THE EFFECT OF PLATELET RICH CONCENTRATE ON THE REGENERATIVE CAPACITY OF MESENCHYMAL STROMAL CELLS AND ITS POTENTIAL APPLICATION IN CARTILAGE REPAIR

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ABSTRACT

The use of regenerative promoters such as mesenchymal stromal cells (MSC) constitutes one of the novel approaches to enhance tissue repair. However, these cells need to be greatly expanded and differentiated prior to their clinical use. Platelet rich concentrate (PRC) has recently emerged as a potentially valuable adjunct that could considerably enhance the therapeutic potential of MSC. Although there are several lines of evidence indicating that PRC enhanced proliferation and differentiation of MSC when combined with lineage-specific culture media, the extent of contribution of the platelet itself has not been previously demonstrated. This thesis aimed to investigate the effect of PRC in serum free medium (without the influence of additional differentiation media) in enhancing the proliferation and differentiation capacity of MSC *in vitro* and their regenerative potential to repair focal cartilage defect *in vivo*. The first study involved optimisation of PRC preparation and investigating the effect of a single application of PRC in inducing the proliferation and differentiation of human bone marrow-derived MSC (hMSC) within a very short time span. The results showed that hMSC proliferation was greatest when the cells were cultured in medium containing 15% PRC. At this optimal dose, PRC was able to induce hMSC differentiation to the common mesenchymal lineages i.e. osteogenic, adipogenic and chondrogenic, within 8 days of culture duration, without the need to be activated and the aid of any external lineage-specific growth factors in the culture medium. The study was further extended to compare the expression of lineage-specific markers both at the mRNA and protein levels of cells undergoing osteogenesis, adipogenesis and chondrogenesis under the influence of PRC to those cultured in the standard lineage-specific differentiation media during 24 days of culture (i.e. typical duration to achieve complete cellular differentiation to the common mesenchymal lineages). The results indicate that PRC induced osteogenesis and chondrogenesis at a

greater extent compared to the respective standard differentiation media. Chondrogenic hMSCs in the PRC group showed lower expression of hypertrophic genes. The final study was conducted to determine the applicability and the potential advantageous role of PRC in promoting chondrogenesis of MSC *in vivo* to enhance cartilage repair in a rabbit model of focal cartilage defect. Rabbits (6-7 months old) were subjected to full thickness focal cartilage defects limited to the chondral surface. At 3 and 6 month post-transplantation, animals in the PRC+allogenic rabbit MSC (rbMSC) group had significantly higher morphological score (ICRS) and histological score (O'Driscoll) compared to rbMSC and PRC group alone. Strong safranin O and collagen type II staining and high glycosaminoglycan content confirmed the formation of a hyaline-like cartilage in the PRC+rbMSC group. Collectively, these results demonstrate that PRC alone in serum free medium enhanced MSC regenerative capacity both *in vitro* and *in vivo*. PRC on its own could be used as an adjunct to provide sufficient pool of pre-differentiated MSC for potential clinical application in musculoskeletal tissue regeneration, particularly for the repair of focal cartilage injury.

ABSTRAK

Penggunaan perangsang regenerasi seperti sel stromal mesenkimal (MSC) merupakan salah satu pendekatan novel untuk meningkatkan pemulihan tisu. Walau bagaimanapun, sel-sel ini perlu berkembang dengan pesat dan mengalami diferensiasi sebelum sesuai digunakan ditahap klinikal. Larutan kaya platelet (PRC) kini muncul sebagai 'adjunct' berpotensi tinggi yang mampu meningkatkan potensi terapeutik MSC. Walaupun terdapat beberapa bukti yang menunjukkan bahawa PRC boleh meningkatkan proliferasi dan diferensiasi MSC apabila digabungkan dengan kultur media 'lineage-specific', namun sejauh mana sumbangan platelet itu sendiri terhadap pemerhatian ini tidak diketahui. Tesis ini bertujuan untuk mengkaji peranan PRC dalam medium bebas serum (tanpa pengaruh dari media diferensiasi tambahan) dalam meningkatkan kapasiti proliferasi dan diferensiasi MSC *in vitro* dan potensi regenerasinya untuk membaikpulih kecederaan tisu rawan *in vivo*. Kajian pertama melibatkan pengoptimuman PRC dan penentuan kapasiti satu aplikasi PRC dalam mendorong proliferasi dan diferensiasi MSC dari tulang sum-sum manusia (hMSC) dalam jangka masa yang singkat. Keputusan kajian menunjukkan bahawa proliferasi hMSC mencapai tahap tertinggi apabila sel dikultur dalam medium yang mengandungi 15% PRC. Pada dos optimum ini, PRC mampu mendorong diferensiasi hMSC kepada beberapa 'lineage' mesenkimal yang umum iaitu 'osteogenic', 'adipogenic' and 'chondrogenic' dalam tempoh kultur selama 8 hari, tanpa keperluan untuk diaktifkan dan bantuan dari faktor lain. Kajian diteruskan untuk membandingkan ekspresi petanda 'lineage-specific'pada tahap mRNA dan juga protein oleh cells yang mengalami 'osteogenesis', 'adipogenesis' dan 'chondrogenesis' dibawah pengaruh PRC berbanding sel yang dikultur dalam media 'lineage-specific' standard dalam tempoh kultur selama 24 hari (iaitu tempoh biasa untuk mencapai diferensiasi sel dengan sempurna kepada 'lineage' mesenkimal umum). Keputusan kajian menunjukkan bahawa PRC mendorong 'osteogenesis' dan 'chondrogenesis' pada tahap yang lebih baik

daripada media diferensiasi standard masing-masing. ‘Chondrogenic hMSC’ dalam kumpulan PRC menunjukkan tahap ekspresi gen ‘hypertrophic’ yang lebih rendah. Kajian yang terakhir dijalankan untuk menentukan kebolehgunaan dan peranan PRC dalam menggalakkan ‘chondrogenesis’ MSC *in vivo* untuk meningkatkan pemulihan tisu rawan dalam model kecederaan tisu rawan fokus pada arnab. Arnab (berumur 5-6 bulan) didedahkan kepada kecederaan tisu rawan berketebalan penuh yang terhad kepada permukaan kondral. Pada 3 dan 6 bulan selepas transplantasi, haiwan didalam kumpulan PRC+alogenik MSC (rbMSC) menunjukkan skor morfologi (ICRS) dan histologi (O’Driscoll) yang tinggi berbanding kumpulan rbMSC dan PRC secara individu. Perwarnaan safarin O dan kolagen jenis II yang kuat serta kandungan ‘glycosaminoglycan’ yang tinggi mengesahkan pembentukan tisu rawan jenis hialin didalam kumpulan PRC-rbMSC. Secara keseluruhan, keputusan kajian menunjukkan bahawa PRC dalam medium bebas serum dapat mempertingkatkan kapasiti regenerasi MSC *in vitro* dan *in vivo*. PRC dengan sendirinya boleh digunakan sebagai ‘adjunct’ untuk membekalkan MSC yang telah didiferensiasi dengan mencukupi untuk aplikasi klinikal dalam regenerasi tisu otot rangka, terutamanya bagi pemulihan kecederaan tisu rawan.

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TABLE OF CONTENTS

CHAPTER 3: OPTIMIZATION OF PRC PREPARATION AND DETERMINATION OF ITS EFFECT ON HUMAN MESENCHYMAL STROMAL CELL PROLIFERATION AND EARLY DIFFERENTIATION IN THE MONOLAYER CULTURE SYSTEM

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
β	Beta
3D	3 dimensional
ACAN	Aggrecan
ACI	Autologous chondrocyte implantation
BMP	Bone morphogenetic protein
BSP	Bone sialoprotein
Ca^{2+}	Calcium
DAB	3, 3’ Diaminobenzidine
DAPI	4', 6-diamido- 2-phenylindole
ECM	Extracellular matrix
EGF	Epidermal Growth Factor
ERK	Extracellular signal-regulated kinase
FGF	Fibroblast growth factor
FITC	Fluorescein isothyocyanate
FSC	Forward-light scatter
GAG	Glycosaminoglycans
GAPDH	Glyceraldehyde-3-phosphate
GP	Glycoprotein
hMSC	Human bone marrow derived mesenchymal stromal cells
HRP	Horseradish peroxide
IGF	Insulin-like growth factor
IL	Interleukin
L-DMEM	Low glucose Dulbecco’s Modified Eagle Medium
mTOR	Mammalian target of rapamycin

MAPK	Mitogen-activated protein kinase
MEK	MAP (Mitogen-Activated Protein) Kinase/ERK (Extracellular Signal-Regulated Kinase) Kinase
MSC	Mesenchymal stromal cells
PDGF	Platelet Derived Growth Factor
PI3K	Phosphatidylinositol 3-kinase
PRC	Platelet Rich Concentrate
PRP	Platelet Rich Plasma
rbMSCs	Rabbit bone marrow derived mesenchymal stromal cells
RGD	Arginine, glycine, aspartic acid
RUNX2	Runt family transcription factor 2
Smad	Small 'mothers against' decapentaplegic
SNAREs	Soluble *N*-ethylmaleimide-sensitive factor Attachment protein Receptors
Sox	Sex-determining region Y-type high motility group box
SPARC	Secreted protein acidic and rich in cysteine
SSC	Side light scatter
TGFβ	Transforming growth factor β
VEGF	Vascular endothelial growth factor

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background of the study

Cell-based therapy is one of the novel therapeutic strategies in the field of regenerative medicine that is increasingly being explored in recent years. It holds great potential for replacing or regenerating damaged tissues, with the ultimate aim of restoring the normal function of the organ (Ajibade, Vance, Hare, Kaplan, & Lesniak, 2014; Mason & Dunnill, 2008). This therapeutic approach is based on the concept of utilising diverse cell types including primary or terminally differentiated cells as well as progenitor cells such as mesenchymal stromal cells, embryonic stem cells and induced pluripotent stem cells (Heathman et al., 2015). These cells induce the repair of damaged tissues either by direct cell replacement and/or through their trophic effect on the surrounding tissues (Buzhor et al., 2014). Mesenchymal stromal cells (MSCs) have been considered as an attractive source for cell-based therapies among the various cell types with regenerative potential, mainly because their use is free from ethical concerns and they have tremendous inherent self-renewal and differentiation potential. These cells are also relatively easy to isolate, have high proliferation rate and can modulate immune responses (Johnstone, Hering, Caplan, Goldberg, & Yoo, 1998; Pittenger et al., 1999). However, in order for MSC treatment to be effective in clinical settings, the cells need to be greatly expanded to obtain a sufficient quantity (Murphy, Moncivais, & Caplan, 2013). In addition, MSCs also need to be pre-differentiated into therapeutically relevant cell type prior to its clinical use. The conventional method of cell expansion and differentiation involves supplementation of the culture media with fetal bovine serum (FBS) and/or other lineage-specific growth factors. The use of FBS however, is often associated with a high risk of pathogen transmission and immunological reactions against the xenogenic serum antigens (Iudicone et al., 2014; Kinzebach & Bieback, 2013). This problem may be circumvented

by using naturally occurring growth factors as an alternative culture media for MSC expansion and differentiation. One such source of biological pool of growth factors is the platelet rich plasma (PRP).

PRP contains three to four fold higher platelet number in the plasma compared to whole blood (Marx, 2001). It contains a mixture of various growth factors, cytokines and chemokines that regulate fundamental mechanisms involved in tissue healing such as cellular migration, proliferation, and angiogenesis (Gawaz & Vogel, 2013; Stellos et al., 2010). PRP treatment delivers supraphysiological levels of platelet-derived angiogenic and mitogenic growth factors to the damaged tissues, which would subsequently promote tissue regeneration (Redler, Thompson, Hsu, Ahmad, & Levine, 2011). Hence, it has increasingly been used in many fields including plastic surgery, dentistry, maxillofacial, and orthopaedics (De Pascale, Sommese, Casamassimi, & Napoli, 2015).

