 1.5 mL microcentrifuge tube. Genomic DNA obtained was used for telomere length analysis and p53 mutation detection.

4.2.10 Telomere length analysis

A TeloTAGGG Telomere Length Assay kit (Roche) was performed to determine the telomere length of hASCs in accordance with the manufacturer's recommendation. Briefly, 1 µg genomic DNA of hASCs was digested with a solution mixture of Hinf I/

RSA I (restriction enzymes) at 37 °C for 2 hrs. These enzymes only digest non-telomeric DNA to low molecular weight fragments and do not digest telomeric DNA. Following DNA digestion, these DNA were subjected to 0.8% (w/v) agarose gel electrophoresis in 1× Tris-acetate-EDTA buffer (Roche) to separate telomeric DNA from the other DNA fragments. These DNA fragments were transferred to a positively charged nylon membrane (Roche) by Southern blotting overnight. Then, the transferred DNA fragments on the membrane were fixed by baking the membrane at 120 °C for 20 mins. These DNA fragments were subjected to DNA hybridization using a solution containing DIG-labelled probes specific for telomeric repeats with gentle agitation at 42 °C for 3 hrs. The hybridized DNA on the membrane was washed with washing buffers followed by incubation in a solution containing anti DIG-specific antibody tagged with alkaline phosphatase enzyme at room temperature for 30 mins with gentle agitation. Following washing, 40 drops of substrate solutions were added and spread evenly on the wet membrane followed by incubation at room temperature for 5 mins. Alkaline phosphatase enzyme metabolized CDP-Star which is contained in a substrate solution to produce chemiluminescence signals. Chemiluminescence signals produced in this assay were detected by a gel documentation imaging system (Vilber Lourmat, Marne la Vallee, France). The terminal restriction fragment (TRF) length of hASCs was determined by comparing the signals relative to a molecular weight standard.

4.2.11 p53 nucleotide sequence mutation detection

Briefly, Platinum® Taq DNA Polymerase (Invitrogen) was used to perform PCR on genomic DNA of hASCs in accordance to the manufacturer's instructions. The sample for PCR was prepared in a 0.2 mL tube, as shown in **Table 4.2** and then the tube was placed into a thermal cycler for PCR. The details of p53 primers (Biobasic, Ontario,

Canada) and thermal cycling profile for PCR were described in **Table 4.3** and **Table 4.4** respectively.

Table 4.2: Sample preparation for PCR in p53 nucleotide sequence mutation detection assay

Component	Volume (μL)
10 \times PCR buffer	5.0
50 mM MgCl_2	1.5
10 mM dNTP mix	1.0
<i>Taq</i> DNA polymerase	0.2
10 μM forward primer	1.0
10 μM reverse primer	1.0
Genomic DNA (up to 500 ng)	Up to 40.3 μL
Nuclease-free water	Top up to a final reaction volume of 50 μL
Total per tube	50

Table 4.3: p53 primer sequences, primer annealing positions, and expected length of PCR products

p53 Primer	Sequence ¹	Annealing position	Product length (bp)
Exon 5 forward	5'-gctgccgtgtccagttgct-3'	12980 - 12999	294
Exon 5 reverse	5'-ccagccctgtcgtctctcca-3'	13254 - 13273	
Exon 6 forward	5'-ggcctctgattcctcactga-3'	13290 - 13309	199
Exon 6 reverse	5'-gccactgacaaccaccctta-3'	13469 - 13488	
Exon 7 forward	5'-tgccacaggttcccccaagg-3'	13943 - 13962	196
Exon 7 reverse	5'-agtgtgcagggtggcaagtg-3'	14119 - 14138	
Exon 8 forward	5'-ccttactgcctcttgcttct-3'	14413 - 14432	225
Exon 8 reverse	5'-ataactgcacccttggcttc-3'	14618 - 14637	

¹European Molecular Biology Laboratory (EMBL) accession X54156.1 (p53 gene sequence)

Reproduced from Yong et al. (2016)

Table 4.4: Thermal cycling profile for PCR in p53 nucleotide sequence mutation detection assay

Process	Conditions (temperature, duration)
Initial denaturation	94 °C, 3 mins
Amplification:	
i. Denaturation	94 °C, 30 secs
ii. Annealing	55 °C, 30 secs
iii. Extension	72 °C, 1 min
} 35 cycles	

About 10 μ L PCR products were subjected to 4% gel agarose electrophoresis for 45 mins to confirm the existence of p53 nucleotide sequence in PCR product by comparing the PCR product length to a molecular weight standard (**Fig. 4.2**). PCR products were then purified using a Purelink® PCR Purification Kit (Invitrogen) via a spin column-based method. The spin column contains a silica-based membrane which is selectively bound to double stranded DNA. Briefly, 40 μ L PCR products mixed with binding buffers were loaded into a spin column followed by centrifugation at room temperature at $10,000 \times g$ for 1 min. Following washing and centrifugation to remove the impurities and residual washing buffer, purified PCR products were eluted in 50 μ L elution buffer (10 mM Tris-HCl, pH 8.5) into a 1.5 mL microcentrifuge tube.

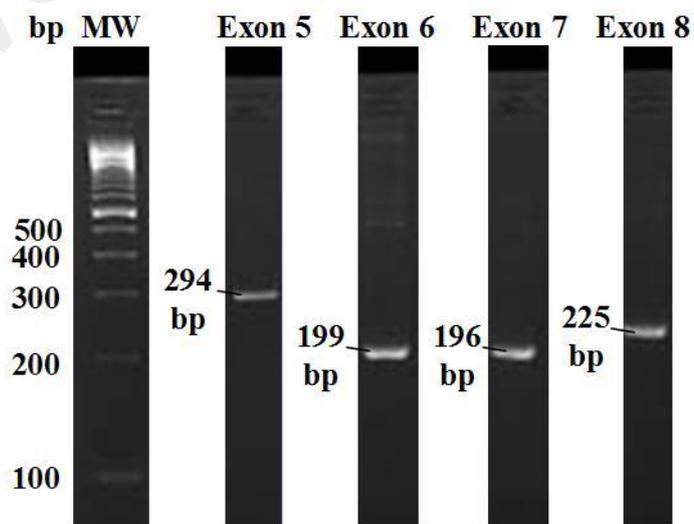


Figure 4.2. Electrophoresis revealed that exons 5 to 8 of p53 nucleotide sequences with the correct base pair (bp) respectively, were amplified successfully using PCR. MW: molecular weight standard.

A TOPcloner™ TA core kit (Enzymomics, Daejeon, Korea) was used to clone the purified PCR products in accordance with the manufacturer's instruction. Briefly, the purified PCR products were ligated with vectors (Plasmid DNA) followed by incubation at 4 °C for overnight. Sample preparation for DNA ligation is shown in **Table 5.5**. After being held overnight, the ligated DNA was inserted into DH5α chemically competent *Escherichia coli* (Enzymomics) cells via a process called transformation. Briefly, 6 μL ligated DNA was mixed with a solution containing DH5α chemically competent *E. coli* cells in a 1.5 mL microcentrifuge tube followed by incubation on ice for 30 mins. Then, the tube was subjected to heat (a transformation method called heat-shock) in a water bath at 42 °C for 30 secs followed by cooling on ice for 2 mins. About 400 μl SOC media was added and the tube was then incubated in an incubator shaker at 37 °C for 1 hr to ensure maximum recovery of DH5α chemically competent *E. coli* cells. About 200 μL solution containing *E. coli* cells were inoculated onto a pre-warmed Lucia Broth (LB) agar (Sigma) mixed with 20 mg/ml X-gal (Corning Cellgro Manassas, USA), 500 mM IPTG (Corning Cellgro) and 50 mg/ml ampicillin (Sigma). After being kept overnight at 37 °C, examination of colonies was performed. Positive clones, colonies containing ligated DNA (white colonies) (**Fig. 4.3**), were picked and inoculated into a 5 mL pre-warmed LB broth (Sigma) mixed with 100 μg/mL ampicillin, followed by incubation overnight at 37 °C.

Table 4.5: Sample preparation for DNA ligation

Component	Volume (μl)
6 × TOPcloner™ buffer	1
Vector (pTOP TA V2) (10 ng/μl)	1
PCR products	Up to 4 μl
Nuclease-free water	Top up to a final reaction volume of 6 μl
Total per tube	6

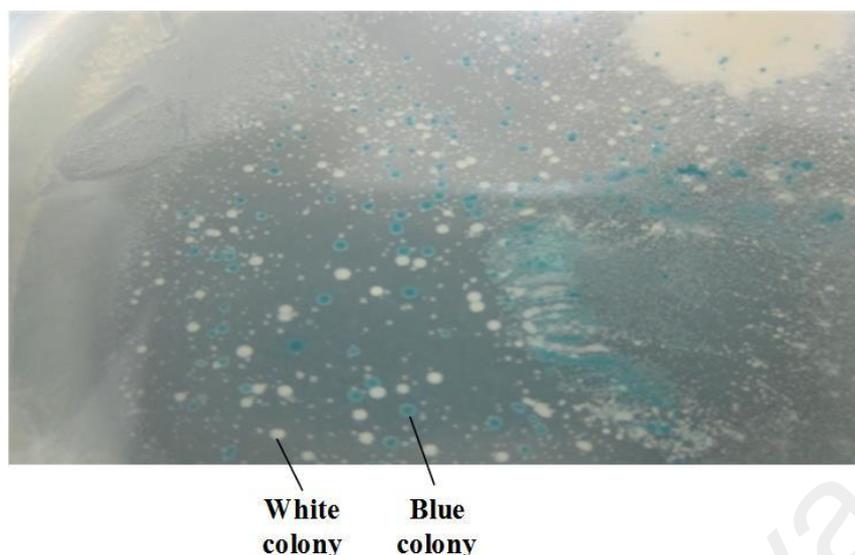


Figure 4.3. Blue/white colony screening. White colony represent the positive clone which contains plasmid DNA ligated with PCR products of p53, while blue colony represent the negative clone which contains only plasmid DNA without PCR products of p53.

After being held overnight, the positive clones were confirmed with PCR using specific primers for p53 exons respectively (**Table 4.3**) with the PCR profile shown in **Table 4.4**, followed by 4% agarose gel electrophoresis (**Fig. 4.2**). Following confirmation of positive clones, a Purelink® Quick Plasmid Miniprep Kit (Invitrogen) was used to extract plasmid DNA from *E. coli* cells via a spin column method. A spin column contains a silica-based membrane which selectively binds to plasmid DNA. Briefly, 5 mL of the overnight LB-culture containing *E. coli* cells were centrifuged to obtain cell pellets followed by resuspension in 250 μ l resuspension buffer containing RNase A in a 1.5 mL microcentrifuge tube for the removal of RNA. About 250 μ L lysis buffer was added into the tube and the tube was then incubated at room temperature for 5 mins to lyse the cells. The lysates obtained were mixed with 350 μ L precipitation buffer and then centrifuged at $12,000 \times g$ at room temperature for 10 mins. Supernatant was loaded into a spin column followed by centrifugation at $12,000 \times g$ at room temperature for 1 min. Following washing and centrifugation to remove impurities and residual washing buffers, plasmid DNA was eluted in 75 μ L Tris-EDTA (TE) buffer into a 1.5 mL microcentrifuge tube.

To confirm the existence of p53 nucleotide sequences in Plasmid DNA, 10 μ L Plasmid DNA was subjected to EcoR1 (Invitrogen) restriction followed by 4% gel electrophoresis (**Fig. 4.4**). Finally, the rest of the plasmid DNA was sent to Bio Basic Inc, Canada, for DNA sequencing. The nucleotide sequences of plasmid DNA were aligned with the wild-type p53 DNA sequences to detect p53 mutation, using online software called Basic Local Alignment Search Tool (BLAST).

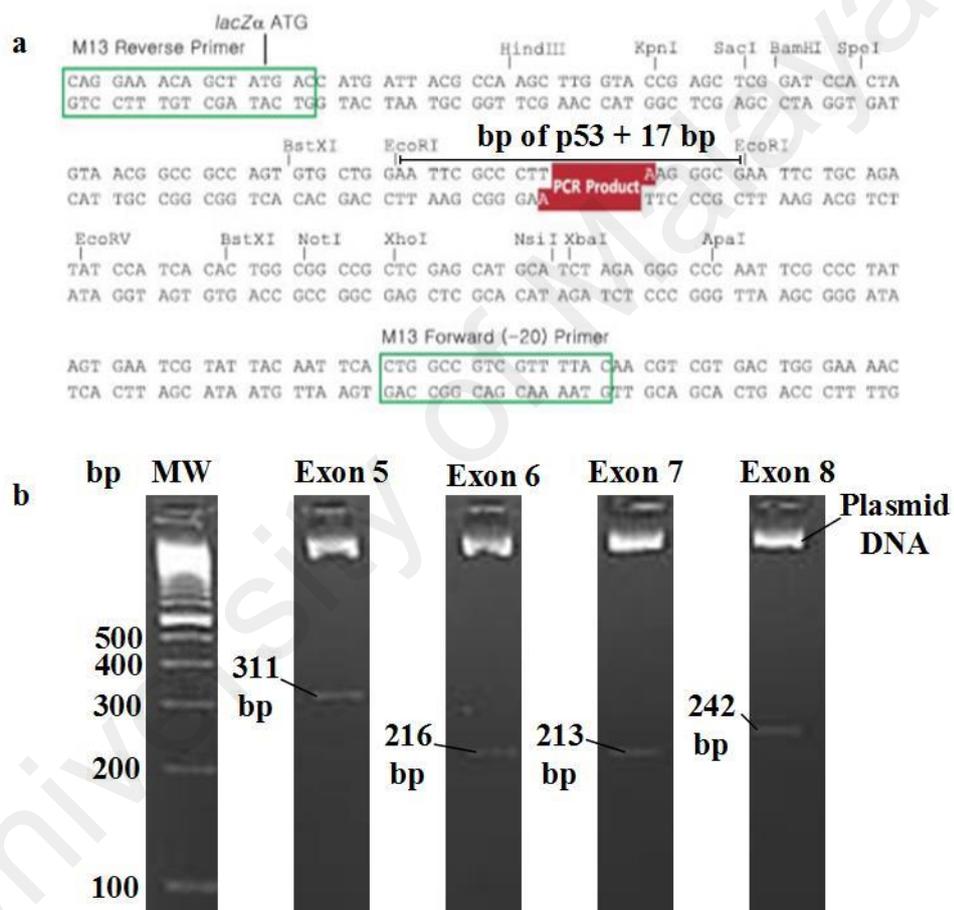


Figure 4.4. EcoR1 restriction. In brief, 10 μ L of plasmid DNA mixed with 1 μ L restriction enzyme EcoR1, 2 μ L reaction buffer and 7 μ L deionized water were incubated at 37 $^{\circ}$ C for 1 hr. **a)** EcoR1 was used to cut plasmid DNA at EcoR1 restriction site to release the p53 nucleotides. **b)** The resulting products are p53 nucleotides + the remaining 17 base pair (bp) of plasmid DNA which were not been cut. MW: molecular weight standard.

4.2.12 Comet assay

A Comet assay was performed in an alkaline condition using a Comet Assay Kit (Trevigen, Gaithersburg, MD, USA) in accordance with the manufacturer's recommendation. Briefly, 1×10^5 hASCs in 50 μ L PBS were mixed with 500 μ L pre-warmed low melting point agarose. About 50 μ L cell-laden agarose was spread evenly onto a frosted microscope slide followed by gelling at 4 °C for 30 mins in the dark. All of the following steps were carried out in the dark. The slide was incubated in a pre-cooled lysis solution at room temperature for 45 mins for cell lysis to generate DNA. The slide was then incubated in an alkaline unwinding solution at room temperature for 20 mins followed by electrophoresis using a pre-cooled alkaline electrophoresis solution for 30 mins. The slide was washed twice with deionized water for 5 mins and then 70% alcohol for 5 mins. Following drying, DNA in the gel was stained with ethidium bromide (Invitrogen) at room temperature for 30 mins followed by observation using an inverted fluorescence microscope (Nikon ECLIPSE TI-S, Tokyo, Japan). The images of DNA were then captured by a digital camera connected to the inverted fluorescence microscope. A software called Open Comet (Gyori et al., 2014) was used to analyze 100 nuclei for each sample. The parameters tested include tail moment, tail DNA % and tail length.

4.2.13 Statistical analysis

One-Way ANOVA with tukey post hoc test was employed to compare data among cryopreserved and fresh hASC groups. Data before and after the differentiation induction in the gene expression study were compared using a paired *t*-test. Each datum was expressed as mean \pm standard error of mean of six different donors ($n = 6$). Each experiment was conducted in triplicates per donor. Statistical significance was accepted at $p < 0.05$.

4.3 Results

4.3.1 The effect of cryopreservation on phenotype of hASCs

To evaluate the effect of cryopreservation on the phenotype of hASCs, microscopic observation and flow cytometry assessment were performed. Through microscopic observation, it was found that hASCs preserved in various CPAs and fresh hASCs (non-cryopreserved hASCs at passage 3) displayed spindle or fibroblast-like shape and adherent properties (**Fig. 4.5**). These results indicate that there is no alteration in the morphology of cryopreserved hASCs.

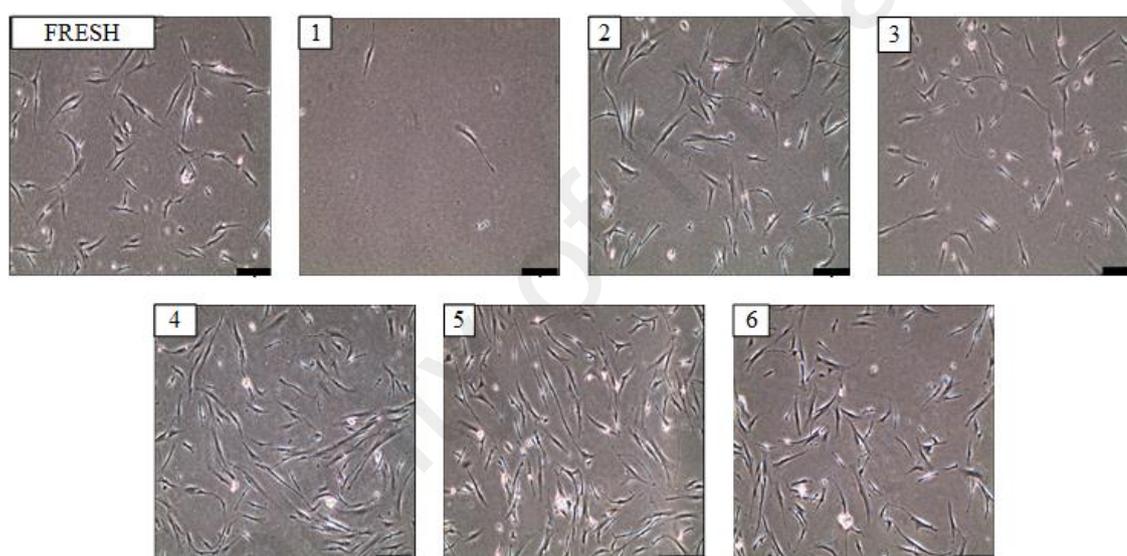


Figure 4.5. Morphology of hASCs was maintained following cryopreservation (magnification 100 ×; scale bar: 100 μm). Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

Flow cytometry analysis revealed that cryopreserved and fresh hASCs are negative for CD14, CD19, CD34, CD45 and HLA DRDPDQ while positive for CD90, HLA ABC, CD44, CD105 and CD73 (**Fig. 4.6**). These results are in accordance with the findings reported by Liu *et al.* (2008) and Gonda *et al.* (2008) Further, there was no significant ($p < 0.05$) changes in the expression of all surface markers among hASCs preserved in various CPAs and fresh hASCs (**Fig. 4.6**), indicating cryopreserved and fresh

hASCs expressed a similar pattern of cell surface markers. Overall, cryopreservation process (freezing and thawing) and the use of CPAs do not affect the phenotypes of hASCs.

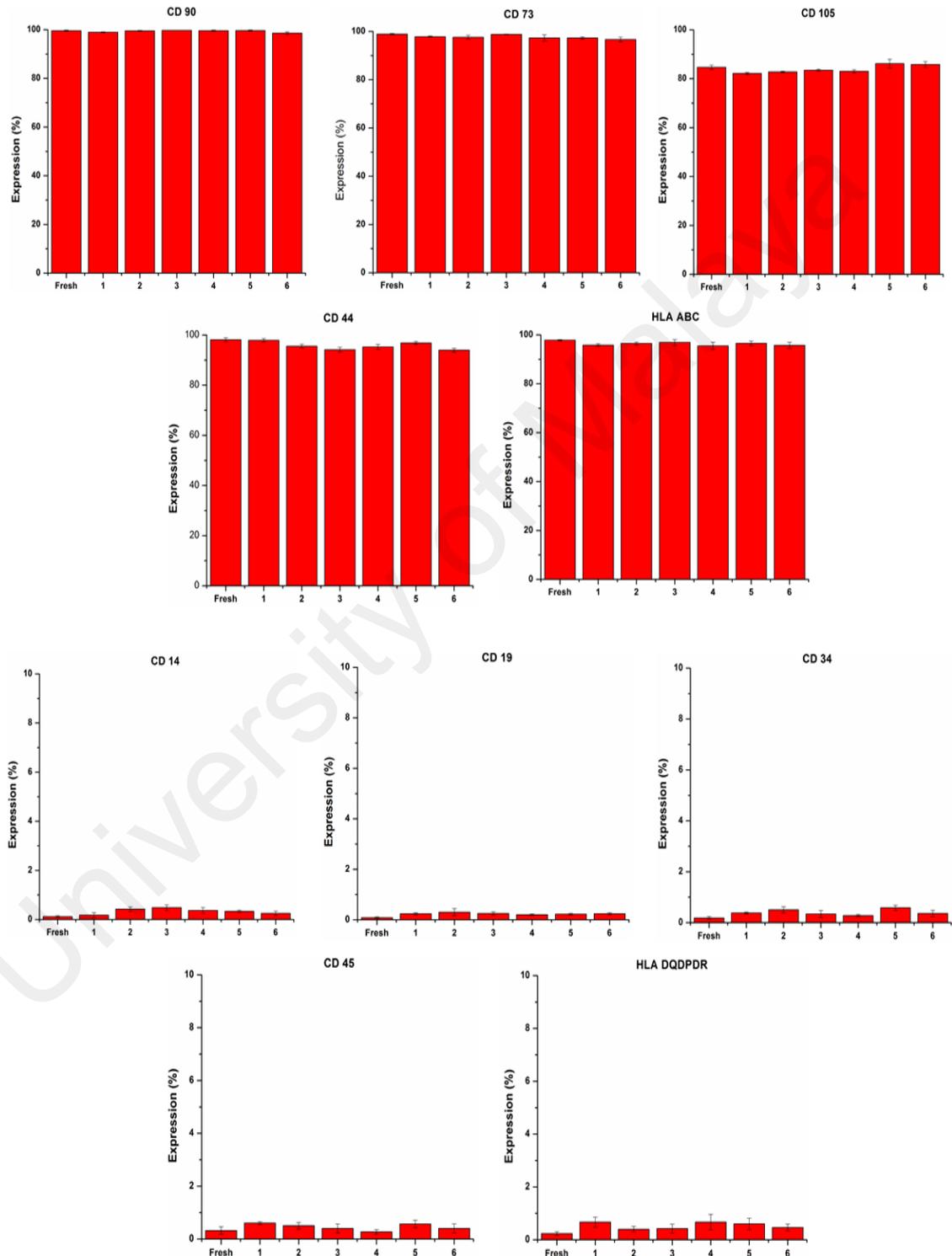


Figure 4.6. Cryopreservation maintain surface markers expression of hASCs. Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.2 The effect of cryopreservation on viability of hASCs

To determine the viability of hASCs preserved in various CPAs, a trypan blue exclusion assay was performed. hASCs preserved in 0.25 M trehalose demonstrated the significantly lowest cell viability ($p < 0.05$, $11.93\% \pm 0.96$) (**Fig. 4.7**). The viability of hASCs preserved in DMSO was significantly ($p < 0.05$) higher compared than those preserved in trehalose, indicating the relatively high efficiency of DMSO in sustaining the survival rate of hASCs throughout the cryopreservation process. Interestingly, it was found that cryopreservation medium containing a reduced concentration of DMSO without FBS (5% DMSO) maintained a high viability of hASCs ($75.96\% \pm 2.62$), as compared ($p > 0.05$) to those preserved in 10% DMSO and 90% FBS (standard cryopreservation medium) ($81.28\% \pm 2.34$).