The potential of PRP could be exploited to enhance the efficacy of cell-based therapy. Hence, there is a resurgence of interest in recent years to investigate the interaction between PRP and MSCs in promoting tissue regeneration in pre-clinical studies. Previous studies have demonstrated that PRP exhibited a strong mitogenic effect on MSCs and induced their proliferation and differentiation to different lineages (Mishra et al., 2009; Parsons et al., 2008; Verrier et al., 2010). However, in these studies, PRP was supplemented in a medium containing serum or differentiation inducing growth factors. It is plausible that the effect of PRP itself may have been overrated. The supplementation of other external growth factors into the culture media in addition to PRP may be a confounding factor, which could obscure the interpretation of the actual effect of PRP itself on cells. These external factors might have produced additive effects, which could largely contribute to the observed enhancement of cell proliferation and differentiation.

It therefore remains unclear to what extent the use of PRP alone is beneficial as a cellular expansion and differentiation medium to promote MSC proliferation and differentiation without any confounding effect of other growth factors in the culture media. Hence, this thesis examines the effect of platelet rich preparation, in particular the platelet rich concentrate (PRC), supplemented in serum-free medium, in enhancing the potential of MSCs to proliferate and differentiate *in vitro*.

In this study, platelet rich concentrate (PRC), which is platelets resuspended in sterile phosphate buffer saline (PBS) was used instead of PRP to avoid practical limitation associated with the use of PRP. The presence of fibrinogen in plasma results in gel formation when supplemented to culture media. This would preclude appropriate data collection and analysis of *in vitro* experiments. Given that the effects of PRC and PRP on cell proliferation and differentiation was previously found to be similar (Zhang, Xie, & Lin, 2015), it can be assumed that the composition of active substances remain similar between the two types of preparation.

To test the potential application of the *in vitro* findings in *in vivo* conditions, the study was further extended to investigate whether PRC can enhance the regenerative potential of MSCs to promote *in situ* tissue repair. In this thesis, a rabbit model of full thickness focal cartilage defects was chosen to prove the potential therapeutic benefit of PRC *in vivo*. Cartilage tissue, unlike other tissues, lacks blood vessels, nerves and lymphatic drainage, and hence, has a limited spontaneous healing potential. Healing is further constrained by the dense cartilage extracellular matrix that prevents the migration of chondrocytes to the site of injury (Dhinsa & Adesida, 2012). Hence, defects limited to the chondral surface do not heal and those extended into the subchondral bone result in the formation of a fibrous tissue, which has weaker mechanical properties compared to

the normal hyaline cartilage tissue (Buckwalter, 1998; Caplan, Elyaderani, Mochizuki, Wakitani, & Goldberg, 1997). Therefore, successful repair of cartilage defects remains a challenge for clinicians. The commonly used cell-based treatment strategy for cartilage repair such as autologous chondrocyte implantation (ACI) is usually preferred only for small defects and is limited by the need to perform two surgeries and dedifferentiation of chondrocytes that have been expanded *in vitro* (Eslaminejad & Poor, 2014). As an alternative to chondrocytes, the use of MSCs has been successfully applied for the repair of cartilage defects in animal models (Dashtdar et al., 2011; Tay et al., 2012). PRP injections following marrow stimulation have also been found to successfully repair cartilage defects in rabbits (Huh et al., 2014). However, the effect of PRP treatment in combination with MSCs on cartilage repair has not been previously investigated. The treatment using combination of PRP and MSCs might result in a much superior repair outcome, as the growth factors in PRP might accelerate the proliferation and differentiation potential of MSCs *in vivo*. Hence, this thesis also examines the effect of PRC in enhancing the regenerative potential of MSCs to repair cartilage tissues in an animal model of focal cartilage defect limited to the chondral surface.

1.2 Hypotheses

1. PRC alone, without the effect of other differentiation inducing growth factors supplemented in the culture media, has the potential to induce proliferation and differentiation of human bone marrow-derived mesenchymal stromal cells (hMSCs) *in vitro*.

2. PRC enhances the regenerative potential of MSCs *in vivo*, thus further improve the quality of cartilage repair in a rabbit model of full thickness focal cartilage defects limited to the chondral surface.

1.3 Objectives

1. To determine the effect of a single application of PRC in serum-free medium on proliferation and directing early differentiation (8 days) of hMSCs without the aid of external lineage-specific growth factors in the culture medium.

2. To investigate the effect of PRC in maintaining cell proliferation during a longer period of culture (24 days) in order to obtain sufficient pool of viable cells for differentiation.

3. To compare the effect of PRC on hMSC differentiation to adipogenic, osteogenic and chondrogenic lineages as reflected by the expression of lineage-specific markers at gene and protein levels, to the cells cultured in respective standard differentiation media during the 24-day culture period.

4. To determine the effect of PRC in enhancing the regenerative potential of MSCs to augment articular cartilage repair *in vivo* in a rabbit model of full thickness focal cartilage defect, as reflected by macroscopic and histological changes as well as concentration of cartilage extracellular matrix protein (glycosaminoglycans) at 3 and 6 months post-injury.

1.4 Thesis outline

This thesis is organized into 6 chapters. **Chapter 1** outlines the background of the research as well as the hypotheses and objectives of the study. **Chapter 2** provides a review of the relevant literature related to the structure of platelets, the effect of mesenchymal stromal cells in tissue repair, as well as cartilage biology, pathology, and repair. A critical review of previous studies on platelet rich plasma, MSC differentiation, and cartilage repair is also presented in this chapter.

The body of research in this thesis is divided into three main parts. **Chapter 3** describes the methods development for optimising the PRC preparation. In this chapter, investigation was carried out to determine the most optimal concentration of PRC that would result in maximum hMSC proliferation within 8 days of culture and the necessity for activation of platelets prior to its use. An initial screening was carried out to determine whether PRC has the potential to direct early differentiation of hMSC into the three most common mesenchymal lineages i.e., adipogenic, osteogenic and chondrogenic lineages within a short time span of 8 days without any influence from the external differentiation-inducing factors that are typically supplemented to the culture media.

Chapter 4 presents the investigation of the comprehensive expression of lineage-specific markers by hMSCs undergoing the tri-lineage differentiation process under the influence of PRC during 24 days of culture. The duration of culture was selected based on previous reports indicating that the entire time frame for completion of cellular differentiation would approximately be between 21-28 days (Barry, Boynton, Liu, & Murphy, 2001; Kulterer et al., 2007; Qian et al., 2010). In this part of the study, the extent to which PRC induced differentiation of MSCs was also compared to the commercially available lineage-specific media (positive control). Osteogenesis and adipogenesis were induced in the monolayer culture system, while chondrogenesis was induced in a 3D culture model (i.e. hMSCs encapsulated in alginate beads).

Chapter 5 presents further evaluation of the biological application of the 3D *in vitro* MSC-PRC model to an *in vivo* setting involving a model of focal cartilage chondral defect in rabbits. In this chapter, the effects of MSC, PRC and a combination of MSC and PRC on cartilage repair were evaluated at 3 and 6 months post transplantation. The repair outcomes were reflected by the gross morphology, histology, immunohistology staining and extracellular matrix protein content such as glycosaminoglycan produced by the regenerated tissue in the joint.

Lastly, **chapter 6** summarises the major findings of all studies, provides a brief discussion of the current work and highlights the clinical implications of the findings. A recommendation for future work and concluding remarks of this study are also presented in this section.

CHAPTER 2: LITERATURE REVIEW

2.1 Stromal cells and Regenerative Medicine

Regenerative medicine offers new therapeutic options to repair, replace or regenerate damaged and diseased organs by combining the principles of cell biology and tissue engineering. In this regard, the potential application of several cell types such as embryonic stem cells, autologous cells derived from donor tissue and mesenchymal stromal cells have been widely investigated (Polak & Mantalaris, 2008). Embryonic stem cells are pluripotent cells that have the ability to differentiate to progeny of ectoderm, endoderm, and mesodermal germ layers. However, the activation of immunological reactions, formation of non-cancerous tumours called teratomas (Baker, 2009) and the embryo-destructive derivation of human embryonic stem cells have raised safety and ethical concerns, hampering their application in tissue engineering (Yalcinkaya, Sittadjody, & Opara, 2014). Autologous cells derived from the donor tissue has been widely used in tissue engineering primarily because they are immune compatible. However, the limited ability of the donor tissue to provide sufficient pool of viable cells for transplantation as well as the subsequent donor site morbidity are major obstacles for their use in regenerative medicine (Bauge & Boumediene, 2015). Mesenchymal stromal cells are undifferentiated cells and are a potentially beneficial alternative cell source for application in regenerative medicine. They can be easily isolated from various tissues such as bone marrow and adipose tissue and their use avoids the ethical issues raised by the use of embryonic stem cells. The other properties of these cells such as high proliferation, immunomodulation and differentiation to multiple lineages make them the ideal candidate for regenerative therapeutic applications (Howard, Buttery, Shakesheff, & Roberts, 2008; Yalcinkaya et al., 2014).

2.2 Mesenchymal Stromal Cells

Mesenchymal stromal cells or mesenchymal stem cells (MSCs) are multipotent unspecialized cells. These cells are characterized by their ability to self-renew, extensively proliferate and differentiate to at least one mature cell type (Figure 2.1). MSCs exist in almost all tissues and can differentiate into various specialized cell types when supplemented with the appropriate growth factors. They can be easily isolated from bone marrow, adipose tissue, umbilical cord, fetal liver, muscle and lung, and can be successfully expanded *in vitro* (Wei et al., 2013). Bone marrow and adipose tissue are the most readily available sources of MSCs because they are easy to harvest, present in abundance and their use is not associated with any major ethical concerns. Although adipose tissue-derived MSCs and bone marrow-derived MSCs have similar properties (Poloni et al., 2012), MSCs isolated from bone marrow have a higher degree of commitment to differentiate into chondrogenic and osteogenic lineages (Gimble, Katz, & Bunnell, 2007). However, MSCs isolated from the bone marrow constitute only about roughly 0.01–0.001% of the total bone marrow derived cell population (Pittenger et al., 1999).

The International Society of Stem Cell Research established a minimum set of criteria for defining MSCs, which include: (1) *ex vivo* plastic-adherent cells; (2) capable of tri-lineage (osteoblast, chondrocyte, and adipocyte) differentiation under proper induction; (3) phenotypically positive for CD105, CD73, CD90, and human leukocyte antigen (HLA) class I; and (4) negative for CD45, CD34, CD11b, CD14, CD79a and HLA-DR (Human Leukocyte Antigen - antigen D Related-MHC class II cell surface receptor) (Dominici et al., 2006).

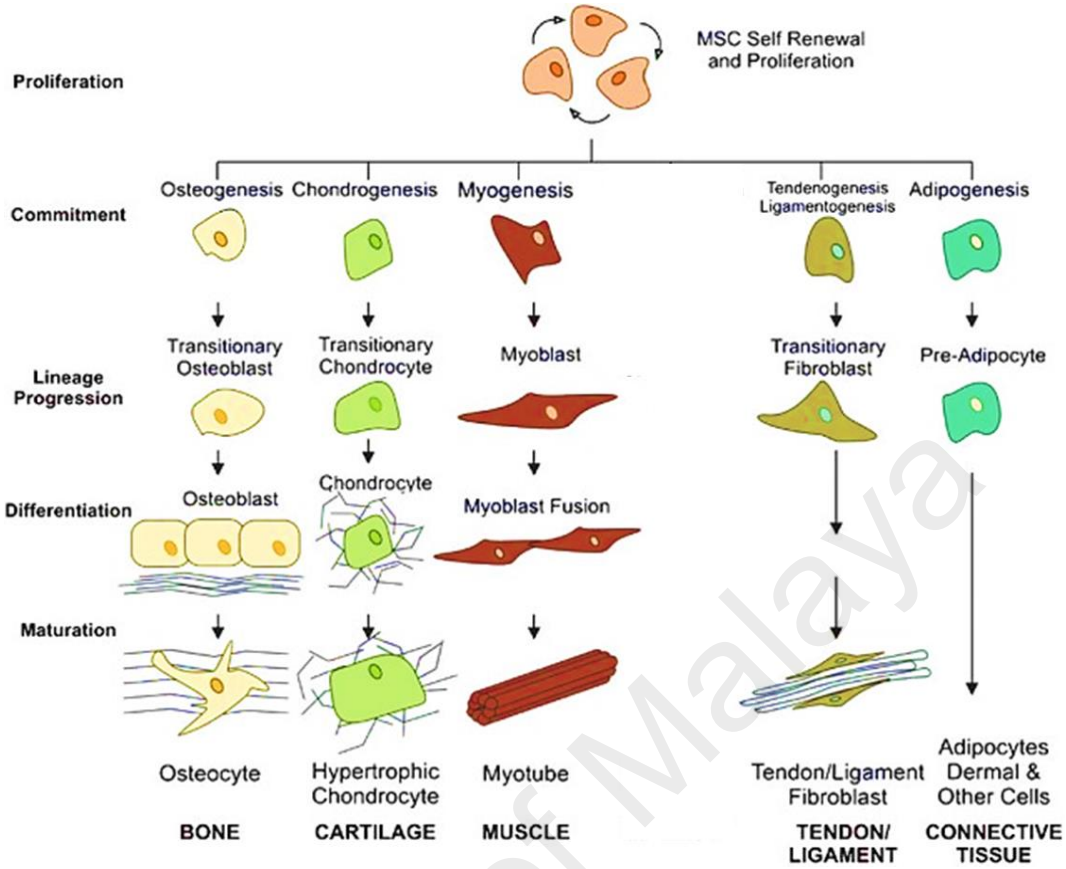

Figure 2.1: Multilineage differentiation potential of MSCs. Mesenchymal stromal cells reside in perivascular niches where they undergo self-renewal and maintain the surrounding cells/tissue. Depending on the stimuli, MSC can undergo differentiation into the osteoblastic, chondrocytic, adipocytic, myotube and, tendon and ligaments. Figure modified from (Firth, Yao, & Yuan, 2011).

2.2.1 Tri-lineage differentiation of MSCs

MSCs have the potential to differentiate to multiple cell types including adipocytes, osteoblasts and chondrocytes. The tendency of MSCs to differentiate to a particular cell lineage depends on the culture conditions.

2.2.1.1 Adipogenesis

Differentiation to the adipogenic lineage is a complex process involving the proliferation of precursor cells followed by their commitment to the adipogenic lineage and terminal differentiation (Ailhaud & Hauner, 2003). PPARG is the master regulator of adipocyte differentiation (Lefterova, Haakonsson, Lazar, & Mandrup, 2014). Increased expression of PPARG triggers the expression of genes important for lipid storage, illustrating the complete transition from primitive precursor cells to terminally differentiated adipocytes (Janderova, McNeil, Murrell, Mynatt, & Smith, 2003). The entire process of adipogenesis is first induced in a confluent population of the cells by exposure to adipogenic inducers as shown in Table 2.1. Dexamethasone, a synthetic glucocorticoid agonist is used to stimulate the glucocorticoid receptor pathway. Insulin is known to act through the insulin-like growth factor receptor 1, and methylisobutylxanthine, a cAMP-phosphodiesterase inhibitor, is used to raise the cAMP level and stimulate the cAMP dependent protein kinase pathway (Menssen et al., 2011). Indomethacin is a non-specific cyclooxygenase (COX) inhibitor that promotes adipogenesis and decreases osteogenesis (Styner, Sen, Xie, Case, & Rubin, 2010). This medium, also being rich in mitogens, induces the entire population of cells to re-enter the cell cycle (G_0 to G_1) and undergo at least two rounds of cell division before proceeding into terminal adipogenesis (Tang, Otto, & Lane, 2003).

Table 2.1: Components present in the adipogenic differentiation medium (Cortes et al., 2013).

Components	Concentration
DMEM-High Glucose	
Fetal Bovine Serum	10%
Penicillin	100 U/mL
Streptomycin	100 µg/mL
Amphotericin B	0.25 µg/mL
Insulin	10 µg/mL
Indomethacin	0.5 mM
Dexamethasone (DEX)	1 µM
3-isobutyl-1-methylxanthine	100 mM

2.2.1.2 Osteogenesis

Bone formation, both *in vivo* and *in vitro*, involves three distinctive stages: chemotaxis and proliferation of osteoprogenitor cells, differentiation into osteoblasts, and mineralization (Huang, Nelson, Smith, & Goodman, 2007). Many studies have investigated the complex mechanisms involved in the differentiation of MSCs into osteoblasts (Birmingham et al., 2012; Langenbach & Handschel, 2013; Xu, Li, Hou, & Fang, 2015). The extracellular matrix (ECM) of the bone is made of two types of proteins: the collagenous proteins, mostly collagen type I, accounting for 90% of the bone matrix proteins (Sroga & Vashishth, 2012) and the non-collagenous proteins, including osteocalcin, osteopontin, bone sialoprotein (BSP) and osteonectin (Florencio-Silva, Sasso, Sasso-Cerri, Simoes, & Cerri, 2015). Osteocalcin, also known as bone gamma-carboxyglutamic acid-containing protein (BGLAP), is a major non-collagenous proteins, synthesized and secreted only by osteoblastic cells during the maturation phase indicating osteoblast differentiation (Sila-Asna, Bunyaratvej, Maeda, Kitaguchi, & Bunyaratavej, 2007). BSP is a highly sulphated phosphorylated protein, which is able to bind to calcium ions due to its high negative charge (Hunter & Goldberg, 1993). It is expressed at the

onset of bone formation and associates with the osteoid matrix in a bone cell culture (Chen, Shapiro, & Sodek, 1992; Kasugai, Nagata, & Sodek, 1992). Osteopontin is also a phosphorylated protein and contains more phosphate than BSP. It is expressed more in mineralized connective tissues at the later stages of bone formation. In bone cell cultures, it is entirely released into the culture medium (Kasugai et al., 1992). Osteonectin, also known as secreted protein acidic and rich in cysteine (SPARC), is abundantly expressed in bone undergoing active remodelling (Sila-Asna et al., 2007).

Table 2.2: Components present in the osteogenic differentiation medium (Cortes et al., 2013).

Components	Concentration
DMEM-High Glucose	
Fetal Bovine Serum	10%
Penicillin	100 U/mL
Streptomycin	100 µg/mL
Amphotericin B	0.25 µg/mL
Sodium β-glycerophosphate	10 mM
Ascorbic acid	0.05 mM
Dexamethasone (DEX)	100 nM

In the *in vitro* culture, mesenchymal progenitor cells cultured in media supplemented with osteogenic supplements (Table 2.2) express markers specific to the osteoblasts, which secretes bone matrix that undergoes mineralization resulting in bone formation (Caetano-Lopes, Canhao, & Fonseca, 2007; Kang et al., 2015). Osteogenic differentiation of MSCs *in vitro* is induced by the presence of dexamethasone, ascorbic acid and β-glycerophosphate. They are required for the formation and mineralization of the ECM secreted by the bone marrow stromal cells (BMSCs) (Maniatopoulos, Sodek, & Melcher, 1988). Ascorbic acid also promotes the secretion of osteogenic collagen type I matrix (Choong, Martin, & Ng, 1993; Langenbach & Handschel, 2013). The *in vitro* osteogenic differentiation of MSCs can be divided into three stages (Huang et al., 2007). The first is

the cell proliferation stage, during which the number of cells peak within the initial 1-4 days. This is followed by the early cell differentiation from days 5-14 characterised by the transcription and protein expression of alkaline phosphatase (*ALP*) (Aubin, 2001). By this time, the cells attain confluence and undergo growth arrest. Decrease in replication is associated with expression of osteoblast functions like the production of ALP, processing of pro-collagens to collagens, and incremental deposition of collagenous ECM. After the initial peak in the expression of *ALP*, its levels starts to decline (Quarles, Yohay, Lever, Caton, & Wenstrup, 1992). Days 14-28 represent the final stages during which the high expression of osteocalcin and osteopontin is followed by the deposition of calcium and phosphate (Hoemann, El-Gabalawy, & McKee, 2009; Huang et al., 2007).

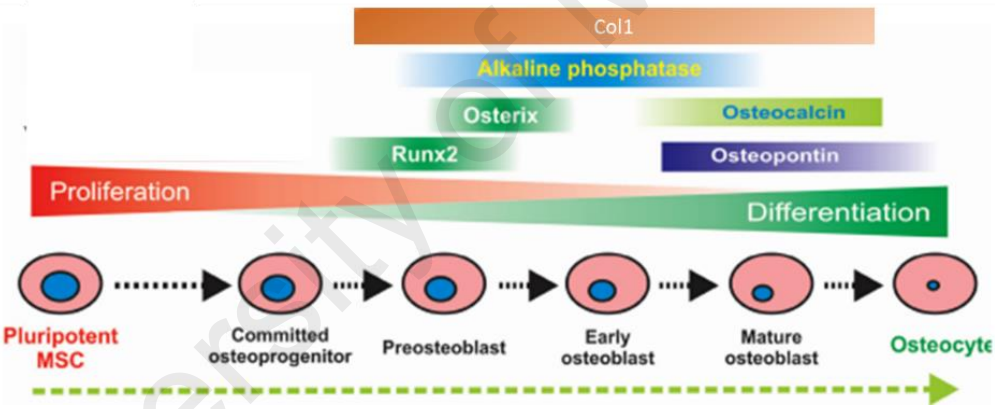

Figure 2.2: Osteogenic differentiation of MSCs. Markers of the osteoblastic differentiation cascade include alkaline phosphatase (*ALP*), Osterix, *RUNX2* (early/middle), osteopontin, osteocalcin, and collagen 1 (late). Modified from (Luther et al., 2011).

Osteogenic differentiation is regulated by the transcription factors runt-related transcription factor 2 (RUNX2), also known as core-binding factor subunit alpha-1 (CBF-alpha-1), and osterix (Figure 2.2). RUNX2 inhibits the differentiation of mesenchymal stromal cells to the other lineages and triggers the expression of major bone matrix protein genes at an early stage of osteoblast differentiation, leading to the cells acquiring an osteoblastic phenotype. The transcription factor osterix (Osx) is the downstream gene of

RUNX2 (Komori, 2006), specifically expressed at a low level by osteoblast and pre-hypertrophic chondrocytes (Nakashima et al., 2002). The Osx transcript is found when MSCs are committed to enter osteoblastic lineage and its expression increases as the osteogenic differentiation progresses. It is required for the expression of osteoblastic markers such as bone sialoprotein, osteonectin, and osteocalcin.