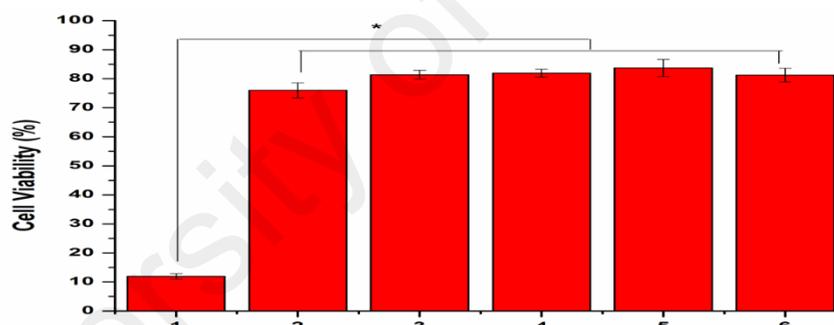


Figure 4.7. The effect of cryopreservation on the viability of hASCs. Cell viability of hASCs preserved in 5% dimethylsulfoxide (DMSO) is similar to those preserved in 10% DMSO + 90% fetal bovine serum (FBS) (standard cryopreservation medium), and significantly ($p < 0.05$) higher than those preserved in 0.25 M trehalose ($*p < 0.05$ relative to 0.25 M trehalose). Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% DMSO; 3) 10% DMSO; 4) 5% DMSO + 20% FBS; 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.3 The effect of cryopreservation on proliferation of hASCs

To determine the proliferation capacity of hASCs preserved in various CPAs, a Resazurin reduction assay was performed and their population doubling time was determined. It was found that hASCs preserved in various CPAs display a similar cell number ($p > 0.05$) to fresh hASCs from day 1 until day 14 (**Fig. 4.8a**). In addition, it was found that population

doubling time of hASCs preserved in various CPAs is similar ($p>0.05$) to that of fresh hASCs (Fig. 4.8b), indicating that cryopreserved and fresh hASCs have a similar proliferation rate. These findings show that the proliferative potential of hASCs is maintained following long-term cryopreservation, as supported by De Rosa *et al.* (2009) and Gonda *et al.* (2008).

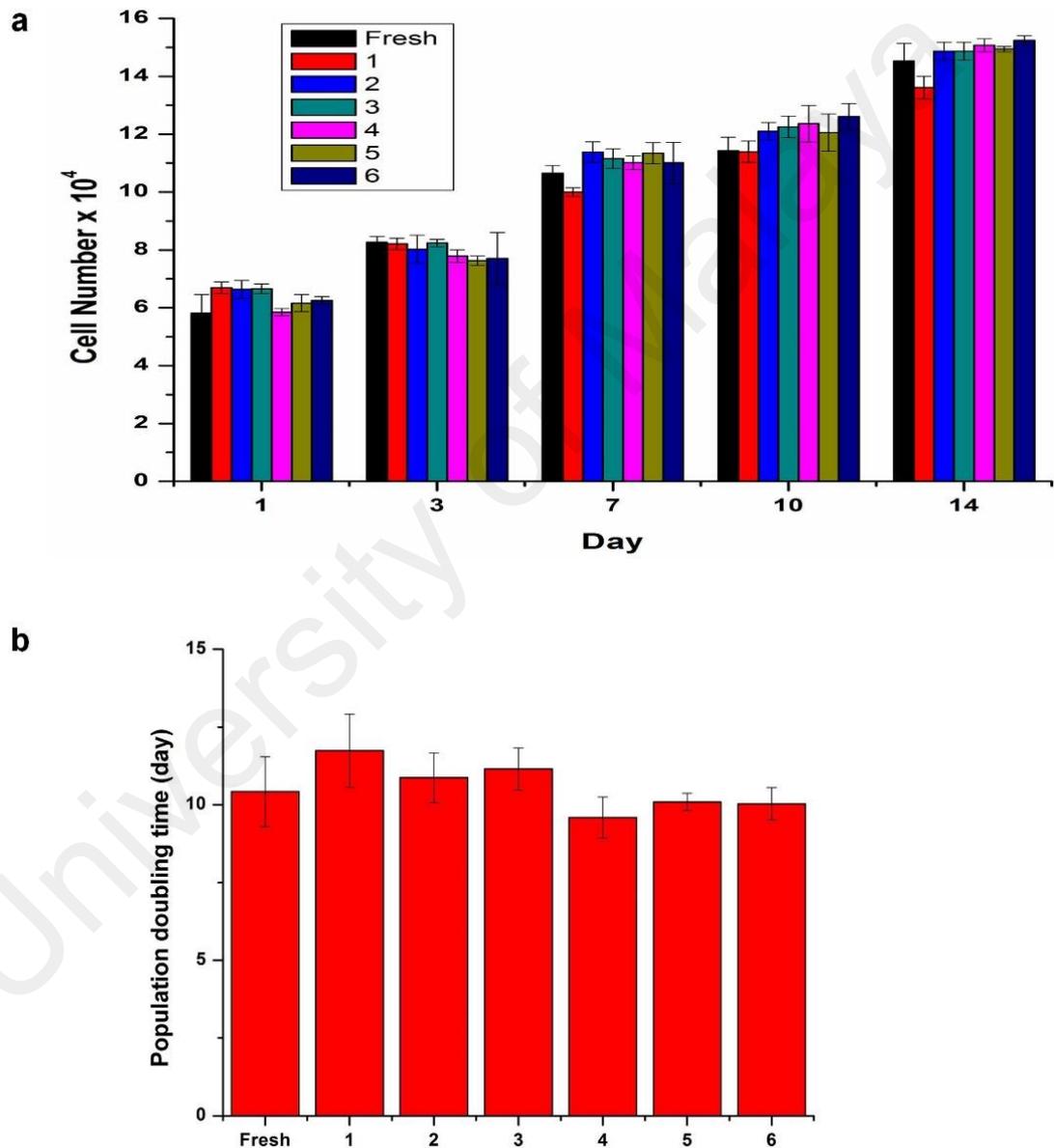


Figure 4.8. Proliferation rate of hASCs was maintained following cryopreservation. a) Fresh and cryopreserved hASCs demonstrated a similar cell number cultured from day 1 to day 14. b) A similar population doubling time was observed in fresh and cryopreserved hASCs. Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% DMSO; 3) 10% DMSO; 4) 5% DMSO + 20% FBS; 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.4 The effect of cryopreservation on adipogenic potential of hASCs

To evaluate the adipogenic potential of hASCs preserved in various CPAs, Oil red O staining and adipogenic gene expression analysis were conducted. It was observed that cryopreserved and fresh ASCs after adipogenic induction showed the formation of round lipid droplets that are positively stained by Oil red O (**Fig. 4.9a**), which is in accordance with the findings reported by Thirumala *et al.* (2010b) and Gonda *et al.* (2008). Further, it was found that both cryopreserved and fresh hASCs display significantly increased ($p < 0.05$) expression levels of adipogenic markers (LPL, FBP4 and PPAR- γ) upon adipogenic induction (**Fig. 4.9b**). These results show that the adipogenic potential of hASCs is maintained after cryopreservation. It was observed that fresh hASCs and hASCs preserved in various CPAs express similar ($p > 0.05$) expression levels of adipogenic markers following adipogenic induction, indicating that they have a similar adipogenic capacity.

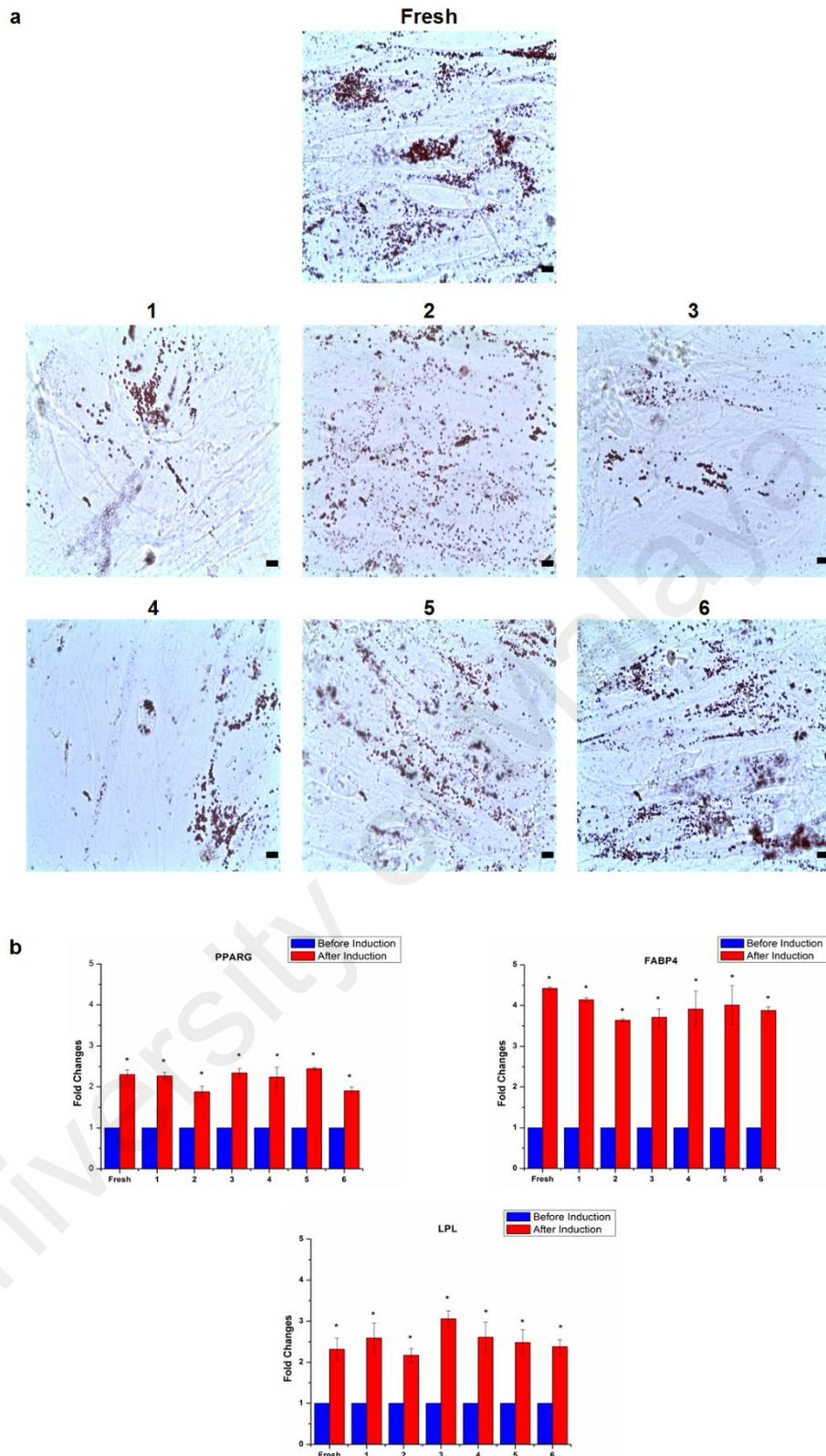


Figure 4.9. Adipogenic potential of hASCs was maintained following cryopreservation. (a) Adipogenesis evaluated by Oil Red O staining (magnification 400×) (scale bars: 20 μm.). (b) hASCs preserved in various CPAs expressed similar level of adipogenic markers (PPAR-γ, FABP4 and LPL) compared to fresh hASCs ($*p < 0.05$ relative to before induction). Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.5 The effect of cryopreservation on osteogenic capacity of hASCs

To assess the osteogenic potential of hASCs preserved in various CPAs, Alizarin red staining and osteogenic gene expression analysis were performed. Many dark red regions stained by Alizarin red were observed, indicating the presence of calcium deposits in cryopreserved and fresh ASCs upon osteogenesis (**Fig. 4.10a**). Similar findings have been reported in literature (Gonda et al., 2008; Liu et al., 2008; Thirumala et al., 2010b). Further, it was found that both cryopreserved and fresh hASCs display significantly elevated ($p < 0.05$) expression levels of osteogenic markers (RUNX2, OSC and ALPL) following the osteogenic induction (**Fig. 4.10b**). These results show that cryopreservation maintains the osteogenic potential of hASCs. Further, similar ($p > 0.05$) expression levels of osteogenic markers among hASCs preserved in various CPAs and fresh hASCs after osteogenic induction were observed, suggesting that they possess similar osteogenic potential.

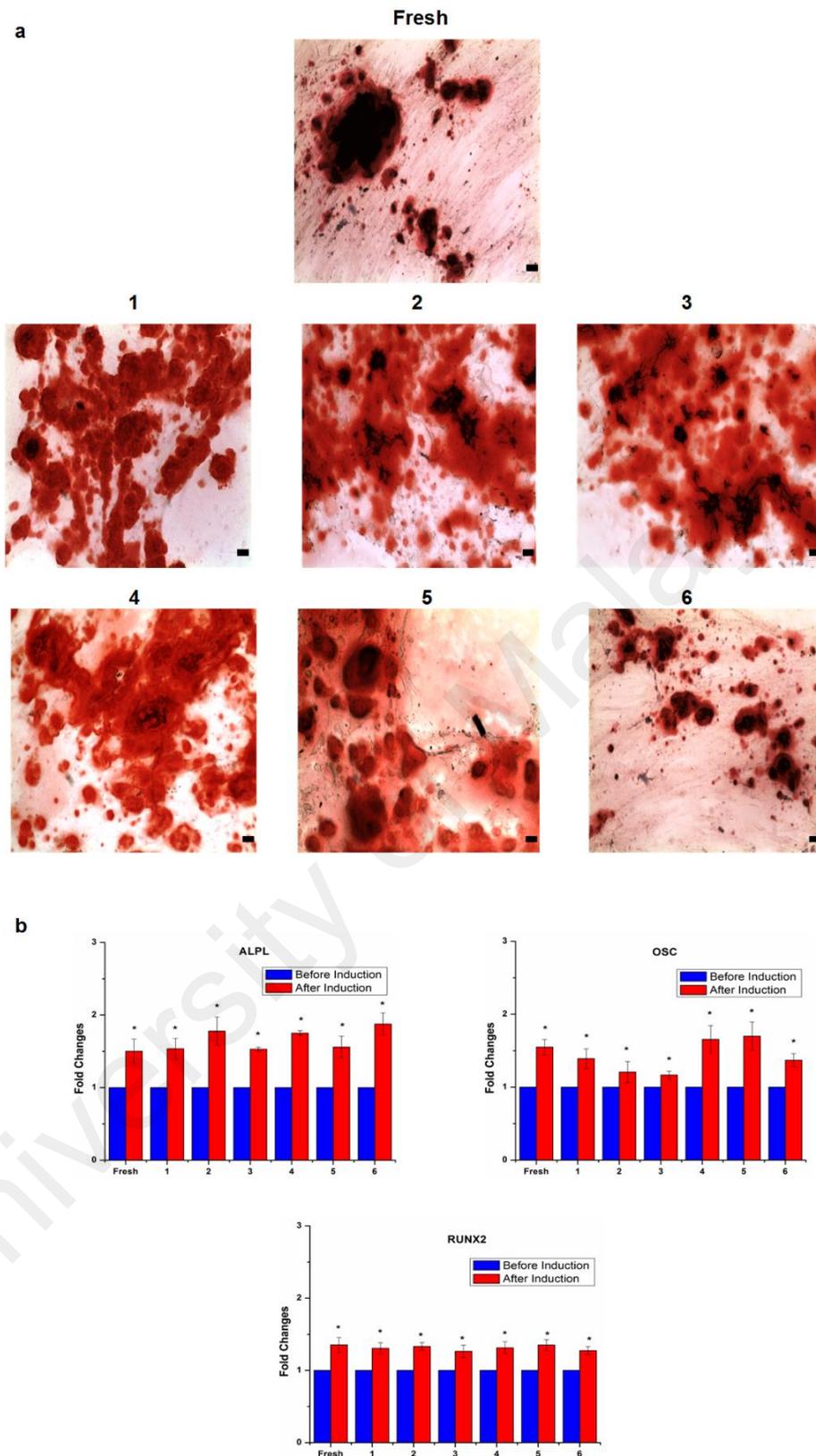


Figure 4.10. Cryopreservation maintained osteogenesis of hASCs. a) Osteogenesis assessed by Alizarin red staining (magnification 100×) (scale bars: 100 μm). **b)** Similar osteogenic gene (ALPL, OSC and RUNX2) expression level in hASCs preserved in various CPAs compared to fresh hASCs (* $p < 0.05$ relative to before induction). Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.6 The effect of cryopreservation on chondrogenic potential of hASCs

To determine the chondrogenic potential of hASCs preserved in various CPAs, Alcian blue staining and chondrogenic gene expression analysis were conducted. It was observed that histological sections of cryopreserved and fresh hASCs after chondrogenic induction display the formation of proteoglycan, that is positively stained by Alcian blue (**Fig. 4.11a**), which is in accordance with the results reported by Gonda *et al.* (2008). Further, it was found that cryopreserved and fresh hASCs show significantly increased ($p < 0.05$) expression level of chondrogenic markers (SOX-9, COL-2 and ACAN) following the chondrogenic induction (**Fig. 4.11b**). These results indicate that the chondrogenic potential of hASCs is maintained following cryopreservation. Further, there was no significant difference ($p > 0.05$) in the expression level of chondrogenic markers among hASCs preserved in various CPAs and fresh hASCs after chondrogenic induction, suggesting that they have similar chondrogenic capacity.

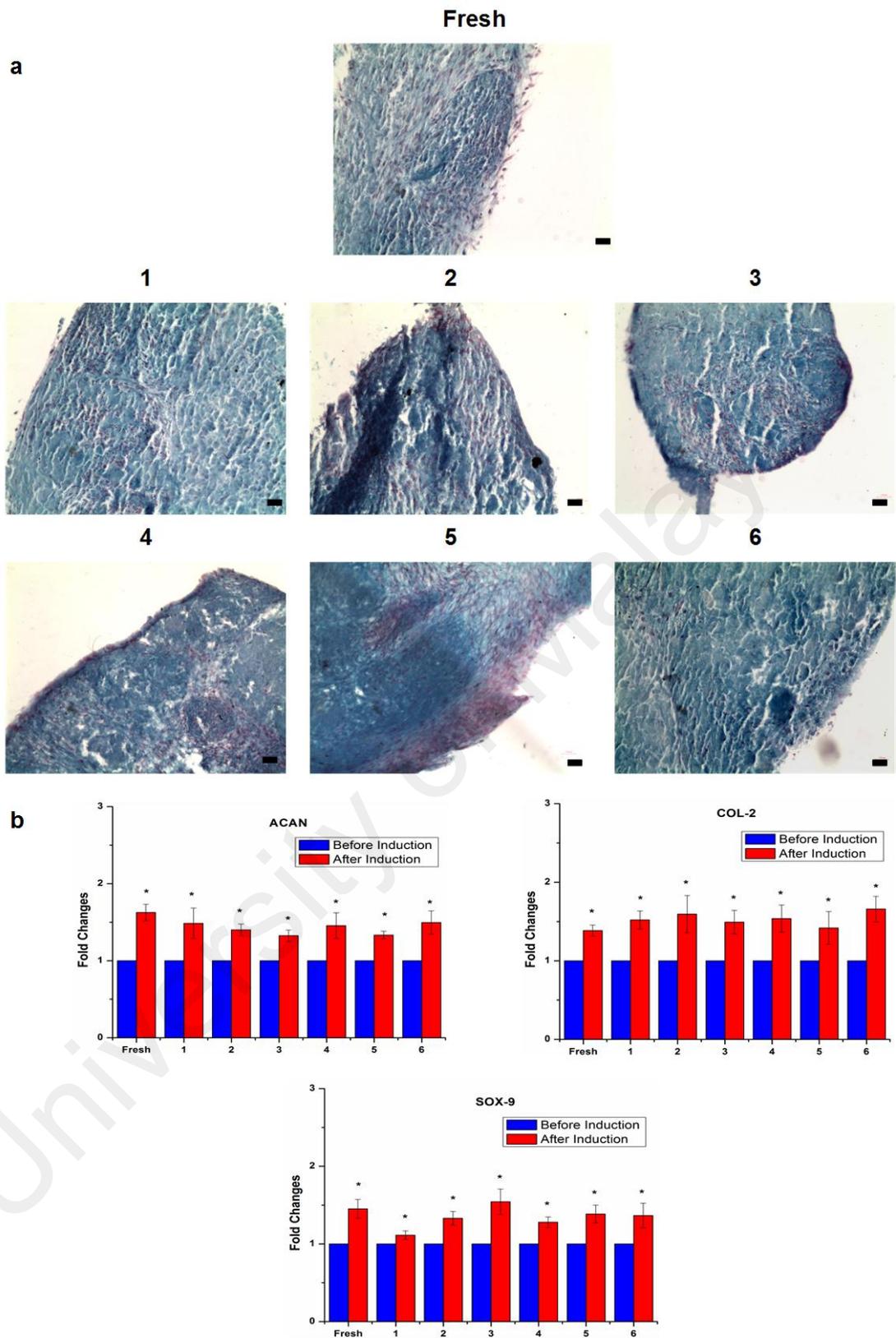


Figure 4.11. Cryopreservation maintained chondrogenesis of hASCs. (a) Chondrogenesis indicated by Alcian blue staining (magnification 100×) (scale bars: 100 μm). **(b)** A similar chondrogenic gene (ACAN, COL-2 and SOX-9) expression level in hASCs preserved in various CPAs compared to fresh hASCs ($*p < 0.05$ relative to before induction). Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.7 The effect of cryopreservation on stemness of hASCs

To date, stemness markers of cryopreserved hASCs have not been evaluated elsewhere. It was found that hASCs preserved in various CPAs display significantly higher ($p < 0.05$) expression level in stemness markers (NANOG, REX-1, SOX-2 and OCT-4) compared to fresh ASCs (Fig. 4.12), suggesting that cryopreserved hASCs have a greater ability to maintain their stemness.