2.2.1.3 Chondrogenesis

Cartilage formation occurs in four stages: cell migration as well as interactions between the epithelial and mesenchymal cells resulting in aggregation, cell condensation, and differentiation of chondroblasts (Hall & Miyake, 2000). Aggregation of chondroprogenitor cells and their subsequent condensation depends on the signals initiated by cell-cell and cell-matrix interactions (Woods, Wang, & Beier, 2007). Before condensation, the prechondrocytic mesenchymal cells synthesize ECM proteins hyaluronan and collagen type I that prevent cell-cell interaction. As condensation commences, there is an increase in hyaluronidase activity with a decrease in hyaluronan, which allows close cell-cell interactions. This is associated with the appearance of cell adhesion molecules, neural cadherin (N-cadherin) and neural cell adhesion molecule (N-CAM), which disappear in differentiating cartilage (Tuan, 2004). The cell-matrix interactions during chondrocyte differentiation are enabled by fibronectin which down-regulate N-CAM and prevent further condensation. The disappearance of type I collagen, fibronectin, and N-cadherin and the appearance of collagen type II, tenascin, aggrecan and cartilage oligomeric protein (*COMP*) initiate the transition from chondroprogenitor cells to a fully committed chondrocyte (Ede, 1983). At the ends of the long bone, chondrocytes undergo maturation, hypertrophy, and calcification. Ultimately the growth

plate region of cartilage is replaced by bone by a process called endochondral ossification (Hidaka & Goldring, 2008).

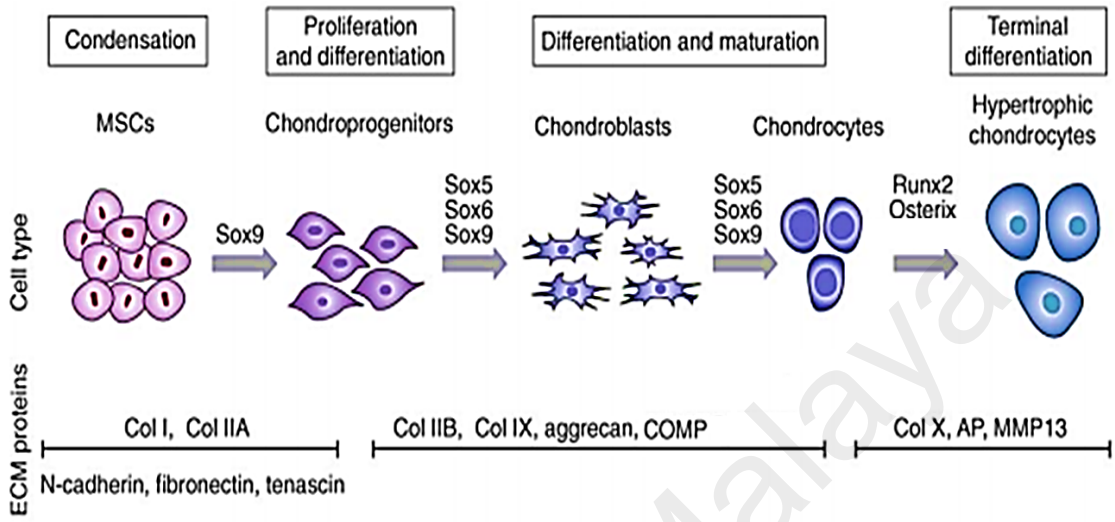

Figure 2.3: Sequence of events involved in the chondrogenic differentiation of MSCs. The temporal expression of different chondrogenic ECM proteins is shown at the bottom. Figure is adapted from (Vinatier, Mrugala, Jorgensen, Guicheux, & Noel, 2009). AP: Alkaline phosphatase; Col: Collagen; COMP: Cartilage oligomeric protein; MMP: Matrix metalloprotease; VEGF: Vascular endothelial growth factor.

The *in vitro* differentiation of MSCs to chondrocytes also occurs in four stages: cell condensation, formation of chondroprogenitors, cell differentiation, and terminal differentiation (Figure 2.3). SOX9 is one of the earliest marker and a key chondrogenic transcription factor. Its expression is elevated in mesenchymal progenitors undergoing condensation. It regulates the expression of other chondrogenic markers including collagen type II and aggrecan. *SOX5* and *SOX6* are not expressed in early mesenchymal condensations, but are co-expressed with *SOX9* during differentiation of MSCs to mature chondrocyte (Akiyama, Chaboissier, Martin, Schedl, & de Crombrugghe, 2002; Giuliani et al., 2013). MSCs cultured in chondrogenic media (Table 2.3) that differentiate to chondroprogenitors are characterized by the deposition of cartilage matrix containing fibromodulin, collagen type II, IX, XI, COMP and aggrecan.

Table 2.3: Components present in the chondrogenic differentiation medium (Cortes et al., 2013).

Components	Concentration
DMEM High Glucose	
Penicillin	100 U/mL
Streptomycin	100 µg/mL
Amphotericin B	0.25 µg/mL
Ascorbate – 2 phosphate	50 µg/mL
Insulin-Transferrin-Selenium (ITS) premix	10%
Pyruvate	1 mM
Dexamethasone (DEX)	0.1 µM
TGF-β1	8 ng/mL

The early differentiated cell expresses fibromodulin mRNA (first 24 hours of differentiation), representing the first matrix component being upregulated, followed by the appearance of COMP mRNA. At this stage, aggrecan, versican and decorin are expressed along with initiation of glycosaminoglycan (GAG) accumulation. Fibromodulin, versican and decorin form the earliest extracellular components during chondrogenesis (Barry et al., 2001). Versican plays a role in extracellular matrix assembly and in cell-cell adhesion (Wu, La Pierre, Wu, Yee, & Yang, 2005). Other proteins such as fibromodulin and decorin are important for organization of collagen fibrillogenesis. Approximately 7 days after initiation of differentiation, the expression of type II collagen and chondroadherin gradually increases until day 21. Chondroadherin mediates signals between chondrocytes and the cartilage matrix (Camper, Heinegard, & LundgrenAkerlund, 1997) and also plays a major role in regulating the formation of the collagen fibrillar network. Accumulation of GAG increases until day 21, indicating the maturation of chondrocytes (Barry et al., 2001). Interactions between SOX9 and RUNX2 control the transition of mature chondrocytes to hypertrophic chondrocytes. Recent studies have shown that SOX9 requires the presence of RUNX2 for the formation of hypertrophic chondrocytes and for the subsequent endochondral bone formation (Bi et

al., 2001; Dy et al., 2012). However, SOX9 is also known to play a role in preventing chondrocyte hypertrophy by inhibiting the expression of RUNX2 (Yamashita et al., 2009).

Various model systems have been used to investigate the chondrogenic differentiation potential of MSCs. The most commonly used include micromass culture (Ahrens, Solursh, & Reiter, 1977; Scharstuhl et al., 2007), pellet culture (Chang et al., 2008; Zhang et al., 2010), and alginate culture (Dashtdar et al., 2016; Xu et al., 2008). All models are based on high-density cell cultures, preventing the adhesion of MSCs and increasing the cell-cell interactions, and thereby creating a suitable environment for the cells to differentiate and maintain a chondrocytic phenotype (Ruedel, Hofmeister, & Bosserhoff, 2013).

In the micromass culture system droplets of resuspended cells are placed in each well of a tissue culture plate. The cells are allowed to adhere in chondrogenic medium and after 24 hours, the cells coalesce and become spherical (Zhang et al., 2010). Pellet culture system provides a 3D environment allowing cell-cell interactions. In this system, cells are first centrifuged in 15 mL polypropylene conical tubes and then the pelleted cells are incubated with loosened caps to permit gas exchange. The cells form a spherical aggregate at the bottom of the tube within 24 hours of incubation (Zhang et al., 2010). The use of alginate culture system is a novel model system for 3D chondrogenic differentiation (Herlofsen, Kuchler, Melvik, & Brinchmann, 2011). Alginate is a naturally occurring anionic polymer derived from brown algae and is composed of repeating units of β-D-mannuronic acid and α-L-guluronic acid (Amsden & Turner, 1999). It has low toxicity, relatively low cost, and forms an ionic gel in the presence of divalent cations such as Ca^{2+} (Wee & Gombotz, 1998). The formation of a hydrogel by

the ionic crosslinking of the guluronic residues in the presence of the divalent cation, permits the diffusion of nutrients that are essential for cell growth (Amsden & Turner, 1999; Wee & Gombotz, 1998). These properties make it an attractive biomaterial and hence it has been widely used in tissue engineering (Lee & Mooney, 2012). The use of alginate provides a well-controlled system for studying cell-cell and cell-matrix interactions. Studies have reported that chondrocytes encapsulated in alginate remain differentiated while those cultured in the monolayer become fibroblastic (Hauselmann et al., 1994; Mhanna et al., 2014) indicating that alginate helps to maintain a chondrocytic phenotype. In this culture system, the cell pellet is resuspended in alginate and this cell suspension is dropped slowly using a micropipette tip into calcium chloride solution. The cell encapsulated beads formed are washed and cultured in low attachment tissue culture plates. The cells can be easily retrieved from the beads by dissolving the alginate in sodium citrate solution.

2.2.2 Basic mechanisms and limitations of application of MSCs in tissue repair

The potential of MSCs to differentiate into various specialized cell types has made them the major focus in cell-based therapies. Recently, the therapeutic potential of MSCs has been demonstrated for regeneration of various tissues including bone (Arinzeh et al., 2003; Kadiyala, Jaiswal, & Bruder, 1997; Kumar, 2014) and cartilage (Dashtdar et al., 2015; Nam et al., 2013; Tay et al., 2012). However, the mechanisms by which transplanted MSCs contribute to the repair of injured tissues is not clearly understood. Previously, it was thought that MSCs repair damaged tissues by differentiating to the resident cells found in the damaged tissues and thus replacing the damaged cells. However, it has recently been shown that in response to an injury, MSCs secrete large quantities of bioactive molecules, which provide paracrine signals to repair injured tissues

(Li, Whyte, & Niyibizi, 2012; Prockop & Olson, 2007). MSCs can also regulate immune responses. At the site of injury, they modulate the inflammatory micro-environment by decreasing the levels of interleukin-1β (IL-1β), IL-6 and tumour necrosis factor-α (TNF-α) (Granero-Molto et al., 2009; Wang et al., 2013). MSCs thus reduce inflammation and facilitate angiogenesis contributing to tissue repair (Shao, Zhang, & Yang, 2015). MSCs also has the ability to reside at the injury site and release soluble mediators, which can promote angiogenesis, regeneration, remodelling, immune cell activation or suppression and cellular recruitment (Dimarino, Caplan, & Bonfield, 2013; Wu, Liu, Wang, & Li, 2014). The most important effect of transplanted MSCs is their immunomodulatory role, through crosstalk with the immune cells or the paracrine actions (Wu et al., 2014). The various mechanisms proposed for the observed effect of MSCs are shown in Figure 2.4.