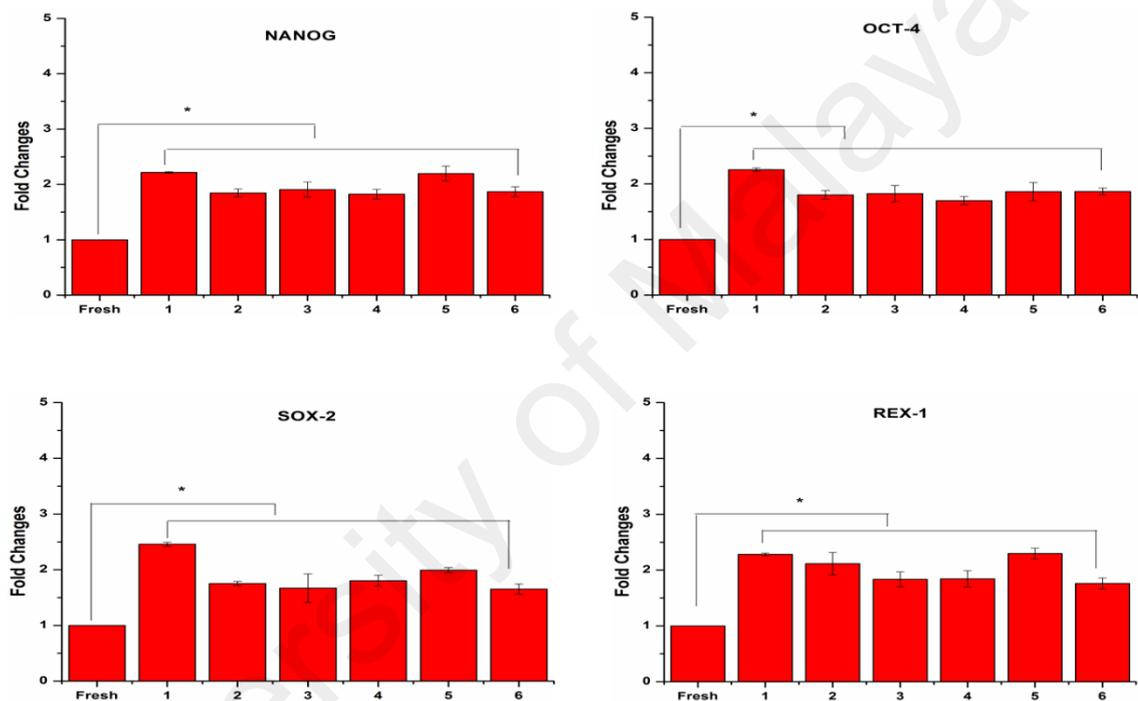


Figure 4.12. Cryopreservation maintained stemness of hASCs. hASCs preserved in various CPAs displayed significantly ($p < 0.05$) higher expression level of stemness markers (NANOG, OCT-4, SOX-2 and REX-1) ($*p < 0.05$ relative to fresh hASCs). Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.8 The effect of cryopreservation on expression of tumor suppressor markers in hASCs

Tumour suppressor activity in the cryopreserved hASCs was evaluated by analyzing the gene expression level of tumour suppressors p53, p21, p16 and pRb. It was found that there was no significant ($p > 0.05$) difference in terms of expression levels of p53, p21,

p16 and pRb among hASCs preserved in various CPAs and fresh hASCs (**Fig. 4.13**), suggesting that there is no indication of an uncontrolled proliferation in cryopreserved hASCs. This result is supported by the cell proliferation data, where a similar proliferation rate in fresh and cryopreserved hASCs was observed.

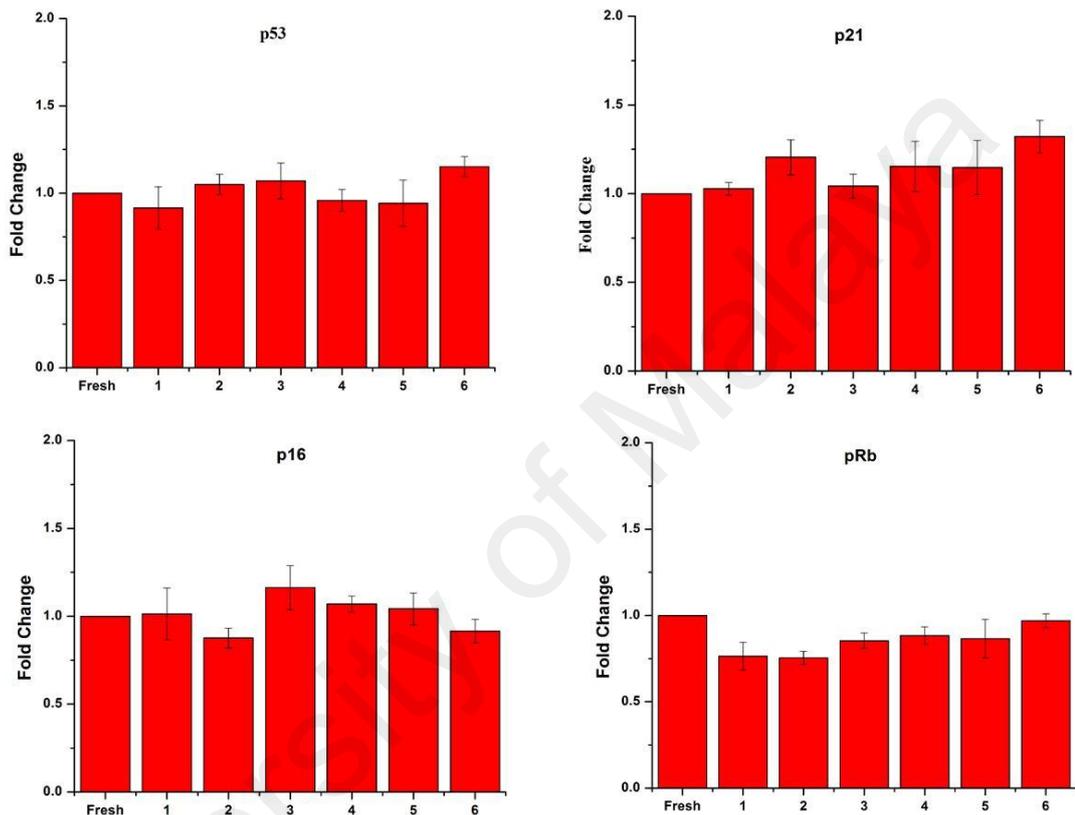


Figure 4.13. Expression of tumour suppressor markers (p53, p21, p16 and pRb) were maintained following the long-term cryopreservation. Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.9 The effect of cryopreservation on hTERT expression, telomerase activity and telomere length in hASCs

No detectable expression of hTERT was observed in fresh hASCs and hASCs preserved with various CPAs (Fig. 4.14a), indicating the extremely low levels of hTERT in fresh and cryopreserved hASCs. Further, it was observed that hASCs preserved in various CPAs and fresh hASCs display a similar ($p>0.05$) relative telomerase activity (Fig. 4.14b) and TRF length (Fig. 4.14c), suggesting that fresh and cryopreserved hASCs have a similar telomere shortening rate.

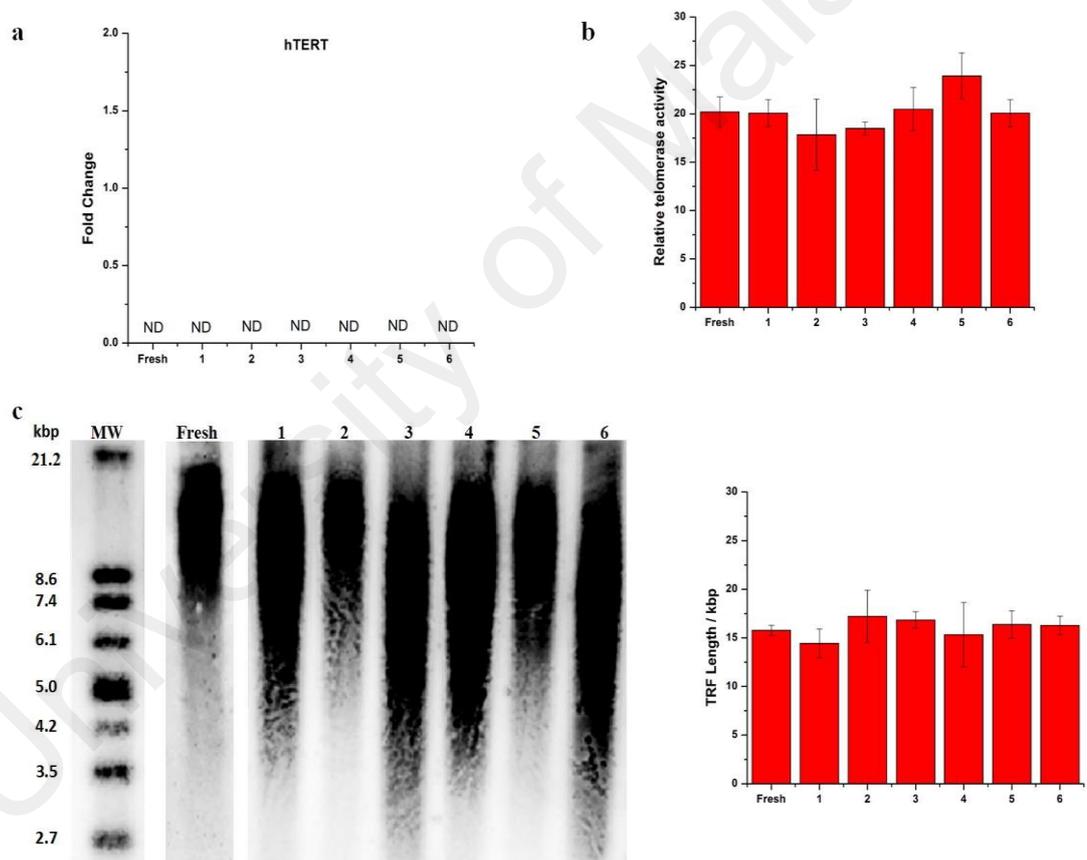


Figure 4.14. Cryopreservation maintained hTERT expression, telomerase activity and telomere length of hASCs. a) No detectable expression level of hTERT was observed in cryopreserved and fresh hASCs. b) Cryopreserved and fresh hASCs displayed a similar relative telomerase activity. c) By comparing the chemiluminescence signal of sample to molecular weight (MW) standard through Southern blotting, it was found that telomere length of hASCs is maintained following the long-term cryopreservation. Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.3.10 The effect of cryopreservation on p53 nucleotide sequence mutation in hASCs

DNA sequence analysis was performed to detect p53 mutation in cryopreserved hASCs, Exons 5 to 8 of p53 were analyzed, as 95% - 98% of p53 mutations exist mainly in these regions (Berloco et al., 2003; Lleonart et al., 1998). BLAST analysis revealed that exons 5 to 8 of p53 nucleotide sequences of fresh hASCs and hASCs preserved in various CPAs are 100% matched with those of wild-type p53 (NCBI accession no. X54156.1) (**Fig. 4.15**), indicating no sign of p53 mutation in cryopreserved and fresh hASCs.

University of Malaya

a. p53 exon 5

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: [embIX54156.1](#) Length: 20303 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
544 bits(294)	3e-157	294/294(100%)	0/294(0%)	Plus/Plus
Query 637	GCTGCCGTTCCAGTTGCTTTATCTGTTCACTTGTCCTGACTTTCAACTCTGTCTCC			696
Sbjct 12980	GCTGCCGTTCCAGTTGCTTTATCTGTTCACTTGTCCTGACTTTCAACTCTGTCTCC			13039
Query 697	TTCTCTTCTACAGTACTCCCTGCCCTCAACAAGATGTTTTGCCAACTGCCAAGACC			756
Sbjct 13040	TTCTCTTCTACAGTACTCCCTGCCCTCAACAAGATGTTTTGCCAACTGCCAAGACC			13099
Query 757	TGCCCTGTGACAGCTGTGGGTTGATTCCACACCCCGCCCGCACCCGCTCCGCGCCATG			816
Sbjct 13100	TGCCCTGTGACAGCTGTGGGTTGATTCCACACCCCGCCCGCACCCGCTCCGCGCCATG			13159
Query 817	GCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGAGGCGCTGCCCCACCATGAG			876
Sbjct 13160	GCCATCTACAAGCAGTCACAGCACATGACGGAGGTTGAGGCGCTGCCCCACCATGAG			13219
Query 877	CGCTGCTCAGATAGCGATGGTAGCAGCTGGGCTGGAGAGACACAGGGCTGG			930
Sbjct 13220	CGCTGCTCAGATAGCGATGGTAGCAGCTGGGCTGGAGAGACACAGGGCTGG			13273

b. p53 exon 6

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: [embIX54156.1](#) Length: 20303 Number of Matches: 2

Score	Expect	Identities	Gaps	Strand
370 bits(200)	7e-105	200/200(100%)	0/200(0%)	Plus/Plus
Query 886	GGCCTCTGATTCCTCACTGATTGCTCTAGGCTCTGCCCCCTCCTCAGCATCTTATCCGAG			945
Sbjct 13290	GGCCTCTGATTCCTCACTGATTGCTCTAGGCTCTGCCCCCTCCTCAGCATCTTATCCGAG			13349
Query 946	TGGAAGAAATTTGCGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGG			1005
Sbjct 13350	TGGAAGAAATTTGCGTGGAGTATTTGGATGACAGAAACACTTTTCGACATAGTGTGG			13409
Query 1006	TGGTGCCTATGAGCCCGCTGAGGCTCTGGTTTCACTGGGGTCTCTGGGAGGAGGGTT			1065
Sbjct 13410	TGGTGCCTATGAGCCCGCTGAGGCTCTGGTTTCACTGGGGTCTCTGGGAGGAGGGTT			13469
Query 1066	AAGGGTGGTTGTCAGTGGCC			1085
Sbjct 13470	AAGGGTGGTTGTCAGTGGCC			13489

c. p53 exon 7

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: [embIX54156.1](#) Length: 20303 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
363 bits(196)	1e-102	196/196(100%)	0/196(0%)	Plus/Plus
Query 1070	TGCCACAGGTCCTCCCAAGGCGCACTGGCCCTCATCTGGGCTGTGTTATCTCCTAGGTT			1129
Sbjct 13943	TGCCACAGGTCCTCCCAAGGCGCACTGGCCCTCATCTGGGCTGTGTTATCTCCTAGGTT			14002
Query 1130	GGCTCTGACTGTACCACCATCCACTACAACATGTTAACAGTTCCCTGATGGGCGGC			1189
Sbjct 14003	GGCTCTGACTGTACCACCATCCACTACAACATGTTAACAGTTCCCTGATGGGCGGC			14062
Query 1190	ATGAACCGGAGGCCATCCTCACCATCATCAGACTGGAAGACTCCAGGTCAGGAGCCACT			1249
Sbjct 14063	ATGAACCGGAGGCCATCCTCACCATCATCAGACTGGAAGACTCCAGGTCAGGAGCCACT			14122
Query 1250	TGCCACCCTGCACACT			1265
Sbjct 14123	TGCCACCCTGCACACT			14138

d. p53 exon 8

Homo sapiens p53 gene for transformation related protein p53 (also called transformation-associated protein p53, cellular tumor antigen p53, and non-viral tumour antigen p53)
Sequence ID: [embIX54156.1](#) Length: 20303 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
416 bits(225)	1e-118	225/225(100%)	0/225(0%)	Plus/Plus
Query 1037	CCTTACTGCCTTGTCTCTTTTCTATCCTGAGTAGTGGTAATCTACTGGGACGGAA			1096
Sbjct 14413	CCTTACTGCCTTGTCTCTTTTCTATCCTGAGTAGTGGTAATCTACTGGGACGGAA			14472
Query 1097	CAGCTTTGAGTGGCTGTTTGTGCTGTCTGGGAGAGACCGGCGCACAGAGGAAAGAA			1156
Sbjct 14473	CAGCTTTGAGTGGCTGTTTGTGCTGTCTGGGAGAGACCGGCGCACAGAGGAAAGAA			14532
Query 1157	TCTCCGCAAGAAAGGGGAGCCTCACCAGAGCTGCCCCAGGGAGCACTAAGCGAGGTTAA			1216
Sbjct 14533	TCTCCGCAAGAAAGGGGAGCCTCACCAGAGCTGCCCCAGGGAGCACTAAGCGAGGTTAA			14592
Query 1217	GCAAGCAGGACAAGAGCGGTGGAGGACCAAGGGTGCAGTTAT			1261
Sbjct 14593	GCAAGCAGGACAAGAGCGGTGGAGGACCAAGGGTGCAGTTAT			14637

Figure 4.15. Cryopreserved and fresh hASCs showed no sign of p53 mutation. A representative BLAST data demonstrated that exons 5 to 8 of p53 nucleotide sequences in either cryopreserved or fresh hASCs are 100% matched with those of wild-type p53 (NCBI accession no. X54156.1).

4.3.11 The effect of cryopreservation on DNA damage in hASCs

An Alkaline Comet assay was performed to assess the DNA damage pattern in cryopreserved hASCs. More DNA with short or without comet tail were observed in hASCs preserved in various CPAs and fresh hASCs (**Fig. 4.16a**). Further, hASCs preserved in various CPAs and fresh hASCs displayed a similar ($p>0.05$) comet tail length, DNA %, and moment (**Fig. 4.16b**), indicating low levels of DNA damage in long-term cryopreserved hASCs.

University of Malaya

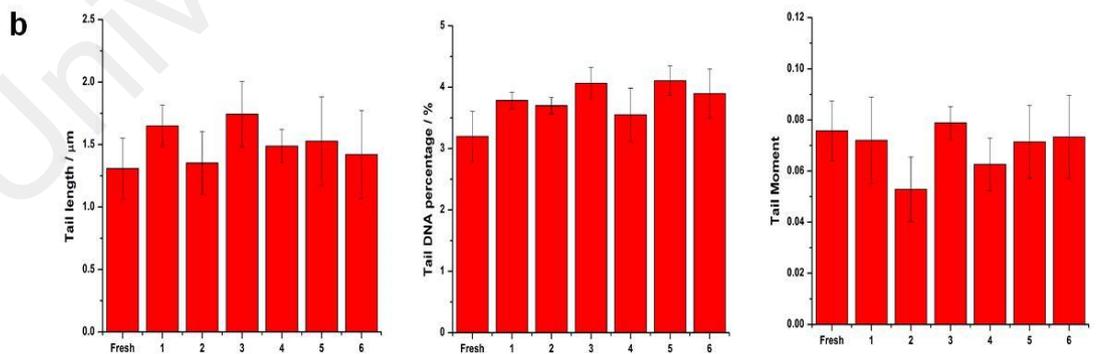
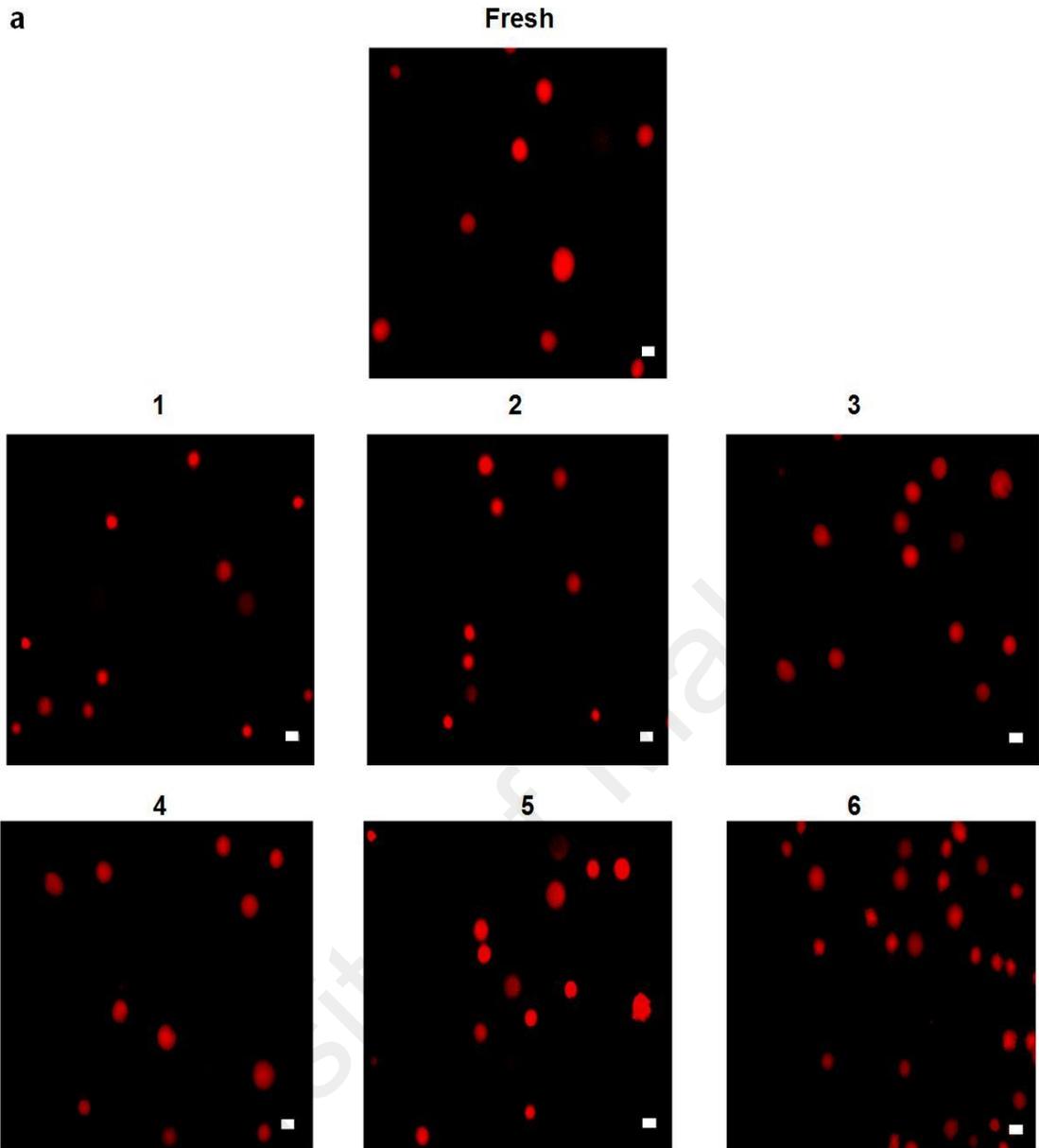


Figure 4.16. Low levels of DNA damage were observed in cryopreserved hASCs. a) Fresh and cryopreserved hASCs displayed more DNA with short or without comet tails (magnification: 100 \times) (scale bars: 100 μm). **(b)** Fresh and cryopreserved hASCs showed a similar comet tail length, tail DNA % and tail moment. Cryoprotective agent: 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS.

4.4 Discussion

In the present study, hASCs were cryopreserved for 3 months (long-term) (Katkov et al., 2006; Lee et al., 2013) using a slow freezing method in various CPAs, including 1) 0.25 M trehalose; 2) 5% DMSO; 3) 10% DMSO; 4) 5% DMSO + 20% FBS; 5) 10% DMSO + 20% FBS and 6) 10% DMSO + 90% FBS. In general, 10% DMSO + 90% FBS which was used to preserve and maintain high viability of many types of cells during slow freezing, acts as a standard cryopreservation medium (Li & Ma, 2012; Zeisberger et al., 2011). However, many adverse effects, *e.g.*, respiratory depression and neurotoxicity have been raised due to the clinical uses of cells preserved with 10% DMSO (Benekli et al., 2000; Windrum & Morris, 2003). Therefore, it is desirable to reduce the concentration of DMSO in cryopreservation medium to 5% to preserve cells for clinical use (Windrum et al., 2005). On the other hand, the use of FBS, an animal-based product, should be minimized or excluded from the cryopreservation media to lower the risk of xenogeneic immune response to recipients after implantation of cryopreserved cells, as suggested by regulatory guidelines (Balci & Can, 2013). So, it is desirable to reduce the concentration of FBS in cryopreservation medium to 20% (FBS concentration used for cryopreservation of many hMSCs) (Liu et al., 2011; Liu et al., 2008; Zhang et al., 2011b) or 0% (xeno-free cryopreservation medium). Trehalose at a concentration of 0.25 M is suggested as an alternative CPA to completely replace DMSO and FBS. It was found to be effective in preserving the functional properties and viability of adipocytes from human adipose tissue, a source from which hASCs are isolated (Pu et al., 2005). hASCs preserved in these 6 groups of CPAs were subjected to evaluation of functional properties and biosafety after thawing and subculture to passage 3.

In general, the maintenance of the viability and functional properties of hASCs in long-term storage is important because the therapeutic effects of hASCs might be affected by reduction in their functional capacity and cell viability (Davies et al., 2014). Therefore, CPA choice is important in order to maintain the functional properties and cell viability when the cells are stored in liquid nitrogen (-196 °C) (Karlsson & Toner, 1996; Xu et al., 2010). Various combinations of general used CPAs were tested in this study, including one permeable agent (DMSO) that penetrates the cell membranes and avoid the formation of intracellular ice crystal (Berz et al., 2007), and two non-permeable agents (trehalose and FBS) that sustain the osmotic pressure and cell membrane without penetrating the cell membranes (Karlsson, 2002). Low viability of hASCs preserved in trehalose was observed, possibly because of the inability of trehalose to penetrate the cell membranes to avoid intracellular ice crystal formation, disrupting the cell membranes and thus inducing cell death due to physical damage and dehydration caused by intracellular ice (Karlsson & Toner, 1996; Muldrew & McGann, 1990). On the other hand, the viability of hASCs preserved in DMSO was higher than those preserved in trehalose, indicating the relatively high efficiency of DMSO in sustaining the survival rate of hASCs throughout the cryopreservation process. This is due to the fact that DMSO can penetrate the cell membranes and prevent the formation of intracellular ice, which can cause rupture of cell membranes, by removing water within the cells (Berz et al., 2007). These findings are supported by Janz *et al.* (2012), which has demonstrated that DMSO is better than trehalose in sustaining the cell viability of amniotic fluid-derived stem cells. Therefore, this suggests that CPA containing trehalose alone is a sub-optimal alternate to DMSO in cryopreservation of hASCs. However, recently, when trehalose encapsulated in genipin-cross-linked Pluronic F127-chitosan nanoparticles was delivered into hMSCs for cryopreservation, it was found that cells are capable to maintain their functional properties and high viability following cryopreservation (Rao et al., 2015). This indicates that

trehalose may be more efficient when they are delivered intracellularly for cryopreservation of hMSCs. Meanwhile, it was observed that hASCs preserved in FBS-free DMSO do not suffer from any harmful effects. Given that cryopreservation medium containing only 5% DMSO without FBS is less cytotoxic than 10% DMSO and induces a low risk of xenogeneic immune responses, this raises the potential of 5% DMSO as a sole CPA in cryopreservation medium to store hASCs for clinical applications.

To date, the evaluation of differentiation potential in cryopreserved hASCs has been performed mostly in a qualitative manner, which was only assessed with a histochemical staining method (Thirumala et al., 2010b). However, the gene expression levels of differentiation markers among hASCs preserved in various CPAs following the differentiation induction has not been compared yet. Therefore, it is essential to analyze the gene expression levels of differentiation markers to observe the molecular changes that may occur after cryopreservation. James *et al.* (2011) reported that osteogenic and adipogenic potential of hASCs were decreased following the cryopreservation, as indicated by downregulation of the expression of osteogenic and adipogenic genes. In the present study, gene expression analysis revealed that hASCs preserved in various CPAs and fresh hASCs display a similar potential in adipogenic, osteogenic and chondrogenic differentiation, suggesting that cryopreserved hASCs maintain their capability to differentiate into adipocytes, osteocytes and chondrocytes. Therefore, cryopreserved hASCs, particularly hASCs preserved in cryopreservation medium containing 5% DMSO as the sole CPA, could be potentially used in various clinical applications, *e.g.*, cartilage and bone regeneration.

Stemness (a typical feature of MSCs) plays a crucial role in coordinating differentiation and self-renewal activity of MSCs (Boyer et al., 2005). It has been reported that knockdown of SOX-2 or OCT-4 significantly lowered multipotency and cell proliferation of MSCs (Greco et al., 2007; Seo et al., 2009; Yoon et al., 2011). Interestingly, the increased stemness markers expression in cryopreserved hASCs is reported for the first time, therefore, this requires further investigation. Overexpression of SOX-2 or OCT-4 in MSCs has been found increasing proliferation rate or differentiation potential (*e.g.*, adipogenesis and osteogenesis) (Fan et al., 2013; Liu et al., 2009). However, in the present study, although the enhanced stemness markers expression has been seen in cryopreserved hASCs, they still showed a similar differentiation potential and proliferation rate as fresh hASCs. Pierantozzi *et al.* (2011) and Choi *et al.* (2014) have demonstrated that the enhanced differentiation potential and proliferation rate of MSCs is not directly associated with the increased expression level of stemness markers. In fact, the differentiation and proliferation activity of MSCs is mainly influenced by other factors, *e.g.*, culture environment (2D or 3D) (Han et al., 2012) and oxygen tension level (Choi et al., 2014). In short, cryopreserved hASCs including hASCs preserved in xeno-free cryopreservation medium consists of 5% DMSO, maintained their stemness.

Besides evaluation of phenotype, viability, and functional properties in hASCs preserved in various CPAs, it is also essential to assess their biosafety in terms of tumorigenic potential. To date, the tumorigenic potential of hASCs preserved in various CPAs has not been evaluated elsewhere. Although tumorigenic transformation of hASCs may not happen immediately after cryopreservation, but observing the early changes in terms of expression of tumour suppressor markers (including p53, p21, p16 and pRb), hTERT expression, telomerase activity, telomere length, p53 mutation status,

and DNA damage of cells in response to cryopreservation is significant to assess their tumourigenesis risk. For instance, lymphocytes displayed a significantly reduced telomere length right after cryopreservation (Jenkins et al., 2012), increasing the risk of chromosomal aberration which might lead to tumourigenesis. This proves that data collected at our time point is valuable to assess the tumourigenic potential of cryopreserved cells (de Lima Prata et al., 2012; Jenkins et al., 2012; Luetzkendorf et al., 2015).

Cellular senescence and cell cycle are regulated by tumour suppressor markers (including p53, p21, p16 and pRb) through 2 main tumour suppressor pathways (*e.g.*, p16-pRb and p53-p21-pRb) (Pelicci, 2004). When cells encounter oncogenic stimuli that trigger uncontrolled cell proliferation (one of the hallmarks of human cancer), the tumour suppressor markers (*e.g.*, p16, p53 and p21) will be upregulated, which in turn actuate their downstream target, pRb. pRb will be maintained in its hypophosphorylated state, resulting in apoptosis and cell cycle arrest to suppress cell proliferation, thus preventing tumour formation (Chuaire-Noack et al., 2010; Pelicci, 2004). In the present study, hASCs preserved in various CPAs and fresh hASCs displayed similar expression levels of tumour suppressor markers, suggesting no sign of uncontrolled cell proliferation in cryopreserved hASCs.

Generally, a telomere-maintenance mechanism is usually engaged through upregulation of telomerase to transform normal cells (including hASCs) to tumour cells (Xu et al., 2013). Telomeres, specialized structures at chromosome end, are important for regulation of cell proliferation and genome stability. Telomere length is maintained by an enzyme called telomerase (Blackburn, 1991; Gomez et al., 2012). Telomere dysfunction or shortening can lead to genome instability which induce tumour initiation (Raynaud et

al., 2008). It has been reported that telomeres in tumour cells are shorter than in normal cells (Blasco & Hahn, 2003). Following tumour initiation, subsequent upregulation of telomerase and hTERT (a catalytic subunit of telomerase for protecting the telomeres), and function restoration of telomere activate tumour progression (Ding et al., 2012; Hu et al., 2012; Xu et al., 2013). During tumour progression, telomere length is not shortened, resulting in uncontrolled cell proliferation (Artandi & DePinho, 2010). Increased telomerase activity has been found in 85% - 90% of all human tumours (Elenitoba-Johnson, 2001; Kim et al., 1994). Therefore, hTERT expression, telomere length and telomerase activity were performed to evaluate tumourigenic potential of hASCs in response to cryopreservation. As a result, hTERT was undetected in fresh and cryopreserved hASCs because it is usually undetectable in normal human cells but easily to be detected in tumour cells (Elenitoba-Johnson, 2001; Murofushi et al., 2006). Generally, the upregulation of hTERT maintains tumour phenotype (*e.g.*, increase telomerase activity) and immortality by blocking cell apoptosis (Lamy et al., 2013). For instance, hMSCs isolated from bone marrow were found to display an overexpressed hTERT after long-term culture, resulting in spontaneous malignant transformation (Rosland et al., 2009). In the present study, fresh hASCs and hASCs preserved in various CPAs have a similar telomerase activity and telomere length, indicating no changes in those parameters in response to cryopreservation. Taken together, cryopreserved hASCs are at a low risk of telomere dysfunction-induced tumourigenesis.

Mutation at p53 nucleotide sequences can alter the cell growth regulation, accelerating cell proliferation which may lead to tumour formation (Muller & Vousden, 2013). For instance, in the absence of p53, MSCs derived from bone marrow displayed spontaneous malignant transformation and accelerated cell proliferation (Armesilla-Diaz et al., 2009). It has been reported that p53 mutation was occurred in 60% of human

malignancies (Kusser et al., 1993). In the present study, there is no sign of p53 mutation in hASCs preserved in various CPAs, suggesting that cryopreserved hASCs are at a low risk of malignant transformation.

Upon encounter with oncogenic stresses, p53 and p21 are activated followed by DNA damage response (DDR) (a guardian of genomic integrity) which in turn promotes apoptosis of tumour cells to suppress uncontrolled cell proliferation (Bartek et al., 2007). On the other hand, many factors in the cryopreservation processes, *e.g.*, osmotic shock, reactive oxygen species or CPA toxicity, may cause DNA damage in cells (Kopeika et al., 2015; Stachowiak et al., 2009), potentially inducing chromosomal aberration which may in turn lead to formation of tumour (van Gent et al., 2001). In the present study, fresh and cryopreserved hASCs showed a low level of DNA damage, indicating no sign of oncogenic stress to hASCs in response to cryopreservation.

4.5 Conclusion

In conclusion, hASCs maintained their cell phenotype, differentiation, proliferation and stemness after long-term cryopreservation. Further, xeno-free 5% DMSO (without FBS), which is less cytotoxic and induce low risk of xenogeneic immune response while maintaining functional properties and high viability of hASCs, may be an ideal CPA in long-term preservation of hASCs for clinical applications. In addition, hASCs preserved in various CPAs including hASCs preserved in 5% DMSO displayed a low risk of tumorigenesis as they maintain normal expressions of tumour suppressor markers and hTERT, telomere length and telomerase activity without significant DNA damage and p53 mutation. Taken together, hASCs preserved in 5% DMSO without FBS could be an ideal cell source for clinical applications.

CHAPTER 5: PARACRINE EFFECTS OF HUMAN ADIPOSE-DERIVED MESENCHYMAL STEM CELLS ON CARDIAC FIBROSIS

5.1 Introduction

Differentiation of cardiac fibroblasts to myofibroblasts is a hallmark of cardiac fibrosis, in which cardiac myofibroblasts actively secrete collagen, resulting in excessive accumulation of ECM that leads to permanent scarring of the heart and impact heart function (van den Borne et al., 2010; Weber et al., 2013). Cardiac fibrosis is a substantial problem that is difficult to manage, as it cannot be effectively halted or reversed by surgery or drug therapies (*e.g.*, anti-fibrotic agents) once it has begun (Daskalopoulos et al., 2012). In order to treat cardiac fibrosis, it is important to understand and explore the regulation mechanism of cardiac myofibroblast differentiation, which may offer possible targets for intervention in cardiac fibrosis, leading to the development of an effective treatment for cardiac fibrosis (Yong et al., 2015a).

Cardiac myofibroblasts are likely to be derived from quiescent cardiac fibroblasts in response to biochemical cues, *e.g.*, TGF- β 1, endothelin-1 and angiotensin II (Leask, 2010). Over the past decade, it was realized that the mechanical properties of ECM (*e.g.*, stiffness) also plays an important role in cardiac myofibroblast differentiation (Yong et al., 2015a). For instance, polyethylene glycol diacrylate (PEGDA) hydrogels with physiologically relevant stiffness (10-20 kPa) inhibit myofibroblast differentiation, better maintaining the inactivated phenotype of cardiac fibroblasts, whereas PEGDA hydrogels with stiffness mimicking fibrotic cardiac tissues (40 kPa) promote cardiac myofibroblast differentiation (Zhao et al., 2014). However, recent literature suggests that the mechanical resistance provided by ECM in planar culture, that results in cell signaling and differentiation, could arise from matrix protein (*e.g.*, collagen) tethering apart from substrate stiffness (Chaudhuri & Mooney, 2012; Trappmann et al., 2012). For instance,

collagen tethering can be manipulated through modulation of the distance between two adjacent anchoring points for collagen, by changing substrate pore size or concentration of protein-substrate linker (Trappmann et al., 2012; Wen et al., 2014), which could induce different cell behaviors of human epidermal stem cells (*e.g.*, cell spreading and differentiation) on substrates with various stiffness (Trappmann et al., 2012). Therefore, it is important to develop a model which decouples the effect of ECM stiffness and pore size on cardiac myofibroblast differentiation.

To date, significant effort has been put into cardiac fibrosis therapy. For instance, treatment with TGF- β 1 antibodies that block the activation of TGF- β 1 has been proposed as a potential therapeutic method. However, this non-specific treatment has not been routinely used in clinics due to the potential issue of its adverse effects on other cell populations (*e.g.*, immune cells such as T cells and B cells) (Arslan et al., 2011). Therefore, more promising strategies are needed to attenuate the secretion of TGF- β 1 or TGF- β 1 signaling in a cell-type specific manner rather than blocking the active TGF- β 1 (Hinz & Gabbiani, 2010). To this end, angiotensin II type 1 receptor (AT₁R) and Smad7, two signaling pathways that are located upstream and downstream of the TGF- β 1 receptor, respectively, are selected as targets for implementing such strategies. It was suggested that undifferentiated MSCs which display paracrine effects on the behavior of cardiac cells (*e.g.*, myofibroblast differentiation and cardiomyocyte regeneration) through secretion of soluble factors (*e.g.*, HGF and IGF-1) (Ramkisoensing et al., 2014), may work through AT₁R and Smad7 to reduce cardiac fibrosis. However, to date, the inhibitory mechanism of MSCs on cardiac myofibroblast differentiation still remains elusive. To better understand the therapeutic use of MSCs in cardiac fibrosis, the investigation of the paracrine effects of MSCs on cardiac myofibroblast differentiation should be performed in the conditions mimicking the stiffness of *in vivo* normal and

fibrotic cardiac tissues. In addition, the paracrine effects of cryopreserved hASCs on cardiac myofibroblast differentiation was also investigated as intensive clinical settings such as cardiac fibrosis therapy might require a large number of cryopreserved cells for off-the-shelf use.

In the present study, a mechanically tunable cell culture system developed by Engler's group (Wen et al., 2014) was used to independently investigate the effect of substrate stiffness and pore size on cardiac myofibroblast differentiation. Normal cardiac tissue models and cardiac fibrosis models were developed, based on evaluation of cardiac myofibroblast differentiation markers such as α -SMA, Col I, Col III and TGF- β 1, in cardiac fibroblasts cultured on collagen coated polyacrylamide (PA) hydrogels with various stiffness (4 kPa, 13 kPa and 30 kPa) and pore sizes. Following the development of such models, conditioned medium of fresh and cryopreserved hASCs was applied to the models to determine the role and inhibitory mechanism of hASCs on cardiac myofibroblast differentiation and fibrosis. The aim of this work is more focused on the reduction of interstitial fibrosis (development of myofibroblasts) rather than scar elimination, as scar elimination effect can only be observed in animal-based cardiac fibrosis model upon treatment with MSCs. The development of such normal cardiac tissue models and cardiac fibrosis models would encourage research into cardiac myofibroblast differentiation *in vitro* in an accurate and controlled manner. The findings from this study could promote the establishment of the therapeutic use of hMSCs in cardiac fibrosis in future.

5.2 Materials and methods

5.2.1 Isolation and culture of cardiac fibroblasts

Cardiac fibroblasts were isolated from the hearts of neonatal Sprague-Dawley rats (1-3 day old). First, heart tissues of the rats were excised following euthanasia by cervical dislocation. The heart tissues were washed with PBS (Sigma) and minced into small pieces. Digestion of 2 mL of tissue was performed using 5 mL of 0.8% (w/v) collagenase type II enzyme (MP Biomedicals, Aurora, Ohio) solution at 37 °C with agitation for 20 min. The digested heart tissues were centrifuged and washed to obtain the pellets. The pellets were resuspended with cell culture medium composed of DMEM/Ham F-12, 10% FBS and 1% Penicillin/Streptomycin (Gibco), and plated for 45 min in a cell culture plate at 37 °C and 5% CO₂. This allows preferential attachment of cardiac fibroblasts to the cell culture plate. Then, cell culture medium containing cells (mainly cardiomyocytes) were removed and replaced with fresh cell culture medium. Cardiac fibroblasts cultured on day 1 to day 9 after isolation, were characterized using α -SMA immunofluorescence staining. Based on the results of characterization, cardiac fibroblasts on day 4 after isolation were selected, and seeded on PA substrates for evaluation of cardiac myofibroblast differentiation.

5.2.2 Isolation, culture and cryopreservation of hASCs

The method for isolation and culture of hASCs was described in section 3.2.1. Cryopreservation of hASCs was performed following the method as described in section 4.2.2. Passage 3 fresh hASCs and cryopreserved hASCs in 5% DMSO were seeded with a cell number of 3×10^5 into a tissue culture plate. Then 3 days after seeding, medium was collected and used as a conditioned medium of hASCs for evaluating the paracrine effects of fresh and cryopreserved hASCs on cardiac fibrosis.

5.2.3 Fabrication of PA hydrogels

Glass coverslips and slides were cleaned of organics with a dish washing detergent (30 min), 100% acetone (30 mins), 100% methanol (30 mins) and 0.05 N NaCl (1 hr) successively, then rinsed with water and dried in an oven. The surface of a clean coverslip was functionalized with 2% [3-(trimethoxysilyl)propyl methacrylate] (Sigma) in ethanol to enhance the hydrophilic property of the surface for facilitating the attachment of PA hydrogel substrate to glass. Meanwhile, the surface of a glass slide was treated with dichloromethylsilane (DCMS) (Sigma) to enhance the hydrophobic properties of glass for facilitating easy detachment of PA hydrogel after polymerizing on the above treated coverslip. Fabrication of PA hydrogels was performed following the protocols as described elsewhere (Wen et al., 2014). In brief, a polymer solution containing acrylamide (monomer) (MP Biomedicals, Aurora, Ohio), *N,N* methylene-bis-acrylamide (crosslinker) (MBA) (Sigma), 1/100 volume of 10% (w/v) aluminium persulfate (APS) (Sigma) and 1/1000 volume of *N,N,N',N'*-tetramethylethylenediamine (TEMED) (Sigma) was prepared. This solution was sandwiched between a DCMS-treated slide and a functionalized coverslip, and allowed to polymerize at room temperature for 5 min. Following polymerization, PA hydrogel was incubated in 1 mg/mL *N*-sulphosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) (Pierce, Rockford, IL) activated with ultraviolet (UV) light for 10 min, washed with 50 mM HEPES at pH 8.5 (Sigma), and then incubated in 50 µg/mL rat tail collagen type I (Corning, Manassas, USA) in deionized water overnight at room temperature. Sulfo-SANPAH acts as a protein-substrate linker to couple collagen type I to the surface of PA hydrogel to facilitate cell adhesion. Collagen-coated PA substrate was kept in PBS at 4 °C and UV sterilized prior to being used for cell culture. The ratio of acrylamide (%) / MBA (%) was varied in order to adjust PA hydrogel substrate stiffness (4, 13 and 30 kPa) and pore size. Ratios of 4/0.4, 6/0.06, and 10/0.02 were used for 4 kPa gels. For 13 kPa gels,

ratios of 6/0.45, 10/0.1, and 20/0.03 were used. Ratios of 8/0.55, 10/0.3, and 20/0.15 were used for 30 kPa gels. Fabrication of PA hydrogel substrates was demonstrated in brief in **Fig. 5.1**.

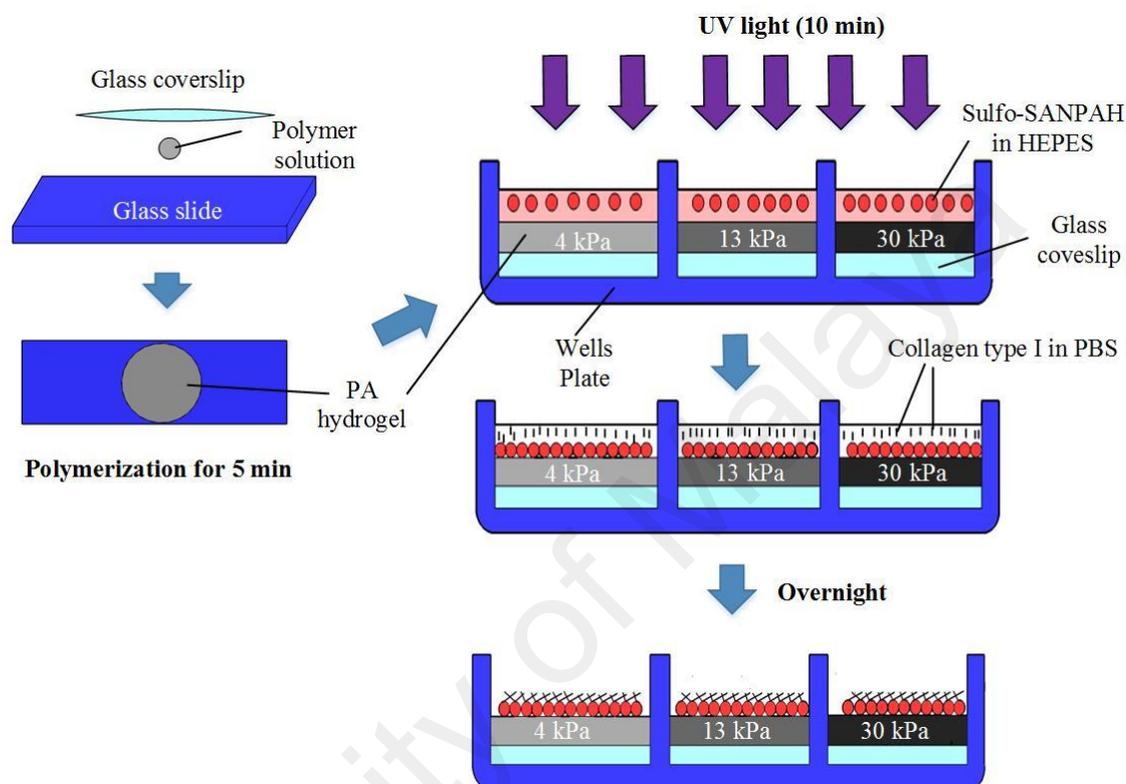


Figure 5.1. Polyacrylamide (PA) hydrogel substrate fabrication. Polymer solutions containing various ratios of acrylamide (%)/MBA (%) were allowed to polymerize and adhere on coverslips followed by crosslinking with Sulfo-SANPAH in the presence of ultraviolet (UV) light and collagen coating. MBA: *N,N* methylene-bis-acrylamide.

5.2.4 PA hydrogel characterization: atomic force microscopy (AFM) and scanning electron microscopy (SEM)

An AFM (Innova, Veeco, Santa Barbara, USA) was used to determine the stiffness of PA hydrogel by indentation. PA hydrogels on glass coverslips were swollen to equilibrium in PBS and indented at a velocity of 2 $\mu\text{m/s}$ until a trigger of 2 nN was detected. All AFM data were analysed using SPIP 6.3.3 software (Image Metrology, Denmark) to determine the Young's modulus, which represents the stiffness of PA hydrogel.

The PA hydrogels polymerized on glass coverslip were left to swell in deionized water overnight, followed by freezing in liquid nitrogen. Then, the frozen hydrogel was lyophilized at overnight -20 °C using a freeze dryer (Heto PowerDry LL 1500, Thermo Scientific, Rockford, IL). Lyophilized samples were sputter coated with Iridium followed by observation using a SEM (S-3000N, Hitachi, Japan). The images were taken at 700× at 15 kV.

5.2.5 Evaluation of cardiac myofibroblast differentiation in cardiac fibroblasts cultured on collagen coated PA hydrogels

To determine the effect of substrate stiffness and pore size on cardiac myofibroblast differentiation, cardiac fibroblasts were seeded at a concentration of 2×10^5 cells/cm² on collagen-coated PA hydrogels with different ratios of acrylamide (%)/MBA (%). Cardiac fibroblasts cultured on a cell culture plate (with stiffness of ~1 GPa) were used as a positive control. Following the 5-day culture on PA hydrogels, these cells were subjected to evaluation of cardiac myofibroblast differentiation through α -SMA immunofluorescence staining and gene (α -SMA, Col I, Col III, TGF- β 1, AT₁R and Smad7) expression analysis (described later). Further, to explore the role of AT₁R in cardiac myofibroblast differentiation, losartan (AT₁R inhibitor) (Selleckchem, Houston, USA) at 10^{-7} M was given to cardiac fibroblasts cultured on PA hydrogels on day 4. The treatment lasted for 1 day. The cells were then subjected to cardiac myofibroblast differentiation assessment.