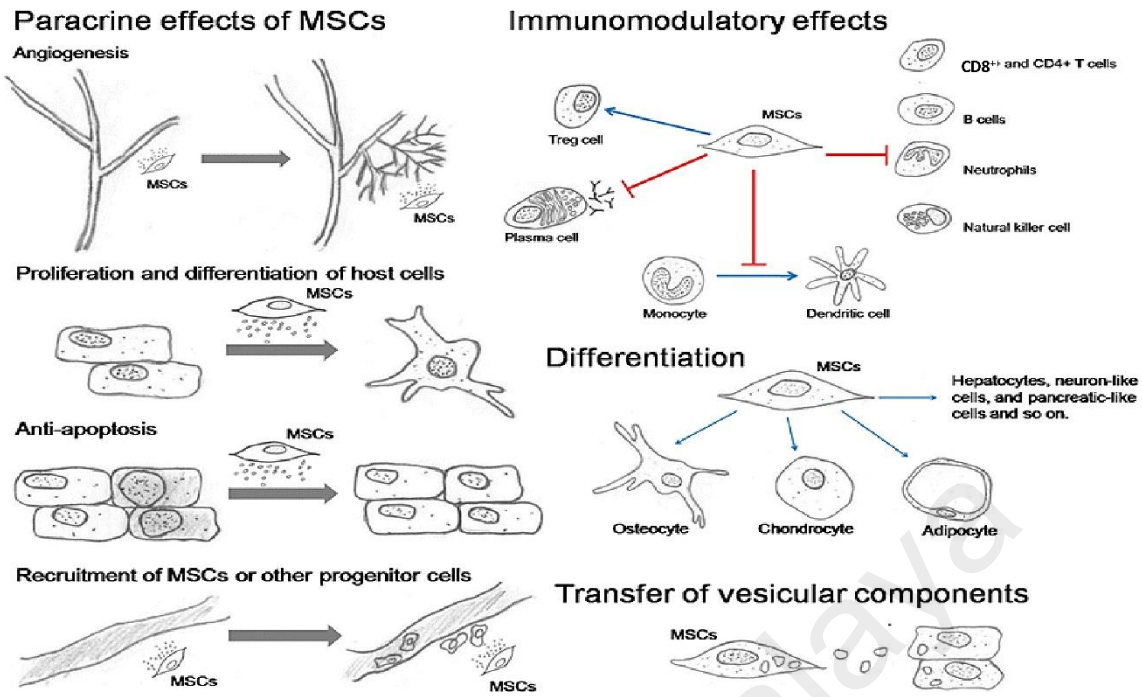

Figure 2.4: Different mechanisms of action of transplanted MSCs. MSCs might act in a paracrine mode and stimulate angiogenesis, protect cells from apoptosis, recruit host MSCs or other progenitor cells and stimulate proliferation and differentiation (Hocking & Gibran, 2010; Meirelles Lda, Fontes, Covas, & Caplan, 2009). The immunomodulatory effect of MSCs inhibit the proliferation and activity of neutrophils, NK cells (natural killer cells), B cells, $CD4^+$ cells and $CD8^+$ T cells, inhibit the maturation of monocytes into dendritic cells, suppress plasma cell immunoglobulin production but stimulate proliferation of regulatory T cells (Treg cells) (Ben-Ami, Berrih-Aknin, & Miller, 2011). MSCs are also able to differentiate into multiple cell types and transfer vesicles containing mRNA, proteins and mitochondria to the host cells (Ben-Ami et al., 2011; Bieback et al., 2012; Shao et al., 2015; Spees, Olson, Whitney, & Prockop, 2006).

Despite the great potential of MSCs, application of MSC-based therapies in the field of regenerative medicine is still limited due to a number of factors. First, a large number of cells, approximately 2 x 10^6 cells/kg, are required for clinical applications (Binato et al., 2013; Schallmoser et al., 2008). This large number of MSCs can be isolated from the adipose tissue, however, the differentiation potential (Huang et al., 2005) and the anti-inflammatory effects (Elman, Li, Wang, Gimble, & Parekkadan, 2014) of adipose-derived MSCs are low compared to the bone marrow-derived MSCs. The drawback of MSCs derived from bone marrow is that the distribution of MSCs in the bone marrow is very low (0.001–0.01%) (Schallmoser et al., 2008). Hence, they need to be extensively expanded *in vitro* to obtain sufficient numbers for therapeutic application. Cell expansion

is usually done in media supplemented with fetal bovine serum (FBS). FBS, being an animal source product has the potential to transmit animal pathogens and develop antibodies against FBS resulting in the rejection of the transplanted cells (Muller et al., 2006). Studies have also shown that about 20-50% of commercial FBS is virus-positive (van der Valk et al., 2010; Wessman & Levings, 1999) and cells cultured in FBS supplemented media also carry approximately 7-30 mg FBS proteins (Spees et al., 2004). These factors may render the cells cultured in FBS supplemented media less desirable for clinical applications. The second major obstacle in the clinical application of MSCs is their poor viability at the site of transplantation due to being exposed to the harsh microenvironment (Kneser, Schaefer, Polykandriotis, & Horch, 2006). The transplanted cells are exposed to sudden deprivation of oxygen and nutrient at the defective site, which would induce massive cell death (Potier et al., 2007). Wu et al. in their study showed that only 5% cells survived 2 weeks post transplantation (Wu et al., 2014). Thus, it is important to develop methods that can improve cell survival and augment cell function for cell-based therapies.

Recently, platelet derivatives have been increasingly used as regenerative promoters in view of the numerous mitotic and angiogenic growth factors present in the alpha granules of the platelets that could promote tissue repair (Eppley, Woodell, & Higgins, 2004). The presence of these growth factors may make platelet rich preparation a desirable medium for cellular expansion and differentiation. Moreover, MSCs cultured in platelet rich preparation have been found to have delayed expression of senescence phenotype (Crespo-Diaz et al., 2011; Griffiths, Baraniak, Copland, Nerem, & McDevitt, 2013). Hence, the MSC-platelet interaction has been speculated to increase the stemness and also prolong the survival time of the transplanted cells (Tobita, Tajima, & Mizuno, 2015). The

next section briefly describes platelet structure, function and their role in regenerative medicine.

2.3 Overview of platelet structure and function

2.3.1 Platelet Structure

Platelets are anucleate cell fragments that circulate in blood and have a diameter of 2-4 µm. They are derived from fragmentation of precursor cells called megakaryocytes in the bone marrow. Each megakaryocyte can produce 5000–10000 platelets. Platelets have a primary role in regulating haemostasis and a secondary role in tissue repair and regeneration (Gawaz & Vogel, 2013). They usually remain in an inactive state in the circulating blood until exposed to platelet agonists or sub-endothelial components (Ghoshal & Bhattacharyya, 2014; Malara & Balduini, 2012). Under a physiological condition, the platelet count in the peripheral blood is about 150,000 to 400,000 per µL of whole blood, and in their inactive state, platelets have an average lifespan of about 8-10 days (Thon & Italiano, 2012).

Platelets contain a number of distinguishable structural elements including a delimited plasma membrane, specialized membrane systems, a spectrin-based membrane skeleton; an actin-based cytoskeletal network; a peripheral band of microtubules; and numerous organelles including α-granules, dense-granules, peroxisomes, lysosomes, and mitochondria (Figure 2.5) (Thon & Italiano, 2012). The invaginations of the surface membrane form the open canalicular system and a closed-channel network of residual endoplasmic reticulum called the dense tubular system. The open canalicular system permeates the cytoplasmic space of the platelet and functions both as a reservoir of α-

granules products, and as a channel through which the intracellular cytoplasmic granules release their content after platelet activation. The dense tubular system serves as the major site of calcium storage and prostaglandin and thromboxane (TXA_2) synthesis in the platelet (Hartwig & Italiano, 2003; Thon & Italiano, 2012). It regulates platelet activation by either sequestering or releasing calcium (Ebbeling, Robertson, McNicol, & Gerrard, 1992) and by maintaining the cytosolic calcium concentrations in the nanomolar range in the resting platelets (Italiano, 2007).

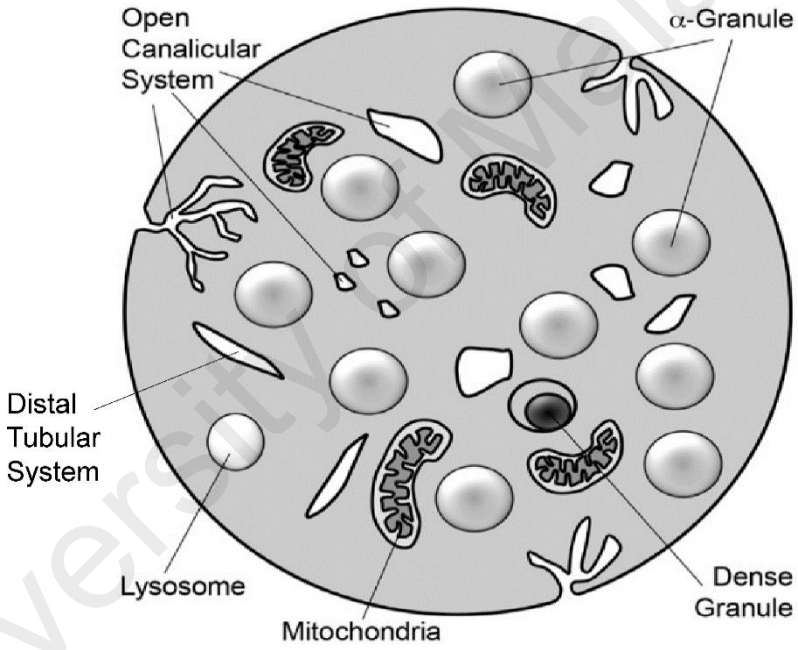

Figure 2.5: Schematic representation of a platelet. Platelets contains α-granules, dense granules, mitochondria and lysosomes. Tunnel invaginations of the plasma membrane forms a complex membrane network, termed the open canalicular system that courses throughout the platelet interior (Fitch-Tewfik & Flaumenhaft, 2013).

Platelets contain a large number of biologically active molecules in their granules. These molecules are secreted and deposited at sites of injury and function to recruit other blood-borne cells. In resting platelets, granules are situated close to the open canalicular system membranes. During platelet activation, the granules fuse and release their content into the open canalicular system via exocytosis. At present, more than 300 distinct molecules have been detected in platelet releasates (Coppinger et al., 2004). Platelets have two major recognized storage granules, which are the α and dense granules. The α granules, which is most abundant, (about 40 per platelet) contain proteins essential for platelet adhesion during vascular repair. These granules are typically 200 to 500 nm in diameter and are spherical in shape with dark central cores. Alpha granules acquire their molecular contents from both endogenous protein synthesis and by the uptake of plasma proteins (Italiano, 2007). The currently known list of alpha-granular proteins, which comprise the bulk of platelet secretome, include various adhesive proteins (e.g. fibrinogen, von Willebrand factor (vWf) and thrombospodin (TSP)), growth factors (e.g. platelet derived growth factor, basic fibroblast growth factor, transforming growth factor-beta), angiogenic factors (e.g. angiogenin, vascular endothelial growth factor), protease inhibitors (e.g. alpha 2-macroglobulin and alpha 2-antiplasmin), proteases (e.g. MMP2, MMP9), necrotic factors (e.g. TNFα, TNFβ), and other cytokines. Other molecules such as fibrinogen, albumin and coagulation factor V are transported into circulating platelets via endocytosis and then incorporated into the α granules. More recently, the inner lining of the alpha-granule unit membrane has been demonstrated to contain a number of physiologically important receptors including glycoprotein IIb/IIIa ($\alpha_{IIb}\beta_3$) and P-selectin (Harrison & Cramer, 1993; Whiteheart, 2011). Dense granules are 250 nm in size and function primarily to recruit additional platelets to the sites of injury. They contain many hemostatically active small molecules that are released upon platelet activation, including serotonin, adenosine-diphosphate (ADP) and calcium. ADP is a strong platelet agonist,

triggering changes in the shape of platelets. Platelets also contain a number of mitochondria, lysosomes and peroxisomes in the cytoplasm. Mitochondria provides the energy source for the platelets to circulate in the blood stream while peroxisomes contain the enzyme catalase and lysosomes contain various degradative enzymes (Italiano, 2007). Platelet activation is required for the release of granular contents from their intracellular reservoir. Collagen, thrombin and adenosine-diphosphate (ADP) are the common agonists known to activate platelets at sites of vascular injury. These agonists activate platelets by binding to specific receptors on the platelet surface, which lead to a series of downstream intracellular signalling that ultimately increases the intracytoplasmic concentration of calcium ions. The increase in platelet intracellular calcium occurs through release from intracellular stores and calcium influx through the plasma membrane (Varga-Szabo, Braun, & Nieswandt, 2009). Upon activation, α – granule membrane fuses with the membranes of open canalicular system or the plasma membrane. This process is coordinated by transmembrane proteins termed SNAREs (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors) (Flaumenhaft, 2003).

2.3.2 Platelet Function

Platelets are involved in the second stage of haemostasis, forming a temporary seal of the injured vessel wall, until a more permanent seal is developed through initiation of the coagulation cascade. Platelets initiate haemostasis by aggregating and adhering to the damaged vessel wall (Ghoshal & Bhattacharyya, 2014). Under physiologic conditions, platelets circulate close to the endothelial cell lining of the vascular wall without actively interacting with the endothelial cells, as the cells maintain an anti-adhesive phenotype (Ware & Heistad, 1993). Platelet adhesion to endothelial cells under normal conditions is prevented by nitric oxide (NO) and prostacyclin (PGI_2) released by endothelial cells.

When the endothelial layer is injured, the sub-endothelial matrix is exposed, activating the platelets and setting in motion a series of events that temporarily seal the injury. Platelet adhesion involves the interactions between GP Ib-IX-V complex and vWF, a multimeric protein synthesized by the endothelial cells and megakaryocytes. Platelet receptors GPVI and $\alpha_2\beta_1$ then mediate the interaction with collagen. After the platelets are adhered, they are activated by a number of agonists such as adenosine diphosphate (ADP) and collagen, which are present at the sites of vascular injury. These agonist bind to specific receptors on the surface of the platelets. Binding to the receptors leads to a series of downstream events that ultimately increases the intracytoplasmic calcium concentration (Rumbaut & Thiagarajan, 2010) and result in platelet activation and further aggregation.

Activation of platelets also leads to the release of a variety of growth factors from their α and dense granules, which act in an autocrine or paracrine manner to modulate cell signalling (Table 2.4). Platelets begin to actively secrete these proteins within 10 minutes after activation, with more than 95% of the presynthesized growth factors secreted within 1 hour. The active proteins secreted, bind to the transmembrane receptors of target cells (e.g., mesenchymal stromal cells, osteoblasts, fibroblasts, endothelial cells, and epidermal cells), initiating an intracellular signalling cascade, which result in the expression of genes that directs cellular proliferation, matrix formation, osteoid production, and collagen synthesis (Gassling, Acil, Springer, Hubert, & Wiltfang, 2009; Marx, 2004).

Due to the fact that the growth factors released from platelets can accelerate chemotaxis, mitogenesis, cell differentiation and angiogenesis, preparation of concentrated platelets have been increasingly used for tissue regeneration. Such concentrated platelet preparations have been referred to as platelet rich plasma (PRP), platelet rich concentrate (PRC), platelet/preparation rich in growth factors (PRGF) or human platelet lysate (HPL).

Platelet-rich plasma (PRP) is defined as a portion of the plasma of autologous blood having platelet concentrations above baseline. Normal platelet counts in blood range between 150,000/ μL and 350,000/μL, with an average of 200,000/μL. Clinically, PRP, which contains about 1 million platelets/μL has been shown to enhance bone and soft tissue healing (Marx, 2001). This concentration of platelets in a small volume of plasma is referred to as PRP. However, some investigators have suggested that PRP must have at least 3-5 fold increase in platelet concentration over baseline (Eppley, Pietrzak, & Blanton, 2006). It has also been proposed that low concentration of platelets in PRP might not have sufficient capacity to produce the desirable effects while highly concentrated PRP might have an inhibitory effect (Marx, 2001; Yamaguchi, Terashima, Yoneyama, Tadano, & Ohkohchi, 2012). Therefore, it is important to determine the optimal dose of PRP prior to its therapeutic applications.

The platelets in PRP is concentrated using a double centrifugation technique. The first spin (called the hard spin) separates the red blood cells from the plasma, which contains the platelets, the white blood cells, and the clotting factors. The second spin (called the soft spin) separates the platelets and white blood cells together with a few red blood cells from the plasma. This soft spin produces the PRP and separates it from the platelet poor plasma. Isolated platelet concentrates are further delineated based on their leukocyte

content. They are termed either pure-PRP (P-PRP) or leucocyte-rich PRP (L-PRP). For the production of P-PRP, upper layer and superficial buffy coat are transferred after the first spin into an empty sterile tube. For the production of leucocyte rich PRP (L-PRP), the entire layer of buffy coat and few RBCs are transferred after the first spin. The second spin step is then performed to produce P-PRP or L-PRP. The upper portion of the plasma that is composed mostly of platelet-poor plasma is discarded and the platelet pellet is resuspended in a small volume of plasma (Dhurat & Sukesh, 2014). The PRP isolated contain concentrated cocktail of growth factors that are released when PRP is activated using either calcium chloride or thrombin or a mixture of both. The concentration of growth factors released from the platelets upon activation are commonly quantified by enzyme-linked immunosorbant assay (ELISA). The role of L-PRP on tissue healing has been controversial. Some studies proposed that leukocytes stimulate the healing process in damaged tissue and simultaneously suppress the growth of some bacteria (Alsousou, Thompson, Hulley, Noble, & Willett, 2009; Cieslik-Bielecka et al., 2009). On the other hand, various reports showed positive correlation between the total number of leukocytes in PRP and increased levels of pro-inflammatory cytokines, indicating that leukocytes in PRP may inhibit the healing process (Dragoo et al., 2012; McCarrel, Minas, & Fortier, 2012; Pavlovic, Ciric, Jovanovic, & Stojanovic, 2016).

Depending on the PRP preparation protocols, the PRP isolated may differ in the cytokine content. The cytokine content also varies with differences in platelet concentrations, type of anticoagulant used, speed of centrifugation, and the mode of activation (Dhurat & Sukesh, 2014; Tamimi, Montalvo, Tresguerres, & Blanco Jerez, 2007; Weibrich, Hansen, Kleis, Buch, & Hitzler, 2004). This would ultimately result in variations in the therapeutic effect of PRP. The optimal protocol used for PRP preparation must not only focus on producing higher platelet number but must also be able to maintain the platelets in the

non-activated form during the preparation process. It is important for the platelets to remain non-activated to ensure that the growth factors that have a short half-life are retained in the platelets until their application. It has also been noted that when PRP is supplemented to the culture medium *in vitro*, it results in the formation of gel due to the presence of fibrinogen in the plasma, and this hinders effective *in vitro* cellular analysis. Hence, to avoid gel formation, platelet pellets can be resuspended in buffered solutions such as phosphate buffered saline (PBS) or tyrodes solution for *in vitro* applications (Verrier et al., 2010). Such preparation is termed platelet rich concentrates (PRC). PRC differs from PRP only with respect to the medium used to resuspend the platelets. In PRP the platelets are resuspended in plasma, and hence, it contains plasma proteins like fibrinogen, albumin and clotting factors like V and XI. PRC, on the other hand does not contain these components (Cazenave et al., 2004) and contains only platelets as the active component. The major components present in PRP is depicted in table 2.4.

In view of the fact that the growth factors released from platelets in platelet preparations have the potential to stimulate proliferation and differentiation of progenitor cells, there is presently an increasing interest to further investigate the interactions between platelet preparations and mesenchymal stromal cells (MSCs) for potential application in regenerative medicine (Kakudo et al., 2008; Kocaoemer, Kern, Kluter, & Bieback, 2007). This is due to the possibility to deliver physiologically natural growth factors and other cytokines in supraphysiologic concentrations directly to the site of injury and the possible paracrine effect of these growth factors on MSCs (Anitua, Andia, Ardanza, Nurden, & Nurden, 2004).

Table 2.4: Major components in platelet rich plasma. Adapted from (Dhurat & Sukesh, 2014; Foster, Puskas, Mandelbaum, Gerhardt, & Rodeo, 2009; Martinez, Smith, & Palma Alvarado, 2015; Steed, 1997; Zhang et al., 2013).

Categories	Specific Molecules	Biologic Activities
Growth Factors	TGF-β	Stimulates proteoglycan and collagen type II synthesis, proliferation of undifferentiated mesenchymal stromal cells, fibroblastic and osteoblastic mitogenesis and angiogenesis
	PDGF	Chemoattractant, cell proliferation, osteoinductive, regulates collagen synthesis
	IGF-I, II	Cell Proliferation, maturation, bone matrix synthesis, enhances proliferation and differentiation of osteoblasts, promotes chondrogenic potential of mesenchymal stromal cells
	FGF	Promotes growth and differentiation of chondrocytes and osteoblasts, cell proliferation
	EGF	Stimulates chemotaxis, cell proliferation
	VEGF	Angiogenesis
	HGF	Tissue regeneration
Adhesive proteins	Fibrinogen Fibronectin	Blood clotting cascade (fibrin clot formation) Binds to cell-surface integrins, affecting cell adhesion, cell growth, migration and differentiation
	Vitronectin	Cell adhesion, chemotaxis
Clotting factors	Factor V, factor XI, protein S, antithrombin	All play a role in thrombin activation and eventual fibrin clot formation
Bioactive Molecules Found in the Dense Granules of Platelets		
	Serotonin	Vasoconstriction, increased capillary permeability, macrophage attraction
	Histamine	Increased capillary permeability, attract and activate macrophages
	ADP	Promotes platelet aggregation
	ATP	Participates in platelet response to collagen Cofactor for platelet aggregation and fibrin formation

TGF: transforming growth factor; PDGF: platelet-derived growth factor; IGF: insulin-like growth factor; FGF: fibroblast growth factor; EGF: epidermal growth factor; VEGF: vascular endothelial growth factor; HGF: hepatocyte growth factor; ADP: adenosine diphosphate; ATP: adenosine triphosphate

2.3.3 Platelet rich plasma and mesenchymal stromal cells

The effect of PRP on MSC proliferation has been widely studied *in vitro*. Most of these studies, which used PRP as a supplement for cell expansion, concluded that PRP has a great potential to increase MSC proliferation (Gottipamula, Sharma, Krishnamurthy, Sen Majumdar, & Seetharam, 2012; Hildner et al., 2015; Iudicone et al., 2014; Shih et al., 2011). The effect of PRP in inducing MSC differentiation was also investigated in a few studies. However, the results on the extent of differentiation induced by PRP remained inconclusive. A previous study by Lange et al. reported diminished effect of platelet lysate on adipogenic differentiation (Lange, Brunswig-Spickenheier, Eissing, & Scheja, 2012), while Cervelli et al. found that PRP in combination with insulin significantly enhanced adipogenic MSC differentiation (Cervelli et al., 2012). PRP also was reported to significantly enhance the osteogenic differentiation of MSCs in few studies (Parsons et al., 2008; van den Dolder, Mooren, Vloon, Stoelinga, & Jansen, 2006; Verrier et al., 2010), while in other studies, it was reported to inhibit the differentiation of MSCs to the osteogenic lineage (Gruber et al., 2004; Ogino, Ayukawa, Tsukiyama, & Koyano, 2005). Few studies also reported enhanced effect of PRP in inducing chondrogenic differentiation of MSCs (Mardani et al., 2013; Mishra et al., 2009; Wang, Chang, Kong, & Wang, 2015). However, studies by Elder et al. and Drengk et al. showed no significant increase in the expression of chondrogenic markers in the PRP treated cells (Drengk, Zapf, Sturmer, Sturmer, & Frosch, 2009; Elder & Thomason, 2014). These studies not only used different culture systems such as pellet culture, alginate or monolayer, but other lineage-specific differentiation factors were also externally added to the culture media to promote chondrogenic differentiation of the MSCs. A summary of these studies is presented in Table 2.5.