5.2.6 Evaluation of paracrine effects of fresh and cryopreserved hASCs on cardiac myofibroblast differentiation

Conditioned medium of fresh and cryopreserved hASCs was given respectively to cardiac fibroblasts following 5 days of culture on PA hydrogels, to determine the paracrine effects of fresh and cryopreserved hASCs on cardiac myofibroblast differentiation. The treatment lasted for 3 days. To explore the potential role of HGF on anti-fibrotic activity of conditioned medium of fresh hASCs, the conditioned medium was incubated with 3 ng/mL HGF antibody (anti-HGF) (R & D System, Minneapolis, MN, USA) at 37 °C for 1 hr prior to being given to cardiac fibroblasts. After 3 day-treatment, myofibroblast differentiation evaluation of these cells was performed. The concentration of HGF in conditioned medium of fresh and cryopreserved hASCs was determined by enzyme-linked immunosorbent assay (ELISA) (described later).

5.2.7 Immunofluorescence staining

Cardiac fibroblasts were subjected to fixation using 10% formaldehyde for 10 mins followed by cell membrane permeabilization with 0.5% triton X-100 for 5 mins. Immunofluorescence staining was performed using Rhodamine Phalloidin (Cytoskeleton, Denver, Colorado, USA), α -SMA-FITC antibody (Sigma Aldrich, St. Louis, USA) and 4'-6-diamidino-2-phenylindole (DAPI) (Southern Biotechnology Associates, Birmingham, Alabama). Following cell membrane permeabilization, cells were incubated in 0.7% (v/v) Rhodamine Phalloidin (stain F-actin) for 30 mins. Then, cells were incubated with 1% (v/v) α -SMA-FITC antibody for 30 mins followed by incubation in 10% (v/v) DAPI (stain nucleus) for 10 mins. Cells were washed three times with PBS after incubation in each immunofluorescence dye to remove the residual dye. The images were captured by an imaging system (Olympus IX81, Tokyo, Japan). Image overlay was performed using ImagePro Plus 6.0 (Media cybernetics. Inc., Bethesda, MD).

5.2.8 RNA extraction, cDNA synthesis and Real-Time PCR

Cardiac fibroblasts were subjected to RNA extraction followed by cDNA synthesis and Real-Time PCR using methods as described in section 3.2.4. The genes to be analyzed include Col I (Rn00584426_m1), Col III (Rn01437681_m1), α -SMA/Acta2 (Rn01759928_g1), TGF- β 1 (Rn00572010_m1), AT₁R (Rn00578456_m1) and Smad7 (Rn00578319_m1). The housekeeping gene used for normalization was GAPDH (Rn99999916_s1). All data were expressed as fold change in gene expression relative to a group as stated in figure captions.

5.2.9 ELISA

ELISA was performed to determine the concentration of HGF in conditioned medium of fresh and cryopreserved hASCs using a human HGF ELISA kit (Neobioscience, Beijing, China) following the manufacturer's instruction. In brief, standards with concentration of 8000 pg/mL, 4000 pg/mL, 2000 pg/mL, 1000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL and 0 pg/mL were prepared. About 100 μ L of each standard and sample (conditioned medium of fresh hASCs or cryopreserved hASCs) were incubated in each well of microplate coated with HGF antibody at 37 °C for 90 mins to enhance binding of standard and sample to antibody. Then, the resulted products were incubated in a solution containing secondary HGF antibody tagged with biotin at 37 °C for 60 mins followed by incubation in a solution containing streptavidin conjugated with horseradish peroxidase (HRP) enzyme at 37 °C for 30 mins. 3', 3', 5, 5'-Tetramethylbenzidine (TMB) substrate solution was added to each well and the microplate was incubated at 37 °C for 15 mins in the dark. Color of solution was changed from blue to yellow when Stop reagent was added to stop the activity of HRP enzyme. Absorbance of standards and samples at 450 nm were measured using a microplate reader (Multiskan G0, Thermo Scientific, Rockford, IL). A standard curve with known concentration of HGF versus absorbance at 450 nm was

plotted. By referring to the standard curve, the concentration of HGF in conditioned medium of fresh and cryopreserved hASCs corresponds to its absorbance at 450 nm was determined.

5.2.10 Statistical analysis

All statistical analysis were performed with One-Way ANOVA with tukey post hoc test, independent t-test, or paired *t*-test accordingly using SPSS 18.0 software. Each datum was expressed as mean \pm standard error of mean of six independent experiments ($n = 6$). Each experiment was conducted in triplicates. Statistical significance was accepted at $p < 0.05$.

5.3 Results

5.3.1 Characterization of the PA hydrogels

AFM and SEM were performed to determine the stiffness and pore size of PA hydrogels, respectively. Through AFM analysis, it was found that by varying the ratio of acrylamide monomer and MBA crosslinker, PA hydrogel substrates with different stiffness, *i.e.*, ~4 kPa, ~13 kPa and ~30 kPa, corresponding to the stiffness of immature (1-6 kPa), normal (10-20 kPa) and fibrotic (30-70 kPa) rat cardiac tissues (Berry et al., 2006; Engler et al., 2008) respectively, were observed (**Fig. 5.2a**). Moreover, SEM images show that the pore size of the PA hydrogel substrate can be adjusted without altering the stiffness. The relative pore size increases with increasing concentration of acrylamide and decreasing concentration of MBA for the 4 kPa, 13 kPa and 30 kPa hydrogel formulations (**Fig. 5.2b**). These results indicate that the stiffness and pore size can be independently controlled for PA hydrogel substrate, which are in accordance with the findings reported by Wen *et al.* (2014). Thus, the above substrate can be applied to decouple the effect of substrate stiffness and pore size on cardiac myofibroblast differentiation.

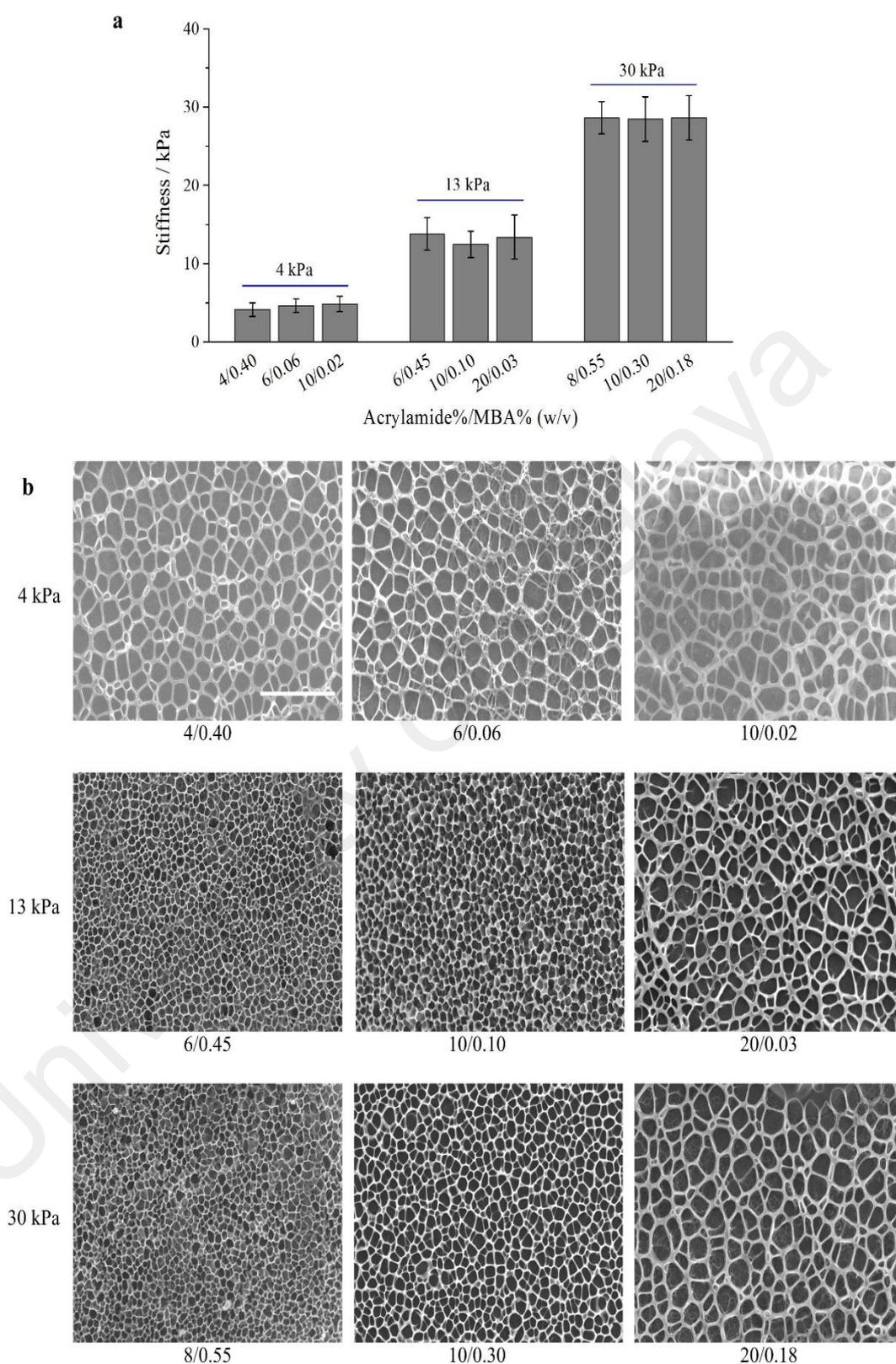


Figure 5.2. Characterization of Polyacrylamide (PA) hydrogels. **a)** Stiffness (4 kPa, 13 kPa and 30 kPa) of PA hydrogels made by various ratios of acrylamide:*N,N* methylene-bis-acrylamide (MBA) determined by AFM. **b)** Relative pore size observed through SEM increases with increasing concentration of acrylamide and decreasing concentration of MBA for the 4 kPa, 13 kPa and 30 kPa hydrogel formulations. Scale bar: 50 μ m.

5.3.2 Characterization of cardiac fibroblasts

Due to limited sources of primary human cardiac fibroblasts, rat cardiac fibroblasts were selected in this study. Since the neonatal heart is less prone to injury compared to the adult rat and heart injury may increase cardiac myofibroblast population (Norris et al., 2008), neonatal rat was used. To characterize cardiac fibroblasts isolated from neonatal rat, microscopic examination and α -SMA immunofluorescence staining were performed. It was observed that the isolated cells display a spindle shape with no spontaneous beating on day 1 until day 9, indicating the absence of cardiomyocytes in the culture. Further, there is absence of α -SMA (defining marker of myofibroblast) positive cells in the culture from day 1 till day 4, suggesting the presence of only cardiac fibroblasts without myofibroblast differentiation (**Fig. 5.3**). However, starting from day 5, cardiac fibroblasts were spontaneously differentiated into myofibroblasts (large cells with spindle shape and protruding dendrite-like processes) (Hinz et al., 2007) on the cell culture plate, as confirmed by the presence of α -SMA positive cells with enhanced assembly of stress fibers (F-actin) (**Fig. 5.3**). These results are in accordance with the findings reported in literature (Santiago et al., 2010; Wang et al., 2003). These findings suggest that cardiac fibroblasts isolated and cultured on cell culture plates should be used before day 5.

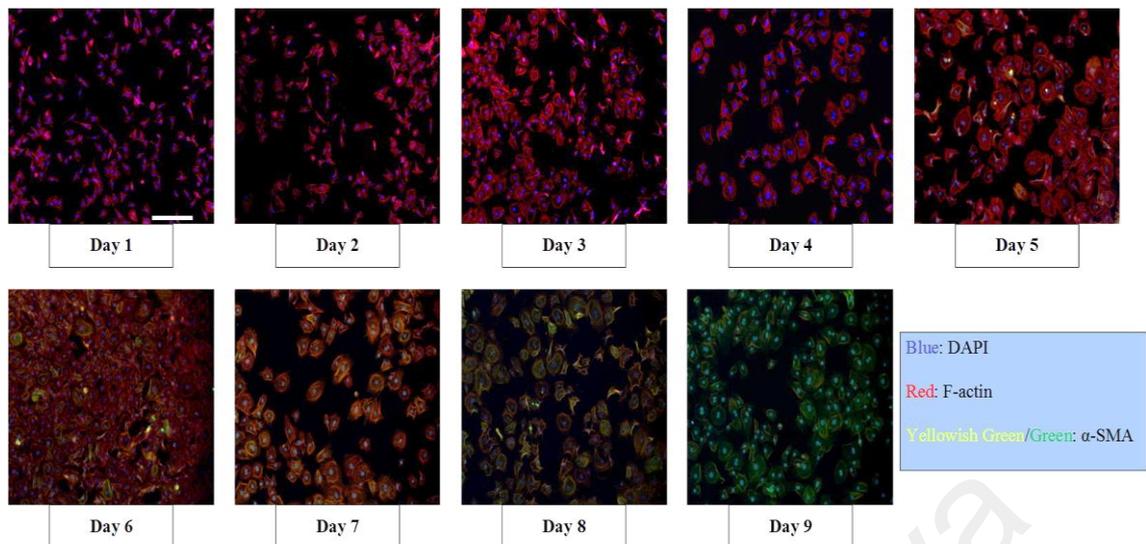


Figure 5.3. Characterization of cardiac fibroblasts and myofibroblasts with immunofluorescence staining. Cardiac fibroblasts cultured on cell culture plate before day 5 were negative to α -SMA, whereas those cultured after day 5 were positively stained by α -SMA. Magnification: 100 \times . Scale bar: 100 μ m.

5.3.3 Effect of pore size and substrate stiffness on cardiac myofibroblast differentiation

To evaluate the effect of pore size and substrate stiffness on cardiac myofibroblast differentiation, α -SMA immunofluorescence staining and gene expression analysis were performed. There is no significant difference ($p > 0.05$) in terms of α -SMA expression among the cells cultured on PA hydrogels with various pore sizes (**Fig. 5.4a-b**). These findings suggest that varying substrate pore size or collagen tethering distance does not seem to affect cardiac myofibroblast differentiation. Similar results that indicate osteogenic and adipogenic differentiation of MSCs are not affected by such changes have been reported in literature (Wen et al., 2014). Taken together, cardiac myofibroblast differentiation, a mechanoresponsive cellular behavior in cardiac fibroblasts, might not be regulated by pore size or collagen tethering in planar culture.

On the other hand, it was found that cardiac myofibroblast differentiation (as indicated by positive α -SMA immunofluorescence staining) was dependent on substrate stiffness, as 30 kPa substrates and cell culture plate (with stiffness of ~ 1 GPa) were positive for α -SMA while 4 kPa and 13 kPa substrates were negative for α -SMA (**Fig. 5.4a**). Gene expression analysis showed that cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate both display significantly ($p < 0.05$) higher expression levels of α -SMA than those cultured on 4 kPa and 13 kPa substrates (**Fig. 5.4b**), further implying that substrate stiffness play essential role in cardiac myofibroblast differentiation. These findings suggest that cardiac myofibroblast differentiation is dependent on ECM stiffness rather than pore size.

Substrates with similar pore size but different stiffness (6/0.06 for 4 kPa, 20/0.03 for 13 kPa, and 20/0.18 for 30 kPa) were selected for further experiments. It was observed that cardiac fibroblasts cultured on 30 kPa substrates show significantly ($p < 0.05$) higher expression levels of Col I, Col III and TGF- β 1 than those cultured on 4 kPa and 13 kPa substrates, but lower than those cultured on cell culture plate (**Fig. 5.5**). These results show that stiff ECM can induce differentiation of cardiac fibroblasts to more active cardiac myofibroblasts, which actively secrete collagen, through upregulation and activation of TGF- β 1 (a potent inducer of myofibroblast differentiation). It has been proven that myofibroblast differentiation and the secretion of collagen and TGF- β 1 were elevated during cardiac fibrosis (Daskalopoulos et al., 2012; van den Borne et al., 2010). These findings suggest that cardiac fibroblasts cultured on 30 kPa substrates mimicking the stiffness of native fibrotic cardiac tissue can be used as an *in vitro* cardiac fibrosis model while those cultured on 13 kPa substrates mimicking native normal cardiac tissue can be used as an *in vitro* normal cardiac model, for pathophysiological and therapeutic studies.

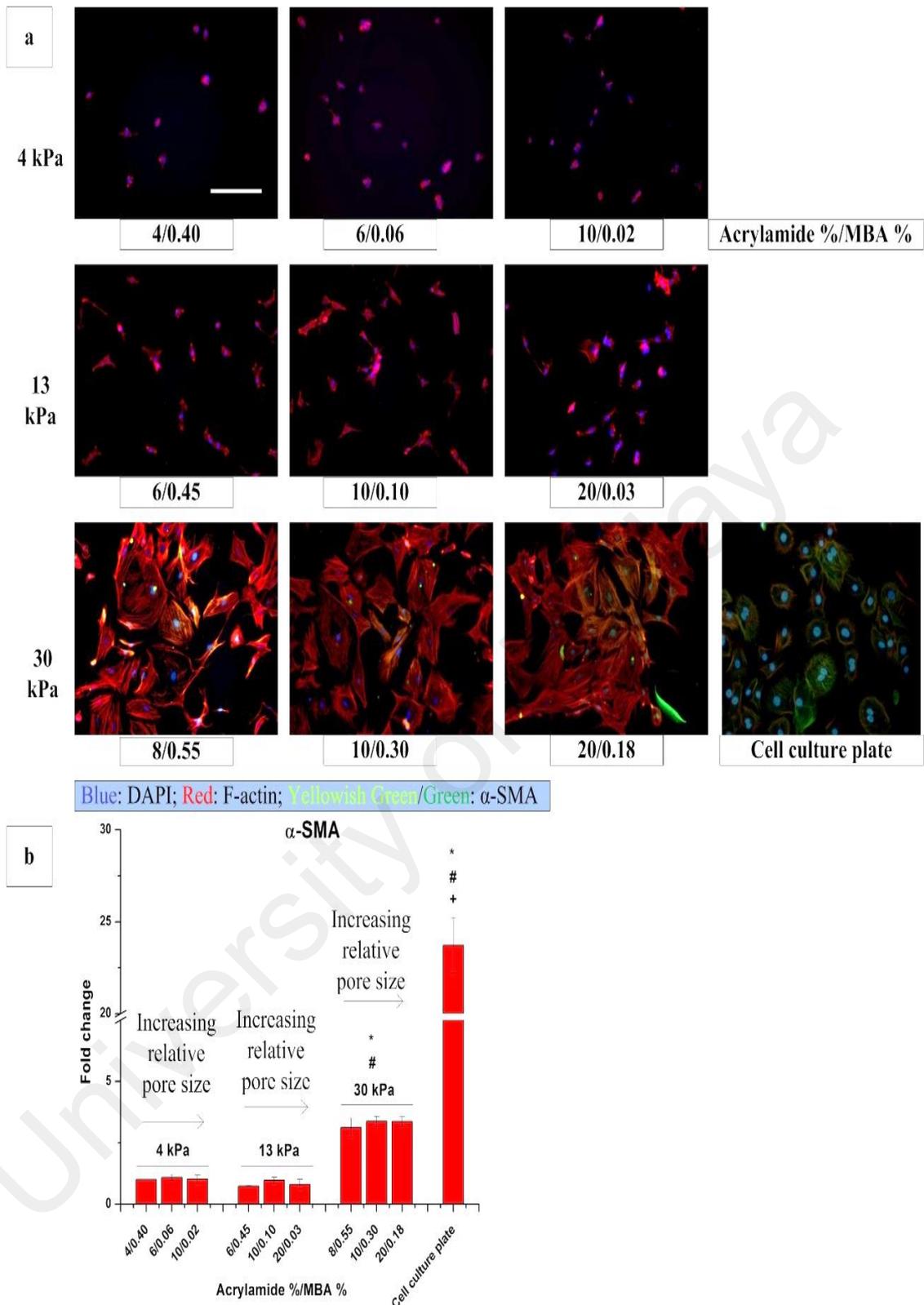


Figure 5.4. Cardiac myofibroblast differentiation was dependent on substrate stiffness instead of pore size. a) Cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate were stained positively by α -SMA. Magnification: 200 \times . Scale bar: 50 μ m. **b)** Varying pore sizes did not affect gene expression levels of α -SMA in cardiac fibroblasts. Cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate display significantly ($p < 0.05$) higher gene expression levels of α -SMA than those cultured on 4 kPa and 13 kPa substrates. Fold change was expressed in relative to 4 kPa substrates with formulation of 4/0.4. MBA: *N,N* methylene-bis-acrylamide.

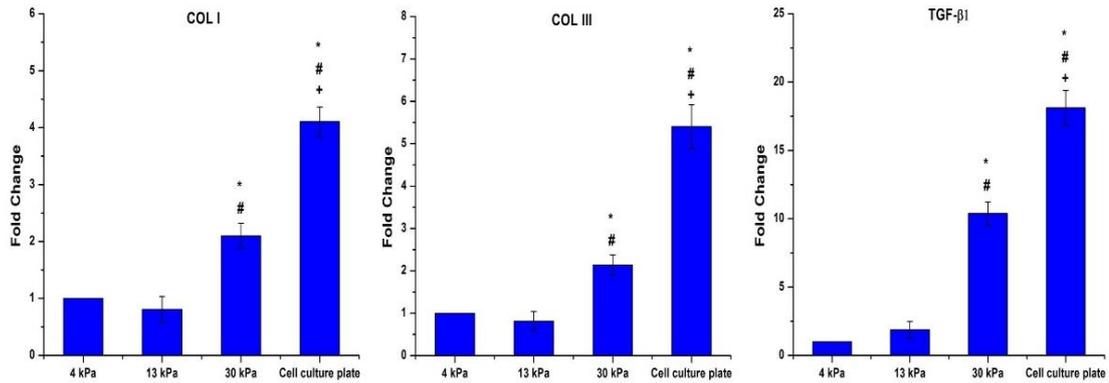


Fig. 5.5. Expression of Col I, Col III and TGF-β1 was dependent on substrate stiffness. A significantly ($p < 0.05$) higher gene expression level of Col I, Col III and TGF-β1 was observed in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate. Fold change was expressed in relative to 4 kPa substrates. * $p < 0.05$ relative to 4 kPa; # $p < 0.05$ relative to 13 kPa; + $p < 0.05$ relative to 30 kPa.

5.3.4 Mechanism of matrix stiffness-induced cardiac myofibroblast differentiation

To date, the comprehensive mechanism of matrix stiffness-induced cardiac myofibroblast differentiation remains elusive, and only TGF-β1 is known to enhance such differentiation in response to stiff ECM (Wells, 2013; Yong et al., 2015a; Zhao et al., 2014). AT₁R, a mechanical-induced signaling pathway (activated in the absence of angiotensin II) (Zablocki & Sadoshima, 2013), has been proven to be activated in cardiac fibroblasts subjected to interstitial fluid shear stress to induce myofibroblast differentiation (Galie et al., 2012). However, the role of AT₁R in matrix stiffness-induced cardiac myofibroblast differentiation is unknown. In the present study, we determined the gene expression level of AT₁R in cardiac fibroblasts in response to various substrate stiffness. It was found that cardiac fibroblasts cultured on 30 kPa substrates display significantly ($p < 0.05$) higher gene expression level of AT₁R than those cultured on soft 4 kPa and 13 kPa substrates, but lowered than those cultured on cell culture plate (**Fig. 5.6a**). These results indicate that AT₁R expression is dependent on substrate stiffness. Further, losartan was used to block AT₁R-mediated signaling to determine the role of this pathway in the cellular responses such as α-SMA expression, secretion of Col I, Col III and TGF-

β 1. Through immunofluorescence staining, it was observed that losartan reduces α -SMA expression in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (**Fig. 5.6b & 5.7**). Moreover, losartan significantly ($p < 0.05$) lowers the gene expression levels of α -SMA, Col I, Col III and TGF- β 1 in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (**Fig. 5.6c**). Blocking of AT₁R did not affect the responses of cardiac fibroblasts cultured on 4 kPa and 13 kPa substrates (**Fig. 5.5c & 5.7**). Taken together, these findings suggest that stiff ECM induces upregulation and activation of AT₁R, which in turn upregulates and activates TGF- β 1 to enhance cardiac myofibroblast differentiation.

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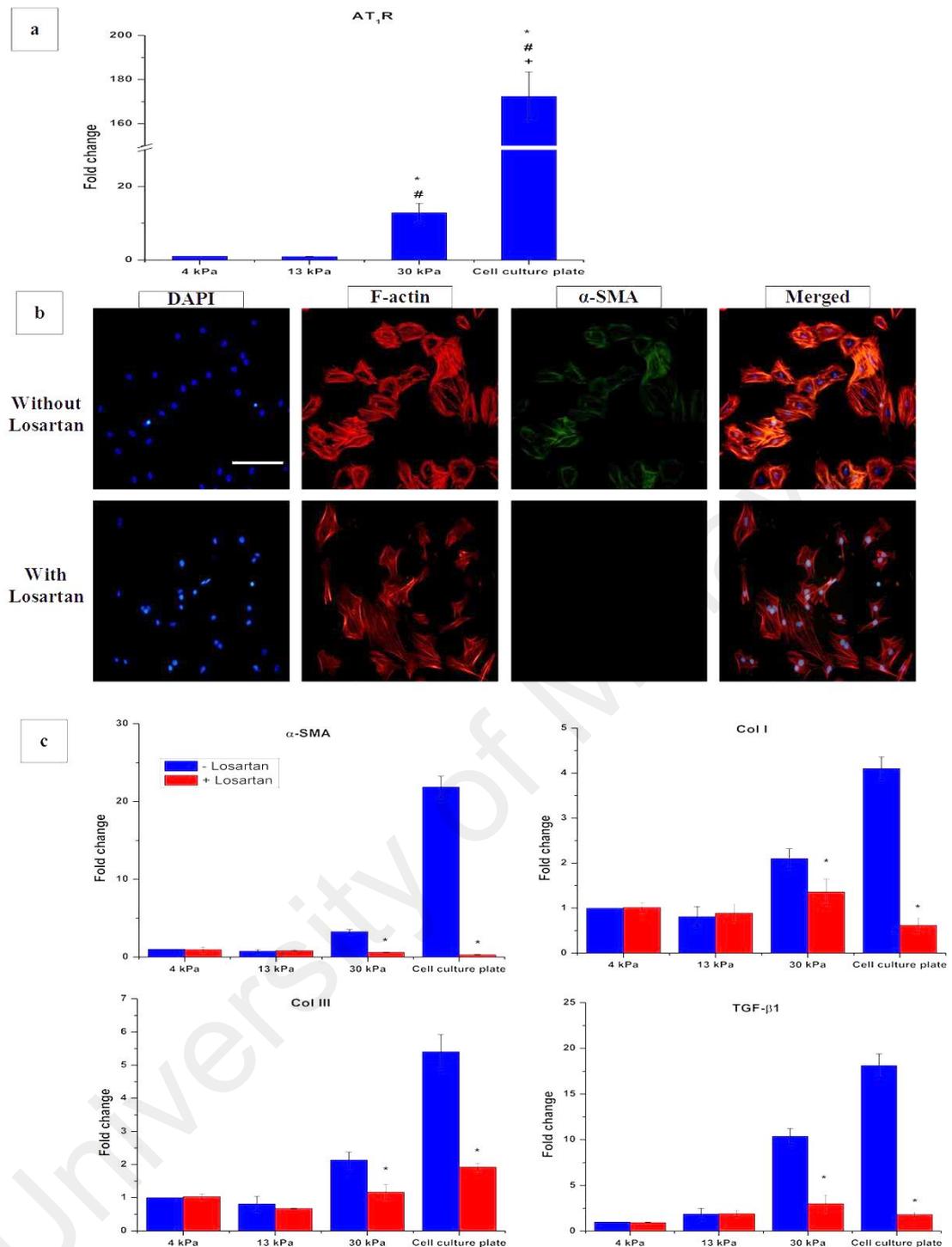


Figure 5.6. Mechanism of matrix stiffness-induced cardiac myofibroblast differentiation. Upregulation and activation of AT₁R induced by stiff substrate enhanced cardiac myofibroblast differentiation. a) A significantly ($p < 0.05$) higher gene expression level of AT₁R was observed in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (* $p < 0.05$ relative to 4 kPa; # $p < 0.05$ relative to 13 kPa; + $p < 0.05$ relative to 30 kPa). Fold change was expressed relative to 4 kPa substrates. **b)** The α -SMA in cardiac fibroblasts cultured on 30 kPa appeared to be reduced in the presence of losartan (AT₁R inhibitor). Magnification: 200 \times . Scale bar: 50 μ m. **c)** Losartan significantly ($p < 0.05$) lowered gene expression levels of α -SMA, Col I, Col III and TGF- β 1 in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (* $p < 0.05$ relative to - losartan). Fold change was expressed in relative to 4 kPa substrates with - losartan.

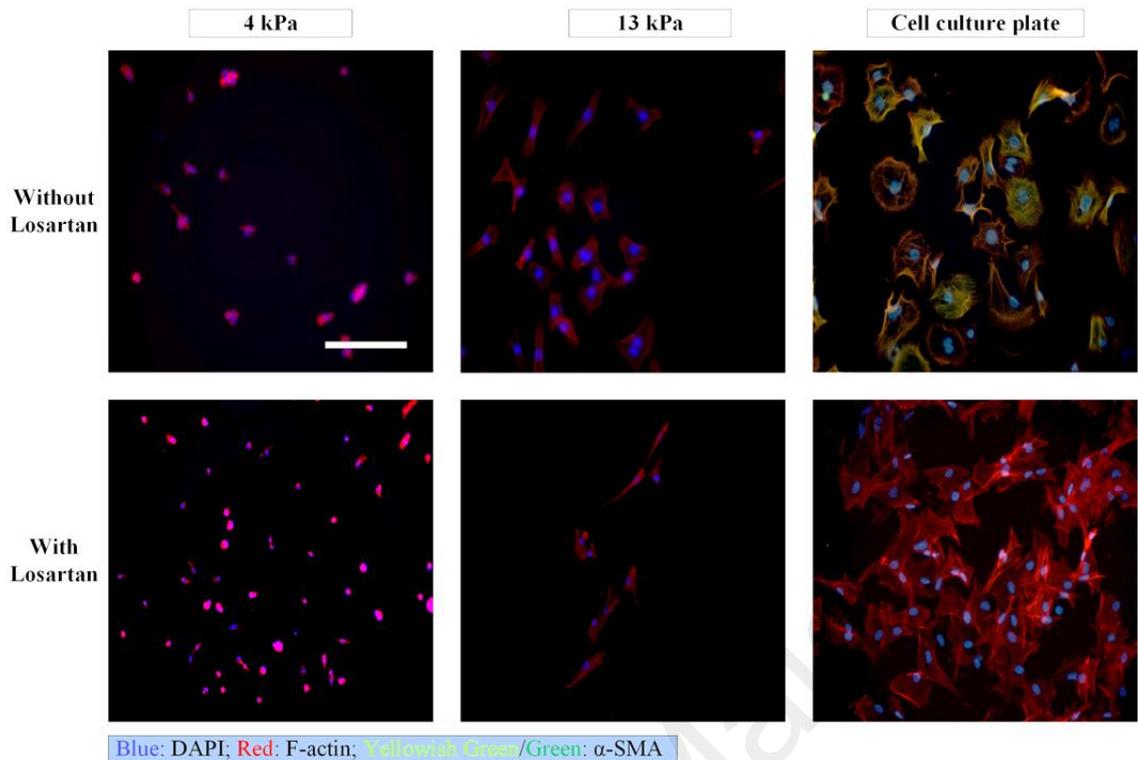


Figure 5.7. Treatment of losartan on cardiac fibroblasts cultured on 4 kPa substrates, 13 kPa substrates and cell culture plate. Blocking AT_1R by losartan did not affect α -SMA expression in cardiac fibroblasts cultured on 4 kPa and 13 kPa substrates. Losartan appeared to reduce expression of α -SMA in cardiac fibroblasts cultured on cell culture plate. Magnification: 200 \times . Scale bar: 50 μ m.

5.3.5 Paracrine effects of fresh and cryopreserved hASCs on cardiac fibrosis

To determine the paracrine effects of fresh and cryopreserved hASCs on cardiac fibrosis, conditioned medium of fresh and cryopreserved hASCs were applied respectively to cardiac fibroblasts for 3 days following 5-day culture on PA hydrogels. It was observed that conditioned medium of both fresh and cryopreserved hASCs reduces the expression of α -SMA in cardiac fibroblasts cultured on 30 kPa substrates (**Fig. 5.8a**) and cell culture plate (**Fig. 5.9**). Further, it was found that conditioned medium of fresh and cryopreserved hASCs significantly ($p < 0.05$) lower the gene expression levels of α -SMA, Col I, Col III and TGF- β 1 in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (**Fig. 5.8b**), while conditioned medium of fresh and cryopreserved hASCs does not have any effect on the responses of cardiac fibroblasts cultured on 4 kPa and 13 kPa substrates

(Fig. 5.8b & Fig. 5.9). These results indicate that conditioned medium of fresh and cryopreserved hASCs contain at least one kind of anti-fibrotic factors that can inhibit matrix stiffness-induced cardiac fibrosis.

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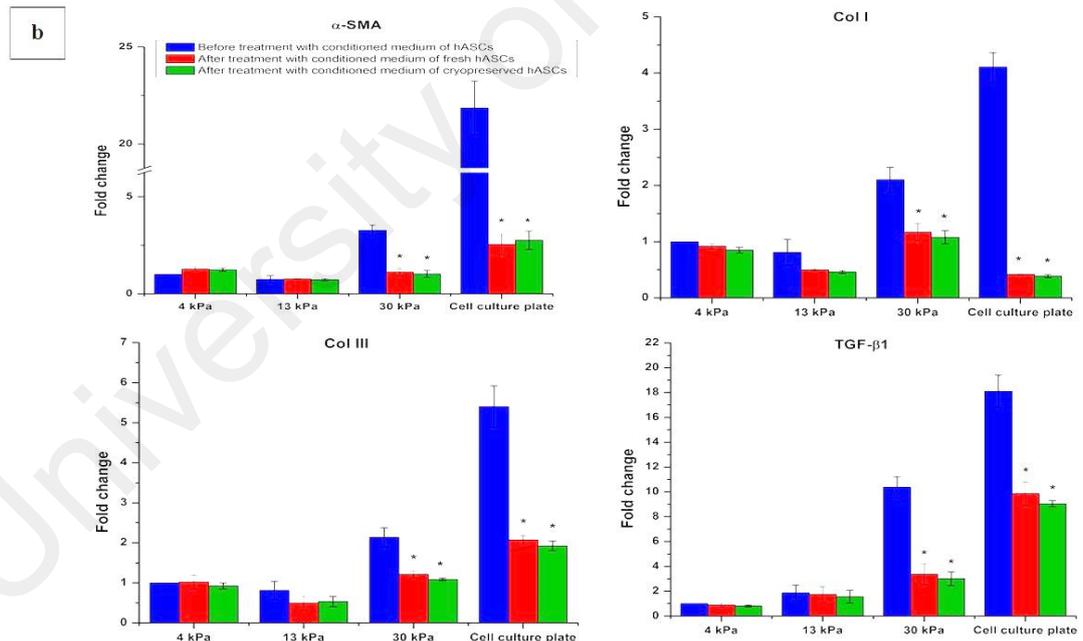
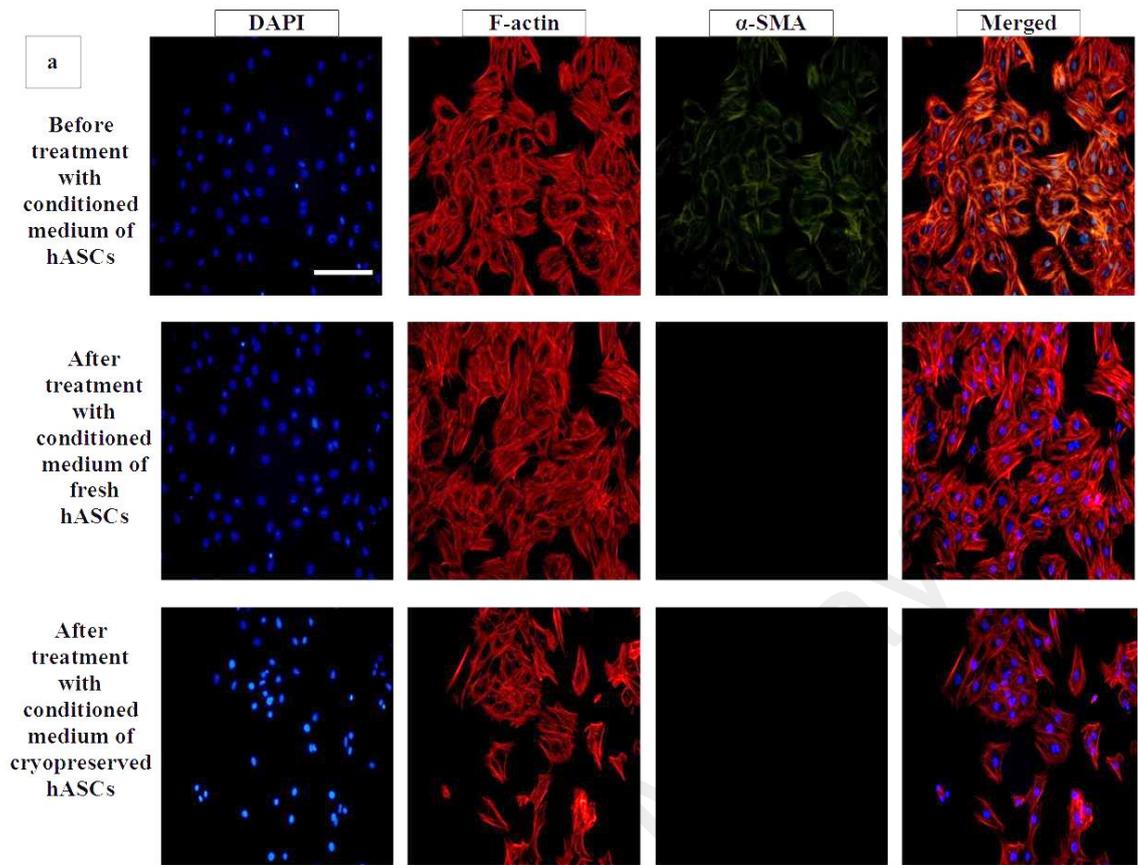


Figure 5.8. Fresh and cryopreserved hASCs inhibited matrix stiffness-induced cardiac myofibroblast differentiation through paracrine effect. **a)** The α -SMA in cardiac fibroblasts cultured on 30 kPa substrates appeared to be reduced in the presence of conditioned medium of fresh and cryopreserved hASCs. Magnification: 200 \times . Scale bar: 50 μ m. **b)** Conditioned medium of fresh and cryopreserved hASCs significantly ($p < 0.05$) lowered gene expression levels of α -SMA, Col I, Col III and TGF- β 1 in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (* $p < 0.05$ relative to before treatment with conditioned medium of hASCs). Fold change was expressed in relative to 4 kPa substrates before treatment with conditioned medium of hASCs.

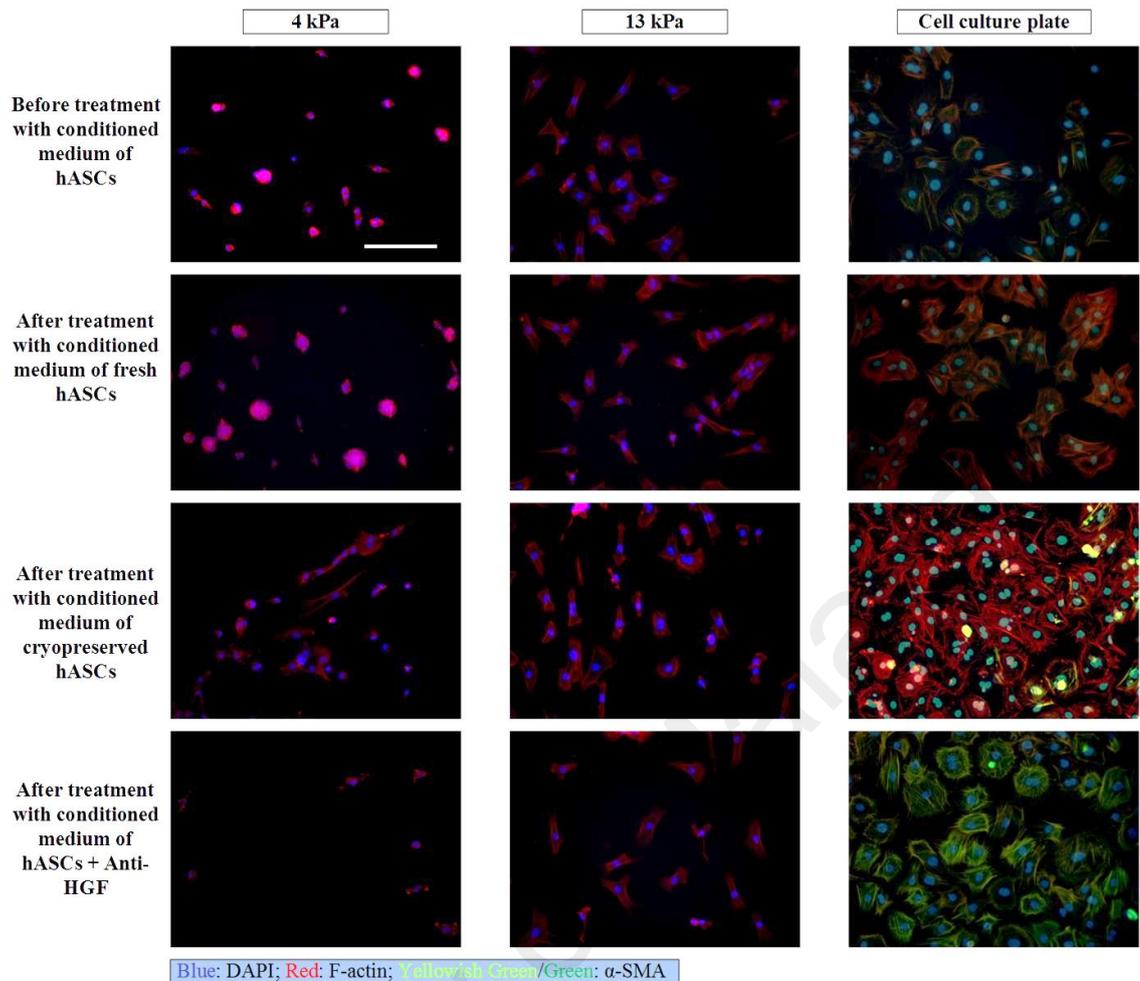


Figure 5.9. Treatment with only conditioned medium of fresh and cryopreserved hASCs or conditioned medium of hASCs + anti-HGF on cardiac fibroblasts cultured on 4 kPa substrates, 13 kPa substrates and cell culture plate. No change of α -SMA expression in cardiac fibroblasts cultured on 4 kPa and 13 kPa substrates. Conditioned medium of fresh and cryopreserved hASCs appeared to reduce expression of α -SMA in cardiac fibroblasts cultured on cell culture plate. Such effect appeared to be abrogated in the presence of anti-HGF. Magnification: 200 \times . Scale bar: 50 μ m.

5.3.6 Inhibitory mechanism of hASCs on cardiac fibrosis

Although significant effort has been put into the study of the anti-fibrotic effects of MSCs (Li et al., 2015; Mao et al., 2013; Mias et al., 2009; Ohnishi et al., 2007; Wang et al., 2011), the mechanism for inhibiting cardiac fibrosis through reduction of cardiac myofibroblast differentiation remains elusive. In the present study, it was found that the conditioned medium of hASCs significantly ($p < 0.05$) lowers the gene expression level of AT_1R in cardiac fibroblasts cultured on stiff substrates (**Fig. 5.10**), indicating that the

conditioned medium of hASCs might downregulate AT₁R which in turn decreases the secretion of TGF- β 1, resulting in lesser cardiac myofibroblast differentiation. In the present study, it was found that cardiac fibroblasts cultured on stiff substrates display a significantly higher gene expression level of Smad7 than those cultured on 4 kPa and 13 kPa substrates. These results demonstrate that Smad7 in cardiac fibroblasts is upregulated in response to substrate stiffness-induced increase in TGF- β 1 to antagonize TGF- β 1 signalling by negative feedback loops. Interestingly, with the addition of conditioned medium of hASCs, cardiac fibroblasts showed a significantly ($p < 0.05$) sharp increase in the gene expression level of Smad7 (**Fig. 5.10**), resulting in lesser cardiac myofibroblast differentiation. Taken together, these findings suggest that the soluble factor in conditioned medium of hASCs might inhibit cardiac myofibroblast differentiation via AT₁R and Smad7.

Among soluble factors secreted by MSCs, HGF, a growth factor which is known to work as an antagonist to decrease TGF- β 1 expression and counteract TGF- β 1 signaling (Inoue et al., 2003; Okayama et al., 2012), may work to inhibit cardiac myofibroblast differentiation. To determine the role of HGF in the conditioned medium of hASCs for inhibition of cardiac myofibroblast differentiation, the active HGF in conditioned medium of hASCs was blocked using anti-HGF, prior to its being given to cardiac fibroblasts. Through immunofluorescence staining, it was observed that cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate with anti-HGF treated conditioned medium of hASCs expressed more α -SMA than those cultured with only conditioned medium of hASCs (**Fig. 5.11a & 5.9**). Further, the gene expression levels of α -SMA, Col I, Col III, TGF- β 1 and AT₁R are significantly ($p < 0.05$) higher, while the expression level of Smad7 is significantly ($p < 0.05$) lower in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate with anti-HGF treated conditioned medium of

hASCs than those cultured with only conditioned medium of hASCs (**Fig. 5.11b**). These results demonstrate that HGF secreted by hASCs plays an essential role on attenuating cardiac fibrosis possibly through the downregulation of AT₁R and upregulation of Smad7.

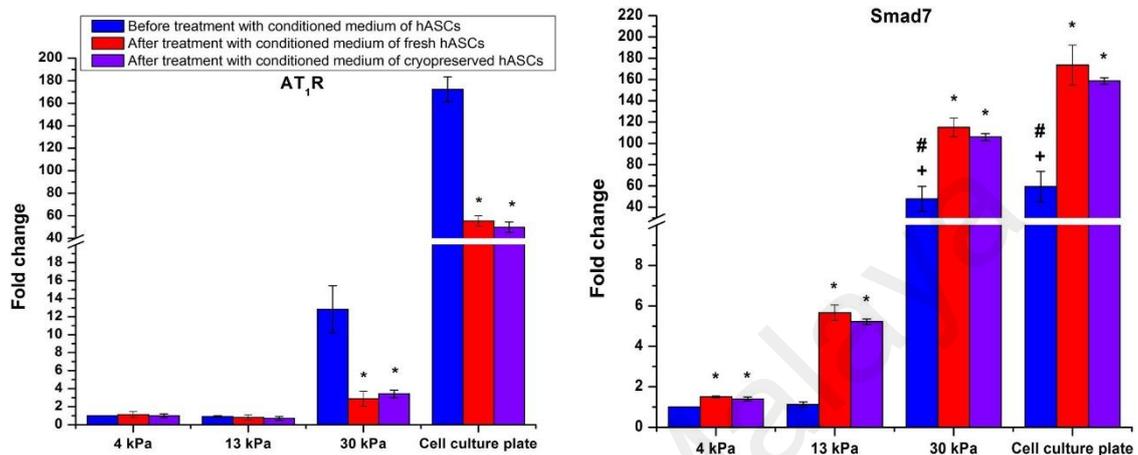


Figure 5.10. Conditioned medium of fresh and cryopreserved hASCs reduced expression of AT₁R while enhanced expression of Smad7. Conditioned medium of fresh and cryopreserved hASCs significantly ($p < 0.05$) lowered the gene expression level of AT₁R and enhanced the gene expression level of Smad7 in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (* $p < 0.05$ relative to before treatment with conditioned medium of hASCs; # $p < 0.05$ relative to 4 kPa; + $p < 0.05$ relative to 13 kPa).