The discrepancy in the results of these studies could be due to various reasons. The use of different protocols for PRP preparation, the type of activator used, differences in the content of platelet concentrates, biological variation of donors, the use of different platelet concentrations and variation in the type of platelet derivatives could all result in the diverse outcomes (Middleton, Barro, Muller, Terada, & Fu, 2012).

Table 2.5: Summary of *in vitro* studies on PRP and MSC differentiation.

No.	Cell type	Cell culture system	Media supplemented with FBS/ADM/ODM /CDM	PRP from single donor or pooled	Treatment	Activator	Outcome	Reference
A. Adipogenic Differentiation								
1	Human bone-marrow derived MSCs	Monolayer	ADM	-	5% Platelet lysate	-	Decreased adipogenic differentiation	(Lange et al., 2012)
2	Human adipose-derived MSCs	Monolayer	-	Pooled	5% PRP + insulin	-	Enhanced adipogenic differentiation	(Cervelli et al., 2012)
B. Osteogenic Differentiation								
3	Rat bone marrow-derived MSCs	Cells seeded on porous collagenous carriers	10% FBS	Pooled	40 µL PRP	$CaCl_2$ and thrombin	Increased proliferation. Inhibited osteogenic differentiation	(Arpornmaeklong, Kochel, Depprich, Kubler, & Wurzler, 2004)
4	Human bone marrow- derived MSCs	Monolayer	ODM and Serum	Pooled	$2x10^8$, $4x10^7$, $8x10^6$,$1.6x10^6$ platelets/mL	Thrombin	Increased proliferation. Inhibited osteogenic differentiation	(Gruber et al., 2004)
5	Rat bone marrow-derived MSCs	Monolayer	15% FBS	Single donor	0.2, 1.0, 2.0, 4.0, and 10 µL PRP /mL	Freeze thaw	Increased proliferation. Inhibited osteogenic differentiation	(Ogino et al., 2005)

Table 2.5: Summary of *in vitro* studies on PRP and MSC differentiation (continued)

	Cell type	Cell culture system	Media supplemented with FBS/ADM/ODM/ CDM	PRP from single donor or pooled	Treatment	Activator	Outcome	Reference
6	Human mesenchymal stromal cells	Monolayer	10% FCS	Single donor	2.5% PRC	-	Increased proliferation and osteogenic differentiation	(Parsons et al., 2008)
7	Human bone marrow MSCs	Monolayer	10% FCS	Single donor	10% PRS	Freeze-thaw	Increased osteogenic differentiation	(Verrier et al., 2010)
8	Rat Muscle satellite cells	Cells seeded on nano-hydroxyapatite/ poly (lactide-co-glycolide)	10% FBS and ODM	-	-	Freeze thaw	Increased proliferation and osteogenic differentiation	(Huang & Wang, 2012)
9	Human Adipose-derived MSCs	Monolayer	ODM	Single	5, 10, 20, 40% PRP. Medium replaced every 2 days.	10% $CaCl_2$	Increased proliferation. Enhanced osteogenic differentiation	(Xu et al., 2015)
C. Chondrogenic Differentiation								
10	Sheep bone marrow-derived MSC	Pellet	-	Pooled	150uL	$CaCl_2$	No significant increase in Col II expression and chondrogenic differentiation	(Drengk et al., 2009)

Table 2.5: Summary of *in vitro* studies on PRP and MSC differentiation (continued)

	Cell type	Cell culture system	Media supplemented with FBS/ADM/ODM/ CDM	PRP from single donor or pooled	Treatment	Activator	Outcome	Reference
11	Human MSCs	Monolayer	FBS+CDM	-	10% Buffered PRP in DMEM .	-	Promoted proliferation, triggered chondrogenic differentiation. Higher expression of Runx2, Sox9, Aggrecan	(Mishra et al., 2009)
12	Human adipose-derived stem cells	Transwell	Differentiation inducing factors	Pooled	5%, 10% and 15% PRP	Thrombin	Induced chondrogenic differentiation. Sox9, Col II, Col X significantly increased	(Mardani et al., 2013)
13	Human adipose-derived MSCs	Monolayer	-	Single Donor	10%, 15% and 20% PRP in DMEM/F12.	Yes. 20% $CaCl_2$	Increased proliferation and expression of chondrogenic markers Col II, Sox9, Aggrecan	(Van Pham et al., 2013)
14	Canine marrow stromal cells	Alginate and PRP beads	Differentiation inducing factors	Pooled	5% PRP every three days for 2 weeks	Freeze Thaw	PRP alone weakly promotes chondroinduction	(Elder & Thomason, 2014)

Table 2.5: Summary of *in vitro* studies on PRP and MSC differentiation (continued)

	Cell type	Cell culture system	Media supplemented with FBS/ADM/ODM/ CDM	PRP from single donor or pooled	Treatment	Activator	Outcome	Reference
15	Rabbit bone marrow-derived MSCs	Pellet culture	-	Single	10% PRP	-	Enhanced chondrogenic differentiation. Higher expression of OCN and Col I	(Wang et al., 2015)
16	Rabbit adipose-derived stem cells	Monolayer	10% FBS	-	10% PRP+10% FBS	-	Col II and aggrecan mRNA was significantly upregulated in the PRP-treated cells	(Tang et al., 2015)

FBS: fetal bovine serum; ADM: adipogenic differentiation medium; ODM: osteogenic differentiation medium; CDM: chondrogenic differentiation medium; PRS: platelet released supernatant; PRP: platelet rich plasma; OCN: osteocalcin

2.3.3.1 Major signalling pathways triggered by growth factors in PRP

Various growth factors in PRP can trigger signalling pathways that regulate MSC proliferation and differentiation. Growth factors such as IGF and FGF released by platelets activate mitogen-activated protein kinase (MAPK) cascades, which have been shown to play a key role in the transduction of extracellular signals to cellular responses such as proliferation and differentiation (Figure 2.6) (Shanmugarajan, Kim, Lee, & Im, 2011; Shaul & Seger, 2007). The signalling via this cascade is usually initiated by activation of small G proteins (e.g. Ras), which transmit the signal further by recruiting Raf kinases to the plasma membrane, where they are activated. Activated Raf subsequently phosphorylates MEK1 and MEK2, which activates ERK1 and ERK2 respectively, thereby regulating cellular proliferation. Phosphatidylinositol 3-kinase (PI-3K) can also activate Ras (Shaul & Seger, 2007) activating the PI-3K-mTOR (mammalian target of rapamycin) pathway. The p85 regulatory subunit of PI-3K stabilizes and protects the p110α subunit from degradation and inhibits its catalytic activity. Consequently, in resting cells, PI3K is inactive. Upon extracellular stimuli, interactions between p85 with phosphorylated tyrosine residues of the receptors, recruit PI3K to the inner surface of the plasma membrane. PI-3K generates PIP3 (phosphatidyl inositol 3,4,5 tri-phosphate), which recruits the protein kinase Akt to the plasma membrane. Active Akt dissociates from the membrane and translocates to the cytoplasm and to the nucleus where it phosphorylates multiple target proteins, implicated in cell proliferation (Aksamitiene, Kiyatkin, & Kholodenko, 2012).

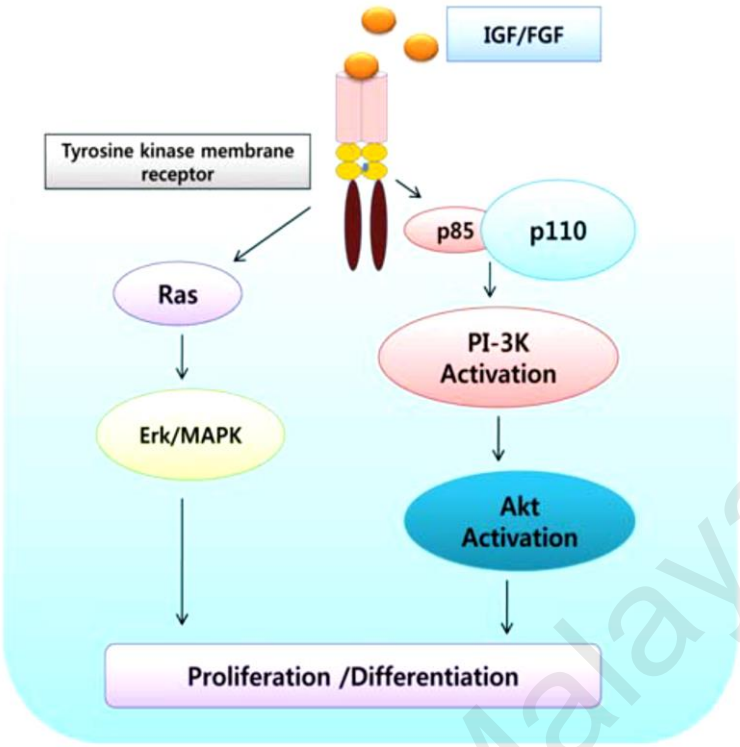

Figure 2.6: Signalling pathways triggered by growth factors leading to cell proliferation and differentiation. Growth factors such as IGF-1 and FGF-2 bind to their receptors and activate signalling molecules like PI-3K and ERK kinase, which regulate cellular functions such as proliferation and differentiation (Shanmugarajan et al., 2011). PI-3K: phosphatidylinositol 3-kinase 3-kinase; ERK: extracellular signal regulated kinase.

The differentiation of hMSCs is also regulated by the growth factors present in PRP, which induce specific intracellular signal pathways that modify the expression and activity of several transcription factors. Runx2, regarded as the master regulator of osteogenesis, is activated by the BMP2 signalling cascade. Activation of BMP signalling during osteogenesis through Smad1/5/8 and MAPK downstream signalling stimulates the Runx2 expression (James, 2013; Lee et al., 2000) (Figure 2.7), which then induces the expression of osteoblastic markers osteocalcin, type I collagen and osteopontin (Jang et al., 2012; Welch et al., 1998).

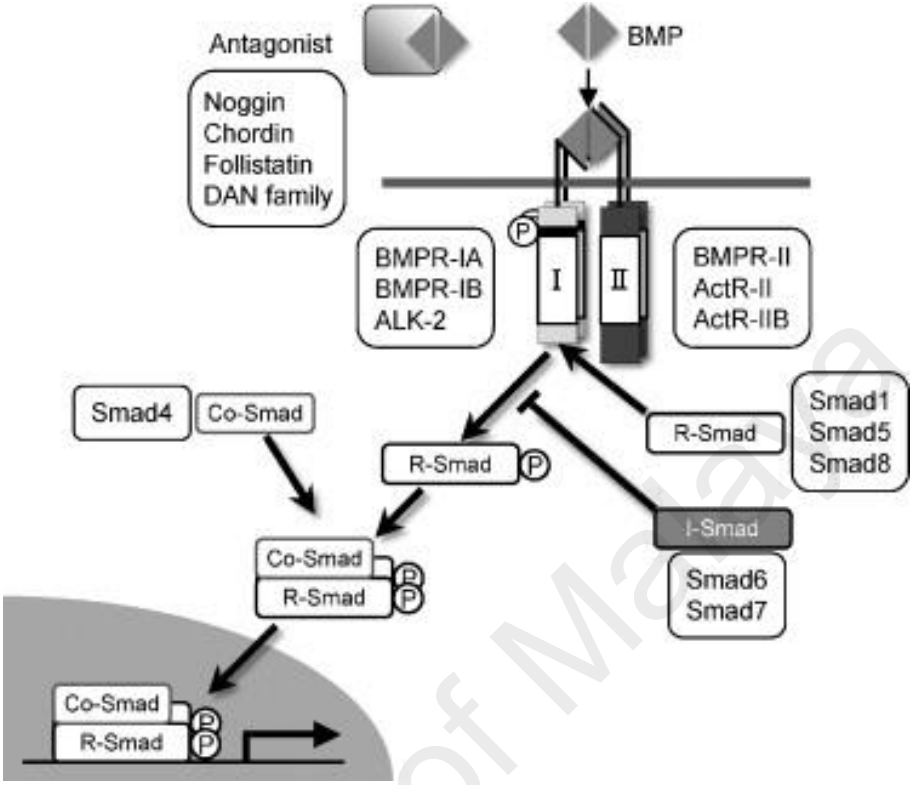

Figure 2.7: Schematic representation of BMP intracellular signal transduction. The BMP-specific R-Smads, Smad1, Smad5, and Smad8 are phosphorylated by the BMP type I receptors. The phosphorylated Smad proteins form complexes with the Co-Smad, Smad4, translocate into the nucleus, bind to the regulatory elements of target genes, and regulate their transcription (figure modified from (Jimi, Hirata, Shin, Yamazaki, & Fukushima, 2010)). BMPR: bone morphogenetic receptor, ALK: activin receptor-like kinase, ActR-II: activin receptor type II, ActRIIB: activin receptor type II B.