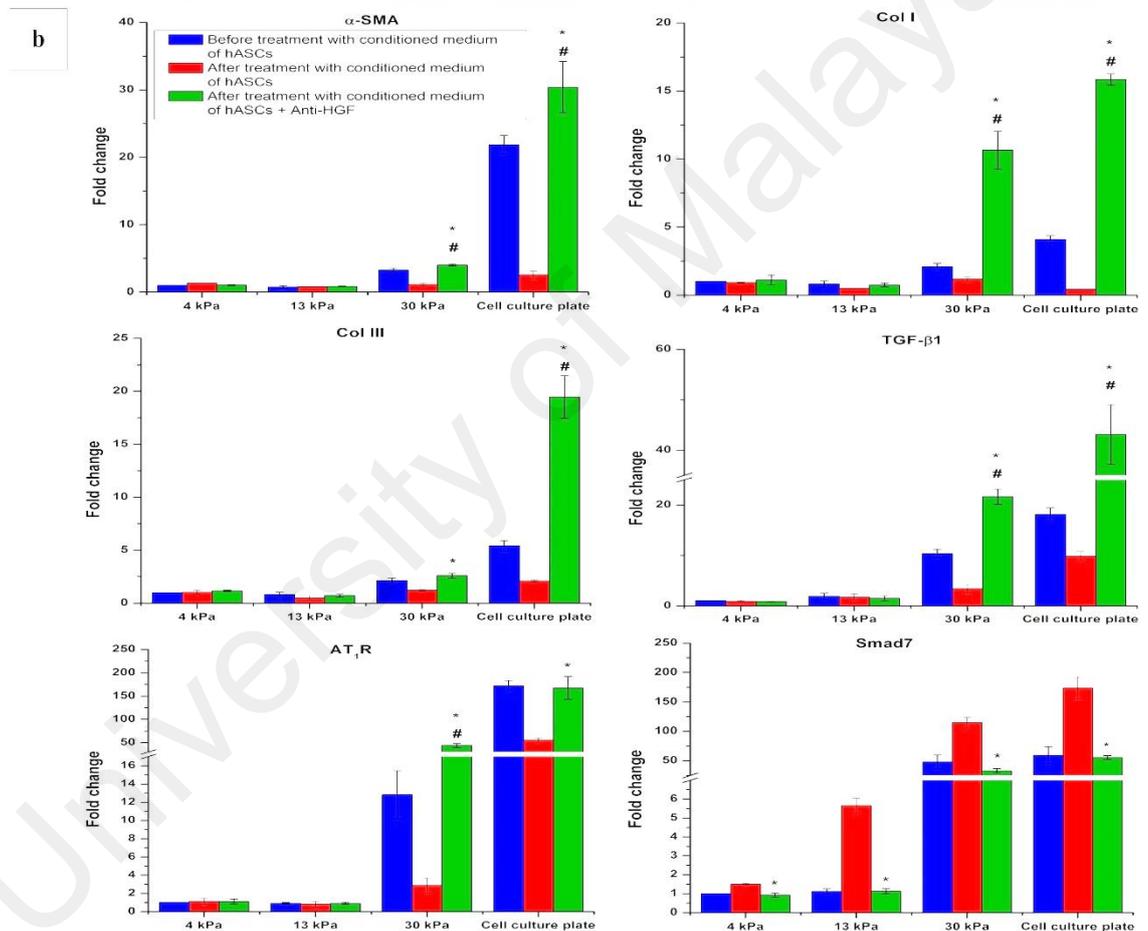
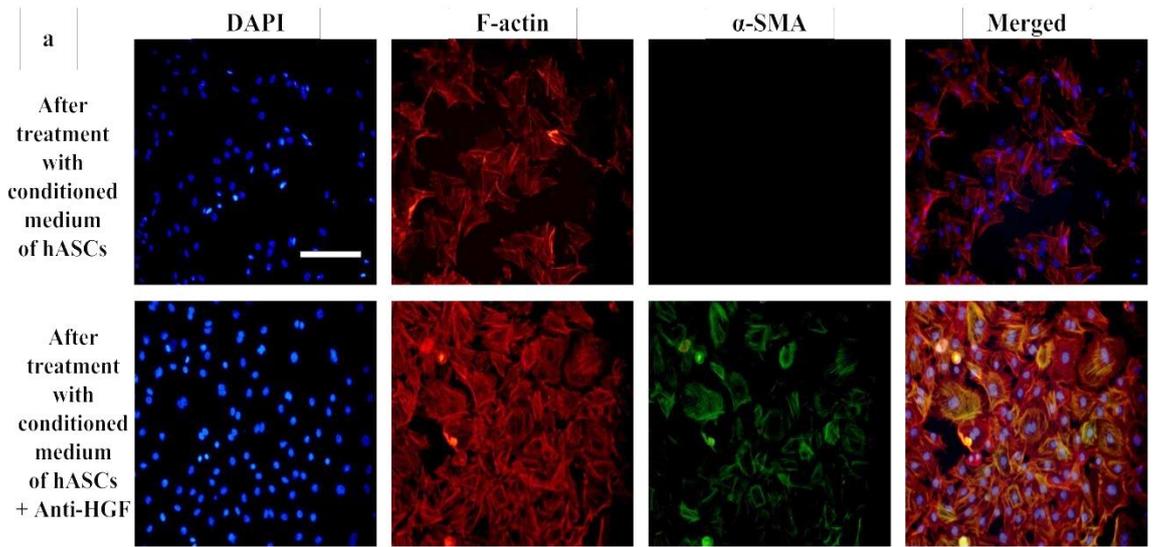


Figure 5.11: hASCs secreted HGF to inhibit cardiac myofibroblast differentiation via AT₁R and Smad7. HGF antibody (anti-HGF) appeared to abrogate the inhibition of conditioned medium of hASCs on the expression of α-SMA in cardiac fibroblasts cultured on 30 kPa substrates. Magnification: 200×. Scale bar: 50 μm. **c)** Anti-HGF negated the anti-fibrotic activity of conditioned medium of hASCs, as indicated by significant ($p < 0.05$) higher gene expression levels of α-SMA, Col I, Col III, TGF-β1 and AT₁R, and lower gene expression level of Smad7 in cardiac fibroblasts cultured on 30 kPa substrates and cell culture plate (* $p < 0.05$ relative to after treatment with conditioned medium of hASCs; # $p < 0.05$ relative to before treatment with conditioned medium of hASCs). Fold change was expressed in relative to 4 kPa substrates before treatment with conditioned medium of hASCs.

ELISA was used to determine the concentration of HGF in the conditioned medium of fresh and cryopreserved hASCs. A reliable standard curve of known concentration of HGF versus absorbance at 450 nm with a high coefficient of determination ($R^2 = 0.99$) was plotted (**Fig. 5.12a**). It was found that the absorbance of conditioned medium of fresh and cryopreserved hASCs at 450 nm is significantly ($p < 0.05$) higher than that of medium without cell and conditioned medium of cardiac fibroblasts (**Fig. 5.12b**), suggesting that conditioned medium of fresh and cryopreserved hASCs contains higher concentration of HGF. There is no significant ($p > 0.05$) difference in terms of absorbance at 450 nm between medium without cell and conditioned medium of cardiac fibroblasts (**Supplemental Fig. 5.12b**), indicating that cardiac fibroblasts did not secrete HGF. Conditioned medium of fresh and cryopreserved hASCs displayed a similar ($p > 0.05$) concentration of HGF (**Fig. 5.12c**).

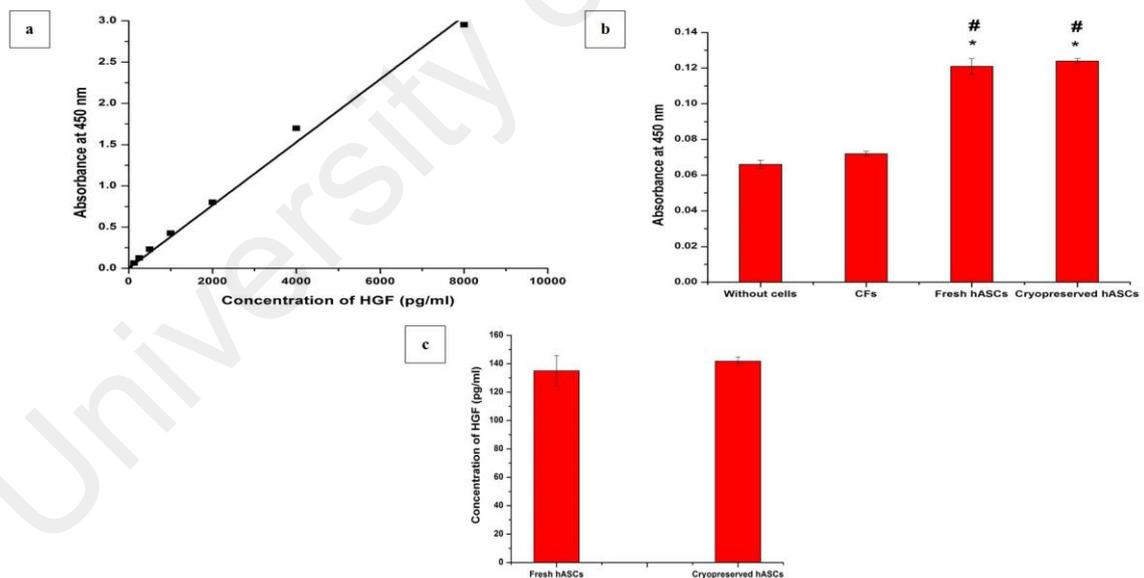


Figure 5.12. A similar concentration of HGF was observed in conditioned medium of fresh and cryopreserved hASCs. a) Standard curve of known concentration of HGF versus absorbance at 450 nm measured through ELISA with coefficient of determination $R^2 = 0.99$. b) Absorbance at 450 nm was significantly ($p < 0.05$) higher in conditioned medium of fresh and cryopreserved hASCs compared to medium without cells and conditioned medium of cardiac fibroblasts (CFs) (* $p < 0.05$ relative to medium without cell; # $p < 0.05$ relative to conditioned medium of CFs). c) By referring to the standard curve, the mean concentration of HGF in conditioned medium of fresh and cryopreserved hASCs are shown to be 135 ± 10.75 pg/mL and 141.7 ± 3.01 pg/mL respectively.

5.4 Discussion

A mechanical tunable cell culture system developed by Engler's group was used in the present study, which allows us to decouple the effect of substrate stiffness and pore size on cardiac myofibroblast differentiation. Col I was selected as the matrix protein coated on PA hydrogels as it represents the majority of ECM (~75%) in the heart (Dobaczewski et al., 2012). As a result, it was demonstrated that cardiac myofibroblast differentiation is dependent on substrate stiffness instead of substrate pore size (represents matrix protein tethering). Substrates mimicking stiffness of native fibrotic cardiac tissues (30 kPa) were found to induce cardiac myofibroblast differentiation, suggesting that cardiac fibroblasts cultured on 30 kPa substrates for 5 days can be used as an *in vitro* cardiac fibrosis model.

Further, it was found that gene expression of AT₁R is dependent on substrate stiffness, which is reported for the first time. AT₁R, a receptor located upstream of TGF- β 1 receptor, is usually activated in response to angiotensin II or shear stress, which in turn increases secretion of TGF- β 1. By blocking AT₁R using losartan, it was found that expressions of TGF- β 1 and α -SMA are reduced, suggesting that losartan might attenuate substrate stiffness-induced increase in TGF- β 1 and α -SMA to inhibit cardiac myofibroblast differentiation through AT₁R. It has been proved that losartan can attenuate AT₁R-mediated signaling induced by angiotensin II or interstitial fluid shear stress to inhibit cardiac myofibroblast differentiation (Campbell & Katwa, 1997; Galie et al., 2012). Taken together, stiff substrate upregulates and activates AT₁R which in turn upregulates TGF- β 1 to promote cardiac myofibroblast differentiation.

Undifferentiated MSCs hold great potential in cardiac fibrosis therapy due to their paracrine functions. MSCs were found to secrete soluble factors that might contribute to anti-fibrotic and endogenous cardiomyogenesis, which aid in cardiac repair and restoring

cardiac function. Among the MSCs derived from different types of human tissue, hASCs, in particular, have attracted special attention due to the ready accessibility and abundance of adipose tissues (Gomillion & Burg, 2006; Kolle et al., 2013; Mizuno et al., 2012). Analyses of the soluble factors released from hASCs have revealed that cultured undifferentiated hASCs at relatively early passages (within passage 5) secrete growth factors (e.g., HGF, IGF-1 and basic fibroblast growth factor (bFGF)), tumor necrosis factor (TNF)- α , cytokines (e.g., interleukin-6 and interleukin-7) and others (Choi et al., 2015; J Salgado et al., 2010; Mizuno et al., 2012). It has been proven that there is a possible cross-species interaction between hASCs and rat cardiac fibroblasts, in which hASCs can reduce rat cardiac myofibroblast differentiation through paracrine effects (Li et al., 2015). However, the inhibitory mechanism of hASCs on cardiac myofibroblast differentiation remains elusive. Following development of an *in vitro* rat cardiac fibrosis model that incorporates stiffness mimicking native fibrotic cardiac tissues, conditioned medium of hASCs was applied to the model to investigate their paracrine effects on cardiac myofibroblast differentiation. Interestingly, it was demonstrated that undifferentiated hASCs can secrete HGF to inhibit matrix stiffness-induced cardiac myofibroblast differentiation via downregulation of AT₁R and upregulation of Smad7, thus reducing cardiac fibrosis. The proposed mechanism of matrix stiffness-induced cardiac myofibroblast differentiation and the inhibitory mechanism of hASCs on such differentiation are depicted in **Fig. 5.13**. It has been reported that conditioned media collected from MSCs cultured on both tissue culture plate and substrates mimicking the stiffness of native fibrotic cardiac tissues contain more HGF than those cultured on substrates mimicking the physiological stiffness of cardiac tissues (Sullivan et al., 2014). These findings suggest that HGF could be secreted by MSCs in response to stiff ECM, which in turn suppresses cardiac fibrosis.

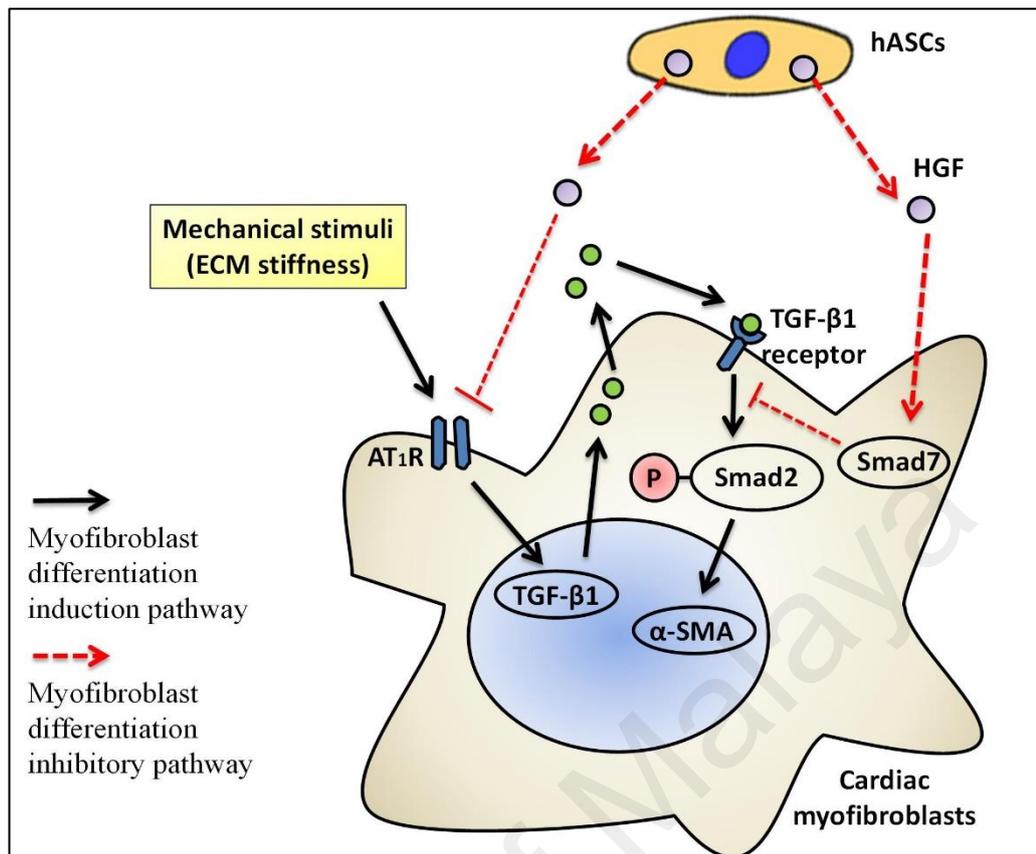


Figure 5.13. Summary of proposed mechanism of matrix stiffness-induced cardiac myofibroblast differentiation and anti-fibrotic effects of undifferentiated hASCs. HGF: hepatocyte growth factor; AT₁R: angiotensin II type 1 receptor; TGF-β1: transforming growth factor beta-1; P-Smad2: phosphorylated Smad2; α-SMA: alpha-smooth muscle actin. ECM: extracellular matrix.

It has been demonstrated that HGF inhibits the increase in AT₁R expression to reduce myofibroblast differentiation of glomerular mesangial cells in the kidney (Iekushi et al., 2011). HGF might bind to HGF receptor (c-met) to attenuate AT₁R expression through inhibition of phosphatidylinositol 3-kinase/Akt signalling pathway (Iekushi et al., 2011). On the other hand, HGF also activates Smad7 to inhibit myofibroblast differentiation of alveolar epithelial cells in lungs (Shukla et al., 2009). Smad7, an inhibitory Smad, is upregulated in response to increasing TGF-β1, and binds to TGF-β1 receptor to prevent interaction of Smad2 with the receptor (Hayashi et al., 1997; Nakao et al., 1997), reducing the expression of α-SMA or myofibroblast differentiation. HGF-induced increase in Smad7 could be contributed by mitogen-activated protein kinase – extracellular-signal-regulated kinase (MEK - ERK) signalling, which in turn blocks

phosphorylation or activation of Smad2 (Hayashi et al., 1997; Shukla et al., 2009). Further studies are needed to explore the roles of these pathways on regulation of AT₁R and Smad7 by HGF in cardiac myofibroblasts. Recently, it has been showed that hASCs may exert anti-fibrotic effects by directly communicating with myofibroblasts (Li et al., 2015). Taken together, hASCs may hold great promise for treating cardiac fibrosis through both paracrine effects and direct cell-to-cell contact.

Although evidence continues to augment that hASCs have great potential as a cardiac fibrosis therapy, but there are many challenges that need to be addressed. First, cardiac fibrosis therapy is an intensive clinical setting which requires a massive amount of cells which *in vitro* cell expansion might not be able to supply in a short duration. Further, hASCs under long-term *in vitro* culture could be prone to contamination, malignant transformation, phenotypic instability and chromosomal aberration (Froelich et al., 2013; Roemeling-van Rhijn et al., 2013). Therefore, a bank of cryopreserved cells is an ideal option which offers a ready off-the-shelf supply of cells for transplant while allowing better timing for therapy. To achieve this, it is essential to cryopreserve hASCs at an early passage (within passage 4) for long-term in order to supply a pool of efficacious and safe cells for transplant, as their therapeutic efficacy might decrease with biosafety concerns raised at late passage (Marquez-Curtis et al., 2015). In the present study, conditioned medium of long-term cryopreserved hASCs (passage 3) also was applied to the *in vitro* cardiac fibrosis model to evaluate their inhibitory effects on cardiac myofibroblast differentiation. It was found that conditioned medium of hASCs cryopreserved in 5% DMSO display a similar potential to inhibit cardiac myofibroblast differentiation as compared to fresh hASCs. Further, a similar concentration of HGF was observed in the conditioned medium of cryopreserved and fresh hASCs, suggesting that cryopreservation does not affect the paracrine function of hASCs. In conjunction with the previous findings

indicating cell functionality of hASCs cryopreserved in 5% DMSO was maintained with a low risk of tumorigenesis, long-term cryopreserved hASCs would be an ideal cell source for cardiac fibrosis therapy in future.

Second, the therapeutic effects of implanted MSCs have been shown greatly reduced under stiff ECM during cardiac fibrosis. For instance, when undifferentiated MSCs were implanted into the heart of rat model of myocardial infarction, they showed bone tissue formation in response to stiff ECM (Breitbach et al., 2007). Last but not least, engraftment or retention of MSCs in the heart is low by any method of delivery (*e.g.*, intramyocardial and intracoronary) (Hou et al., 2005). Therefore, further investigation is needed to assess the delivery time and mode for therapeutic use of hASCs in human body to achieve an efficient and successful cardiac fibrosis therapy.

5.5 Conclusion

Cardiac fibroblasts cultured on 30 kPa hydrogels, which mimic the stiffness of native fibrotic cardiac tissues, can be used as an *in vitro* cardiac fibrosis model. Fresh and cryopreserved hASCs can secrete HGF to reduce cardiac fibrosis and myofibroblast differentiation possibly through the downregulation of AT₁R and upregulation of Smad7. With the development of stem cell delivery method, hASCs may hold great potential for cardiac fibrosis therapy and heart regeneration in future.

CHAPTER 6: CONCLUSION AND FUTURE WORK

6.1 Thesis contribution

This thesis is contributed principally to knowledge. The contributions are as follows:

- i. A cryopreservation method, which adopts an approach using a low concentration of DMSO (5% DMSO), can be used widely to efficiently store hASCs for clinical application.
- ii. Cryopreserved hASCs are at low risk of raising biosafety concerns, which supports their use in various clinical settings.
- iii. Cardiac myofibroblast differentiation is regulated by substrate stiffness independent of substrate pore size.
- iv. An *in vitro* cardiac fibrosis model which incorporates stiffness mimicking native fibrotic cardiac tissues has been developed, allowing the pathophysiological and therapeutic intervention study of cardiac fibrosis in an accurate manner.
- v. hASCs preserved in 5% DMSO could be an ideal cell source for various clinical applications, including but not limited to cardiac fibrosis.

6.2 Conclusion

In chapter 3, the cells isolated from human adipose tissue were characterized. It has been showed that the cells are hASCs, as indicated by their adherent property, positive expression of surface markers of MSCs (CD90, CD73 and CD105 and CD44) and nucleated cells (HLA ABC), negative expression of surface markers of hematopoietic cells (CD14, CD19, CD34, CD45 and HLA DQDPDR) and ability of trilineage differentiation (adipogenesis, osteogenesis and chondrogenesis).

In chapter 4, the viability, functional properties and biosafety of hASCs following the long-term cryopreservation have been evaluated. The findings showed that long-term cryopreserved hASCs maintained their functional properties (*e.g.*, stemness, proliferation and trilineage differentiation potential) and a low risk of tumorigenesis. Among the CPAs, 5% DMSO without FBS which is less cytotoxic and free of xeno-agent while maintaining high viability of hASCs, would be an ideal CPA to cryopreserve hASCs in long-term for clinical applications.

In chapter 5, an *in vitro* cardiac fibrosis model has been developed and used for the evaluation of potential therapeutic application of hASCs in cardiac fibrosis. Cardiac fibroblasts cultured on 30 kPa hydrogels can be used as an *in vitro* cardiac fibrosis model, as such stiffness, which mimics the stiffness of native fibrotic cardiac tissues, induces cardiac myofibroblast differentiation. In addition, fresh hASCs and hASCs cryopreserved in 5% DMSO have been assessed of their potential in reducing cardiac fibrosis using the *in vitro* cardiac fibrosis model. The findings demonstrated that hASCs cryopreserved in 5% DMSO display similar potential as fresh hASCs in reducing cardiac fibrosis through inhibition of cardiac myofibroblast differentiation via downregulation of AT₁R and upregulation of Smad7 in a paracrine manner. hASCs cryopreserved in 5% DMSO could be an ideal cell source for cardiac fibrosis therapy.

Collectively, 5% DMSO could be an ideal CPA for long-term cryopreservation of hASCs for clinical applications. hASCs cryopreserved in such formulation demonstrates their potential therapeutic application in cardiac fibrosis. These findings would help to standardize the cryopreservation protocol of hASCs and optimize the therapeutic benefit of cryopreserved hASCs in cardiac fibrosis.

6.3 Limitation of the study and future work

The viability of cryopreserved hASCs (75% - 85%) can be further improved. Cryopreserved hASCs can be pre-cultured in a hypoxic condition for a short period prior to being used in clinical applications, as hypoxia has been showed to enhance the viability of fresh hASCs in culture. On the other hand, the development of an alternative CPA to completely replace DMSO remains challenging, which requires an extensive investigation. Since hASCs cryopreserved in 5% DMSO have displayed their potential therapeutic application in cardiac fibrosis, their therapeutic potential in other various clinical settings (*e.g.*, diabetes and cancer therapy) should be explored.

The *in vitro* cardiac fibrosis model developed in this study is still lack of paracrine factors arising from cardiomyocytes. In future, current model can be adapted to co-culture cardiac fibroblasts and cardiomyocytes, which allows the mechanistic investigation of their interaction in cardiac fibrosis. Meanwhile, paracrine effects of hASCs on cardiomyocyte regeneration also can be investigated. However, there is a limited assay to distinguish the response of different cells in a co-culture setting. This issue should be addressed prior to the development of an *in vitro* cardiac fibrosis model incorporating both cardiac fibroblasts and cardiomyocytes. With the advance in the development of induced pluripotent stem cells (iPSCs) and hydrogel fabrication techniques (*e.g.*, bioprinting), a 3D *in vitro* cardiac fibrosis model made of human cardiac fibroblasts and cardiomyocytes which both are derived from iPSCs, allowing deep insights into human cell response *in vivo* in an accurate manner. In addition, mechanical strain (*e.g.*, mechanical stretch and interstitial fluid flow), which also play a role in cardiac myofibroblast differentiation, can be incorporated into the model to develop a hypertension-induced cardiac fibrosis model for studying hypertension.

The engraftment or retention of MSCs in the heart is low by any mode of delivery (*e.g.*, intramyocardial and intracoronary). Therefore, the delivery time and mode of cryopreserved hASCs into patients with cardiac fibrosis should be optimized to achieve a successful and efficient cardiac fibrosis therapy.

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LIST OF PUBLICATIONS

1. **Yong, K. W.**, Pingguan-Murphy, B., Xu, F., Abas, W. A., Choi, J. R., Omar, S. Z., . . . Wan Safwani, W. K. (2015). Phenotypic and Functional Characterization of Long-Term Cryopreserved Human Adipose-Derived Stem Cells. *Sci Rep*, 5(9596).
2. **Yong, K. W.**, Wan Safwani, W. K., Xu, F., Wan Abas, W. A., Choi, J. R., & Pingguan-Murphy, B. (2015). Cryopreservation of Human Mesenchymal Stem Cells for Clinical Applications: Current Methods and Challenges. *Biopreserv Biobank*, 13(4), 231-239.
3. **Yong, K. W.**, Safwani, W. K., Xu, F., Zhang, X., Choi, J. R., Abas, W. A., . . . Pingguan-Murphy, B. (2016). Assessment of tumourigenic potential in long-term cryopreserved human adipose-derived stem cells. *J Tissue Eng Regen Med*, 12(10).
4. **Yong, K. W.**, Li, Y., Huang, G., Lu, T. J., Safwani, W. K., Pingguan-Murphy, B., & Xu, F. (2015). Mechanoregulation of Cardiac Myofibroblast Differentiation: Implications for Cardiac Fibrosis and Therapy. *Am J Physiol Heart Circ Physiol*, 309(4), 19.

APPENDIX A

Human Medical Ethic Approval Letter

	UNIVERSITI MALAYA	MEDICAL ETHICS COMMITTEE UNIVERSITY MALAYA MEDICAL CENTRE ADDRESS: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA TELEPHONE: 03-79493209 / 2251 FAXIMILE: 03-79492030
PUSAT PERUBATAN UM		
NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre		ETHICS COMMITTEE/IRB REFERENCE NUMBER: 996.46
ADDRESS: LEMBAH PANTAI 59100 KUALA LUMPUR		
PROTOCOL NO (if applicable): -		VERSION NO.: -
TITLE: Comparison of the effects of different culture conditions on the cardiogenic ability of human adipose derived stem cells		
PRINCIPAL INVESTIGATOR: Dr. Belinda Murphy		SPONSOR: -

The following item have been received and reviewed in connection with the above study to be conducted by the above investigator.

<input checked="" type="checkbox"/> Application for Amendment/Information to Research Project (form)	Ver date: 06 Jun 13
<input type="checkbox"/> Annual Study Report/Study Closure Report	Ver date:
<input type="checkbox"/> Serious Adverse Event Report	Ver date:

Other documents:-
 Change of Title Study (Introduction & Study Protocol)

and the decision is :

<input checked="" type="checkbox"/> Approved
<input type="checkbox"/> Modification requested (item specified below or in accompanying letter)
<input type="checkbox"/> Rejected (reasons specified below or in accompanying letter)
<input type="checkbox"/> Noted

Comments:

Investigator are required to:

- 1) follow instructions, guidelines and requirements of the Medical Ethics Committee.
- 2) report any protocol deviations/violations to Medical Ethics Committee.
- 3) provide annual and closure reports to the Medical Ethics Committee.
- 4) comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and the Declaration of Helsinki.
- 5) obtain permission from the Director of UMMC before starting research that involves recruitment of UMMC patients.
- 6) ensure that if the research is sponsored, the usage of consumable items and laboratory tests from UMMC services are not charged in the patient's hospital bills but are borne by the research grant.
- 7) note that he/she can appeal to the Chairman of MEC for studies that are rejected.
- 8) note that Medical Ethics Committee may audit the approved study.
- 9) ensure that the study does not take precedence over the safety of subjects.

Date of approval : 17th JUNE 2013
 Date of notification: -

c.c Head
 Department of Biomedical Engineering
 Faculty of Engineering, UM

Deputy Dean (Research)
 Faculty of Medicine

Secretary
 Medical Ethics Committee
 University Malaya Medical Centre

.....
PROF. DATUK LOOI LAI MENG
 Chairman
 Medical Ethics Committee

APPENDIX B

Patient consent form

UNIVERSITY MALAYA MEDICAL CENTRE

CONSENT BY PATIENT FOR RESEARCH

I,
Identity Card No.
(Name of Patient)
of
(Address)
hereby agree to take part in the research (lab based/clinical study/questionnaire study/drug trial) specified below:

Title of Study: THE STUDY OF THE ABILITY OF CELLS FROM FAT TISSUE TO TURN INTO HEART CELLS
.....

the nature and purpose of which has been explained to me by
Dr.
(Name & Designation of Doctor)
and interpreted by
(Name & Designation of Interpreter)
..... to the best of his/her ability in language/dialect.

I have been told about the nature of the research in terms of methodology, possible adverse effects and complications (as per patient information sheet). After knowing and understanding all the possible advantages and disadvantages of this research, I voluntarily consent of my own free will to participate in the research specified above. I also understand that there is no additional risk involved in participating in this study (as per patient information sheet).

I understand that I can withdraw from this clinical research at any time without assigning any reason whatsoever and in such a situation shall not be denied the benefits of usual treatment by the attending doctors.

Date: Signature or Thumbprint
(Patient)

IN THE PRESENCE OF

Name)
Identity Card No.)
Designation)
Signature
(Witness for Signature of Patient)

I confirm that I have explained to the patient the nature and purpose of the above-mentioned research.

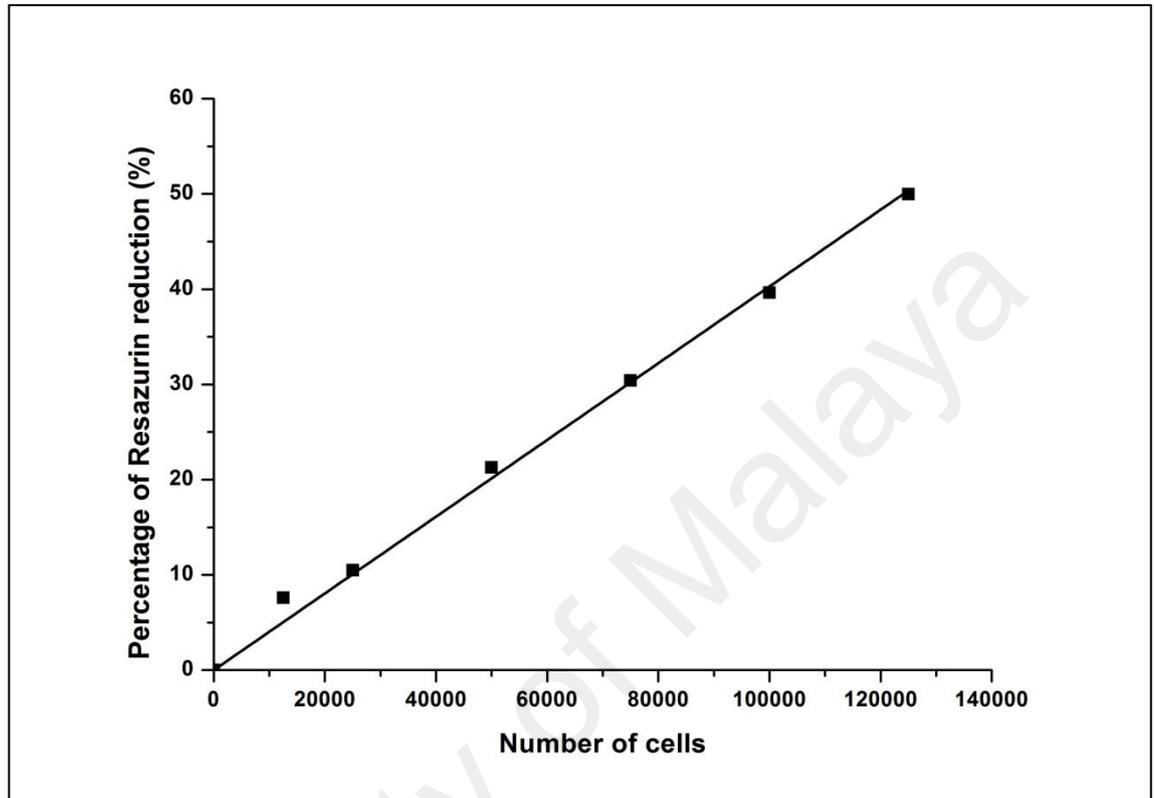
Date Signature
(Attending Doctor)

CONSENT BY PATIENT
FOR
RESEARCH

R.N.
Name
Sex
Age
Unit

APPENDIX C

Standard curve of percentage of resazurin reduction versus cell number ($R^2 = 0.999$)





OPEN

SUBJECT AREAS:
MESENCHYMAL STEM
CELLS
STEM-CELL RESEARCH

Received
28 November 2014

Accepted
4 March 2015

Published
15 April 2015

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Phenotypic and Functional Characterization of Long-Term Cryopreserved Human Adipose-derived Stem Cells

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Cryopreservation represents an effective technique to maintain the functional properties of human adipose-derived stem cells (ASCs) and allows pooling of cells via long-term storage for clinical applications, e.g., cell-based therapies. It is crucial to reduce freezing injury during the cryopreservation process by loading the ASCs with the optimum concentration of suitable cryoprotective agents (CPAs). In this study, human ASCs were preserved for 3 months in different combinations of CPAs, including 1) 0.25 M trehalose; 2) 5% dimethylsulfoxide (DMSO); 3) 10% DMSO; 4) 5% DMSO + 20% fetal bovine serum (FBS); 5) 10% DMSO + 20% FBS; 6) 10% DMSO + 90% FBS. Interestingly, even with a reduction of DMSO to 5% and without FBS, cryopreserved ASCs maintained high cell viability comparable with standard cryomedium (10% DMSO + 90% FBS), with normal cell phenotype and proliferation rate. Cryopreserved ASCs also maintained their differentiation capability (e.g., to adipocytes, osteocytes and chondrocytes) and showed an enhanced expression level of stemness markers (e.g., NANOG, OCT-4, SOX-2 and REX-1). Our findings suggest that 5% DMSO without FBS may be an ideal CPA for an efficient long-term cryopreservation of human ASCs. These results aid in establishing standardized xeno-free long-term cryopreservation of human ASCs for clinical applications.

Stem cells hold great potential for many biomedical applications, particularly cell-based therapies and regenerative medicine^{1,2} due to their capability of self-renewing and differentiating into multiple specific types of cells^{3,4}. Among various stem cells, adipose-derived stem cells (ASCs) have attracted special attention due to their readily accessibility and the abundance of adipose tissue⁵⁻⁷. However, whilst intensive applications require a large number of cells, *in vitro* expansion of ASCs may not yield sufficient cell numbers in a short duration. Therefore, the cells should be capable of being preserved in the long-term with cell functionality maintained for off-the-shelf use⁸. Cryopreservation may be an ideal option for this, which is currently the only method to preserve ASCs with maintained functional properties and genetic characteristics in the long term⁹.

Various methods have been developed for cryopreservation of various stem cells including human ASCs, such as slow freezing and vitrification¹⁰⁻¹⁵. Vitrification only fits well with cryopreservation of human cells in small volumes such as oocytes¹⁶ but is ill suited to large volumes of ASCs¹⁷. Further, it might lead to potential contamination with pathogenic agents due to the direct exposure of cells to non-sterile liquid nitrogen¹⁸, and it involves the potential issue of cell loss due to inefficient cell collection. Thus far, the slow freezing method is the most preferable method of cell cryopreservation in research laboratories today, due to the low risk of contamination and it being an easier process¹⁹. However, slow freezing results in a high risk of freeze injury (e.g., cell death) due to the formation of intra- and extracellular ice during the freezing process²⁰⁻²². To address this issue, optimization of the use of cryoprotective agents (CPAs) is very important to avoid the formation of ice crystal by loading the ASCs with the optimum concentration of suitable CPAs. Among various CPAs, 10% DMSO and FBS

Cryopreservation of Human Mesenchymal Stem Cells for Clinical Applications: Current Methods and Challenges

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Mesenchymal stem cells (MSCs) hold many advantages over embryonic stem cells (ESCs) and other somatic cells in clinical applications. MSCs are multipotent cells with strong immunosuppressive properties. They can be harvested from various locations in the human body (e.g., bone marrow and adipose tissues). Cryopreservation represents an efficient method for the preservation and pooling of MSCs, to obtain the cell counts required for clinical applications, such as cell-based therapies and regenerative medicine. Upon cryopreservation, it is important to preserve MSCs functional properties including immunomodulatory properties and multilineage differentiation ability. Further, a biosafety evaluation of cryopreserved MSCs is essential prior to their clinical applications. However, the existing cryopreservation methods for MSCs are associated with notable limitations, leading to a need for new or improved methods to be established for a more efficient application of cryopreserved MSCs in stem cell-based therapies. We review the important parameters for cryopreservation of MSCs and the existing cryopreservation methods for MSCs. Further, we also discuss the challenges to be addressed in order to preserve MSCs effectively for clinical applications.

Introduction

STEM CELLS ARE IDEAL CANDIDATES for many biomedical applications, particularly cell-based therapies and regenerative medicine.^{1,2} Stem cells are divided into two broad types: embryonic stem cells (ESCs), obtained from the inner cell mass of blastocysts, and adult stem cells, particularly MSCs, found in adult tissues.^{3,4} MSCs hold great potential in clinical applications by avoiding the ethical concerns related to ESCs.^{5,6} MSCs can be isolated from various locations other than bone marrow,^{7,8} including fat,^{9,10} periosteum,¹¹ amniotic fluid,¹² and umbilical cord blood.¹³

MSCs are also capable of differentiating to multiple specific types of cells such those in bone or cartilage,^{14,15} and implantation of MSCs may overcome the problems of the organ shortage crisis. MSCs also have strong immunoregulatory and immunosuppressive effects, which support the use of MSCs in allogeneic transplantation.^{16,17} Implantation of allogeneic MSCs can avoid the graft versus host diseases (GvHD), which are often observed in recipients after allogeneic organ transplantation.^{18,19}

To achieve the large numbers of MSCs required for clinical applications, the cells either need to be expanded in

culture, or MSCs from multiple donors must be pooled. In either case, a method for long-term storage of the cells is required for off-the-shelf availability.²⁰ Cryopreservation is currently the only method to preserve cells, including MSCs, for any considerable period. Cryopreservation maintains cell functional properties and allows pooling of cells to reach the cell numbers required for clinical application.²¹ Without cryopreservation, cells have to be continuously subcultured, which may accumulate genetic changes, resulting in heterogeneity or tumorigenicity.^{22,23}

At -196°C (i.e., in liquid nitrogen), cells have no metabolic demands, thus avoiding biological variation due to genetic drift even when stored for multiple years. Further, cells are protected from infection while cryopreserved.²⁴ Therefore, cryopreservation provides cells with their specific genetic characteristics and intact function at specific passages for clinical and research purposes.²⁵ The modern technique of cryopreservation allows the long-term storage of living cells and tissues that offer a great potential for clinical applications, including bone marrow transplantation,²⁵ bone grafts,²⁶ blood transfusion,²⁷ bone marrow transplantation,²⁵ and *in vitro* fertilization.²⁸ Although preclinical studies have shown that implantation of cryopreserved MSCs is capable of restoring

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Assessment of tumourigenic potential in long-term cryopreserved human adipose-derived stem cells

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Abstract

Cryopreservation represents an efficient way to preserve human mesenchymal stem cells (hMSCs) at early culture/passage, and allows pooling of cells to achieve sufficient cells required for off-the-shelf use in clinical applications, e.g. cell-based therapies and regenerative medicine. To fully apply cryopreserved hMSCs in a clinical setting, it is necessary to evaluate their biosafety, e.g. chromosomal abnormality and tumourigenic potential. To date, many studies have demonstrated that cryopreserved hMSCs display no chromosomal abnormalities. However, the tumourigenic potential of cryopreserved hMSCs has not yet been evaluated. In the present study, we cryopreserved human adipose-derived mesenchymal stem cells (hASCs) for 3 months, using a slow freezing method with various cryoprotective agents (CPAs), followed by assessment of the tumourigenic potential of the cryopreserved hASCs after thawing and subculture. We found that long-term cryopreserved hASCs maintained normal levels of the tumour suppressor markers p53, p21, p16 and pRb, hTERT, telomerase activity and telomere length. Further, we did not observe significant DNA damage or signs of p53 mutation in cryopreserved hASCs. Our findings suggest that long-term cryopreserved hASCs are at low risk of tumourigenesis. These findings aid in establishing the biosafety profile of cryopreserved hASCs, and thus establishing low hazardous risk perception with the use of long-term cryopreserved hASCs for future clinical applications. Copyright © 2016 John Wiley & Sons, Ltd.

Received 8 May 2015; Revised 17 September 2015; Accepted 18 November 2015

Keywords long-term cryopreservation; human adipose-derived stem cells; tumourigenic potential assessment; low risk; biosafety; clinical applications

1. Introduction

Human mesenchymal stem cells (hMSCs) hold great promise in many clinical applications, e.g. cell-based therapies and regenerative medicine, due to their immunomodulation and multilineage differentiation

ability (Doulatov and Daley, 2013; Nauta and Fibbe, 2007). For instance, hMSCs have been successfully applied in clinical trials for graft-versus-host disease (GvHD) treatment and articular cartilage regeneration (Jo *et al.*, 2014; Le Blanc *et al.*, 2008). To fully apply hMSCs in a clinical setting, it is necessary to know the biosafety of these stem cells, e.g. chromosomal abnormalities and tumourigenic potential, in addition to their therapeutic efficacy (Fink, 2009; Goldring *et al.*, 2011).

In general, hMSCs need to be expanded *in vitro* for a long duration to obtain sufficient cells required for clinical applications. During cell expansion, hMSCs may be at risk of chromosomal aberrations and tumourigenesis

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APPENDIX G

Published paper 4

Review

Am J Physiol Heart Circ Physiol 309: H532–H542, 2015.
First published June 19, 2015; doi:10.1152/ajpheart.00299.2015.

Mechanoregulation of cardiac myofibroblast differentiation: implications for cardiac fibrosis and therapy

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Submitted 27 April 2015; accepted in final form 19 June 2015

Yong KW, Li Y, Huang G, Lu TJ, Safwani WK, Pinguan-Murphy B, Xu F. Mechanoregulation of cardiac myofibroblast differentiation: implications for cardiac fibrosis and therapy. *Am J Physiol Heart Circ Physiol* 309: H532–H542, 2015. First published June 19, 2015; doi:10.1152/ajpheart.00299.2015.—Cardiac myofibroblast differentiation, as one of the most important cellular responses to heart injury, plays a critical role in cardiac remodeling and failure. While biochemical cues for this have been extensively investigated, the role of mechanical cues, e.g., extracellular matrix stiffness and mechanical strain, has also been found to mediate cardiac myofibroblast differentiation. Cardiac fibroblasts *in vivo* are typically subjected to a specific spatiotemporally changed mechanical microenvironment. When exposed to abnormal mechanical conditions (e.g., increased extracellular matrix stiffness or strain), cardiac fibroblasts can undergo myofibroblast differentiation. To date, the impact of mechanical cues on cardiac myofibroblast differentiation has been studied both *in vitro* and *in vivo*. Most of the related *in vitro* research into this has been mainly undertaken in two-dimensional cell culture systems, although a few three-dimensional studies that exist revealed an important role of dimensionality. However, despite remarkable advances, the comprehensive mechanisms for mechanoregulation of cardiac myofibroblast differentiation remain elusive. In this review, we introduce important parameters for evaluating cardiac myofibroblast differentiation and then discuss the development of both *in vitro* (two and three dimensional) and *in vivo* studies on mechanoregulation of cardiac myofibroblast differentiation. An understanding of the development of cardiac myofibroblast differentiation in response to changing mechanical microenvironment will underlie potential targets for future therapy of cardiac fibrosis and failure.

cardiac myofibroblast differentiation; mechanical cues; mechanical microenvironment; ECM stiffness; mechanical strain

HEART INJURY from many causes, e.g., ischemic heart diseases and hypertension, can end up with cardiac fibrosis. Cardiac fibrosis is an initial healing process essential for heart repair, but which if dysregulated is liable to cause adverse remodeling of cardiac tissues, leading to the development of congestive heart failure (108, 111). Cardiac fibrosis results from the excessive accumulation of fibrous connective tissues (components of the ECM, such as collagen) deposited by an increased number of cardiac fibroblasts and myofibroblasts around damaged heart tissues, resulting in permanent scarring and impaired cardiac functions (16, 101, 108). The origin of cardiac fibroblasts generated during fibrosis has been determined using various nonuniversal cardiac fibroblast markers (e.g., vimentin, discoidin domain receptor-2, and fibroblast-specific protein-1) and fate-mapping strategies (7, 110). These studies reveal that

cardiac fibroblasts may be derived from endothelial cells [via endothelial mesenchymal transition (EndoMT)], bone marrow-derived precursors, or epicardial cells [via epithelial mesenchymal transition (EMT)] (32, 111, 113). Recently, it has been suggested that the origin of cardiac fibroblasts is dependent on the heart conditions (e.g., whether during postnatal development or injury). With the use of a robust cardiac fibroblast marker (collagen I α 1-green fluorescent protein), it is possible to demonstrate that cardiac fibroblasts generated during fibrosis originate from the activation and proliferation of resident epicardial- and endothelial-derived fibroblasts (1, 7, 60, 61). On the other hand, cardiac fibroblasts during postnatal heart development are formed via EndoMT and EMT. Taken together, this requires further investigations to obtain a universal cardiac fibroblast marker to accurately identify the origin of cardiac fibroblasts, especially for those generated during cardiac fibrosis. Furthermore, while traditional dogma states that cardiac fibroblasts represent the most prevalent cell type in the mammalian heart (~70% of total cell numbers in heart) (71,

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H532

0363-6135/15 Copyright © 2015 the American Physiological Society

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