Signalling pathway triggered by the TGFβ growth factor could affect gene transcription, thereby influencing chondrogenic differentiation. TGFβ binds to the serine – threonine kinase receptors on the cell surface and activates the Smad pathway (Figure 2.8). Type I TGFβ receptors phosphorylate receptor-regulated Smads (R-Smads), which form a complex with common-partner Smad (Co-Smad).

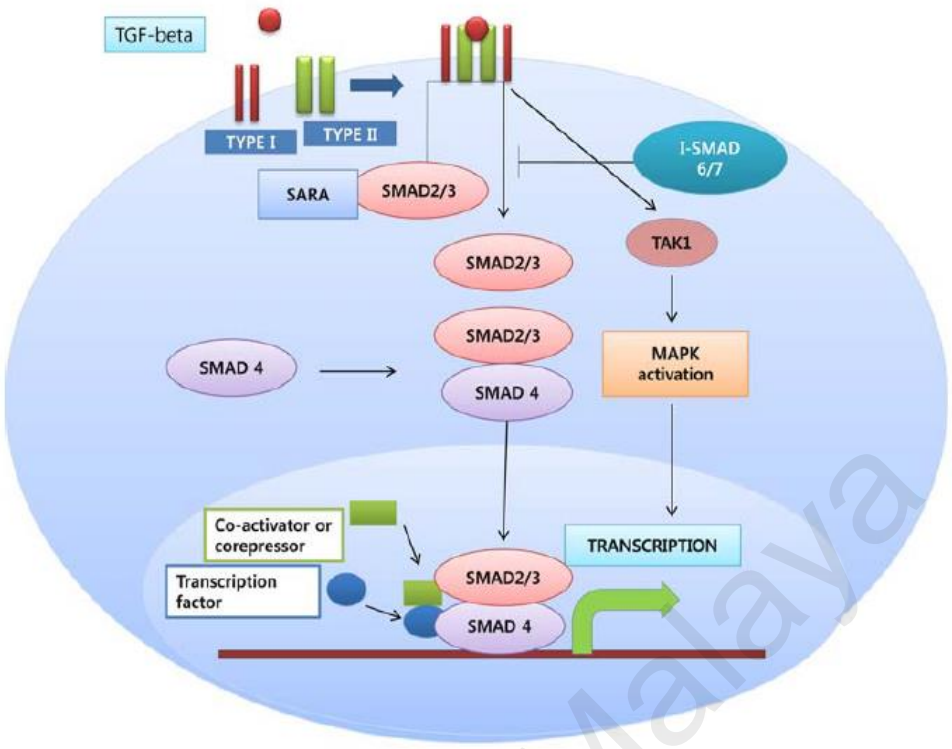

Figure 2.8: Schematic representation of TGF-β signal transduction. Growth factor TGFβ binds to the receptor TGFβII, which then recruits and phosphorylates TGFβI, leading to the activation and phosphorylation of Smad2 and Smad3. Activated Smads combined with Smad4 is translocated into the nucleus and initiates the transcription of chondrogenic-specific genes (Shanmugarajan et al., 2011). SARA: Smad-anchored for receptor activation, MAPK: mitogen activated protein kinase, TAK: Transforming growth factor β-activated kinase 1, I-Smad: inhibitor Smad.

This complex is translocated into the nucleus and it modulates transcription of genes required for chondrogenic differentiation (Song, Estrada, & Lyons, 2009). The other possibility is the activation of mitogen-activated protein kinase (MAPK), which interact with Smads to regulate the proliferation and differentiation process (Arita, Pelaez, & Cheung, 2011). The presence of various chondroinductive growth factors in PRP (Table 2.6) could promote chondrogenic differentiation via the signalling pathways mentioned above.

Table 2.6: Summary of the effect of major growth factors present in PRP on MSC chondrogenesis.

Growth Factor	Effect	References
TGFβ1	Initiator of MSC condensation, increases proliferation and ECM production, downregulates collagen type 1 gene expression, counteracts catabolic cytokines, induce type II collagen expression	(Blaney Davidson, van der Kraan, & van den Berg, 2007; Hui, Rowan, & Cawston, 2001; Ma, Hung, Lin, Chen, & Lo, 2003; Takahashi et al., 2005)
FGF	Increases proteoglycan synthesis and cell proliferation	(Schmal et al., 2007)
BMP2	Increases proliferation, proteoglycan synthesis and ECM production downregulates collagen type 1 gene expression	(Schmitt et al., 2003; Sekiya, Larson, Vuoristo, Reger, & Prockop, 2005; Zhang, Green, & Stott, 2002)

TGFβ1: transforming growth factor β1; FGF: fibroblast growth factor; BMP2: bone morphogenetic growth factor.

In recent years, there has been a resurgence of interest to explore the role of PRP in promoting cartilage repair. Attainment of good healing of cartilage tissues has always been a great challenge, considering that the avascular nature of the tissue often limits proper tissue regeneration following an injury. The abundance of chondroinductive factors within the platelets may synergistically act with the MSC to enhance healing of cartilage tissue. This is a pertinent subject of current research. An understanding of the structure of cartilage and how they undergo matrix destruction during injury and their mechanism of repair will provide further insight to identifying an effective strategy to improve the cartilage repair process. The next section briefly describes the biology of articular cartilage and the potential role of PRP and MSC in the repair of cartilage defects.

2.4 Structure, injury and repair of articular cartilage

2.4.1 Articular cartilage structure and composition

Articular cartilage is a highly specialized connective tissue found at the ends of the articulating bones. It is hyaline in nature, about 2 – 4 mm thick and provides a smooth surface for low-friction movement of the synovial joints. Articular cartilage is avascular, aneural and alymphatic, and hence has a poor self-repair capacity. It is divided into four zones based on the distribution of the collagen fibers and the sparsely populated chondrocytes (Figure 2.9). The thin superficial (tangential) zone protects deeper layers from shear stresses and makes up approximately 10% to 20% of articular cartilage thickness. The collagen fibers of this zone (type II and IX collagen) are aligned parallel to the articular surface. This zone has a high number of flattened chondrocytes and is in contact with the synovial fluid. Below the superficial zone is the transitional (middle) zone which contains 40% to 60% of the total cartilage volume. It is mainly composed of procollagen type II, collagen type IX and collagen type XI (Akkiraju & Nohe, 2015). The chondrocytes in this zone are spherical and occur at a low density (Buckwalter, Mow, & Ratcliffe, 1994; Sophia Fox, Bedi, & Rodeo, 2009).

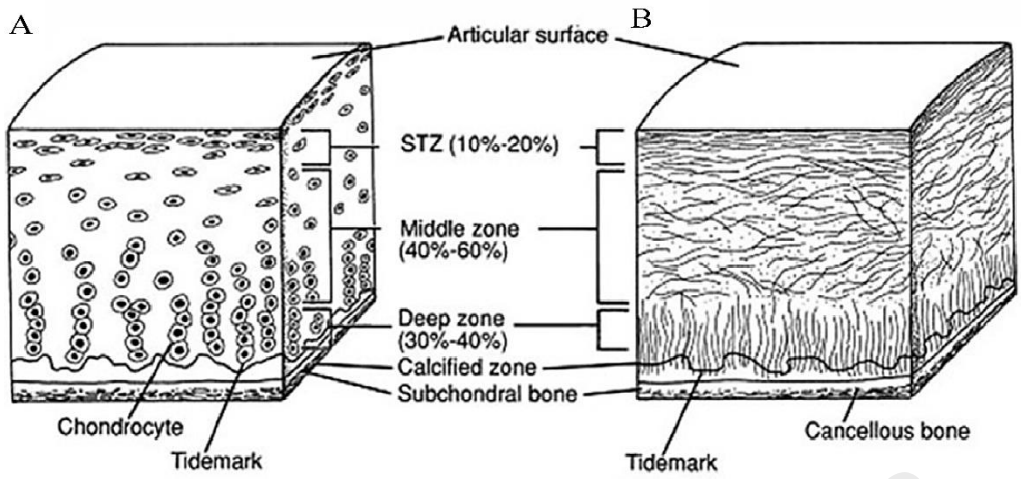

Figure 2.9: Different zones of the articular cartilage. (A) The structure of the articular cartilage can be divided into four zones based on the cellular organization. (B) The zones also differ in the organization of the collagen fibrills (Buckwalter et al., 1994). STZ: superficial tangential zone.

In the deep zone, the collagen fibrils made primarily of procollagen type II and collagen type X (Akkiraju & Nohe, 2015), are arranged perpendicular to the articular surface, providing the greatest resistance to compressive forces. The diameter of the collagen fibres in this zone is the largest and it has the highest proteoglycan content with the lowest water content. The chondrocytes are typically arranged in columnar orientation, parallel to the collagen fibers. The deep zone represents approximately 30% of the articular cartilage volume (Buckwalter, Mankin, & Grodzinsky, 2005; Sophia Fox et al., 2009).

Articular cartilage is composed of a dense ECM with a sparse distribution of highly specialized cells called chondrocytes. The ECM is principally composed of water, collagen, and proteoglycans, with other non-collagenous proteins and glycoproteins present in lesser amounts (Woo & Buckwalter, 1988). Together, these components help to retain water within the ECM, which is critical to maintain its unique mechanical properties (Sophia Fox et al., 2009). About 20% to 40% of the articular cartilage is composed of collagens, proteoglycans and non-collagenous proteins while water

contributes to about 80% of the wet weight (Buckwalter et al., 2005). Interaction of the proteoglycans with water provides a swollen hydrated tissue that helps the cartilage in resisting compressive loading (Lewis, McCarty, Kang, & Cole, 2006).

2.4.1.1 Collagens

Collagen is the most abundant structural macromolecule in ECM making up about 60% of the dry weight of cartilage. About 90% to 95% of the total collagen in the cartilage ECM is comprised of Type II collagen. It forms fibrils and fibers interlinked with the proteoglycan aggregates. Collagen types I, IV, V, VI, IX, and XI are also present but contribute only a minor proportion to the cartilage ECM. These minor collagens help to form and stabilize the type II collagen fibril network. The triple helical structure of the collagen polypeptide chain gives the articular cartilage its shear and tensile properties, which help to stabilize the ECM (Sophia Fox et al., 2009).

2.4.1.2 Proteoglycans

Proteoglycans consist of a protein core to which glycosaminoglycan polysaccharides (chondroitin sulfate and keratan sulfate) are attached to form a bottlebrush-like structure (Figure 2.10). These proteoglycans bind to the backbone of hyaluronic acid (HA) or hyaluronate. Proteoglycans are hydrophilic and they repel each other due to the presence of the negatively charged glycosaminoglycans (Gao et al., 2014).

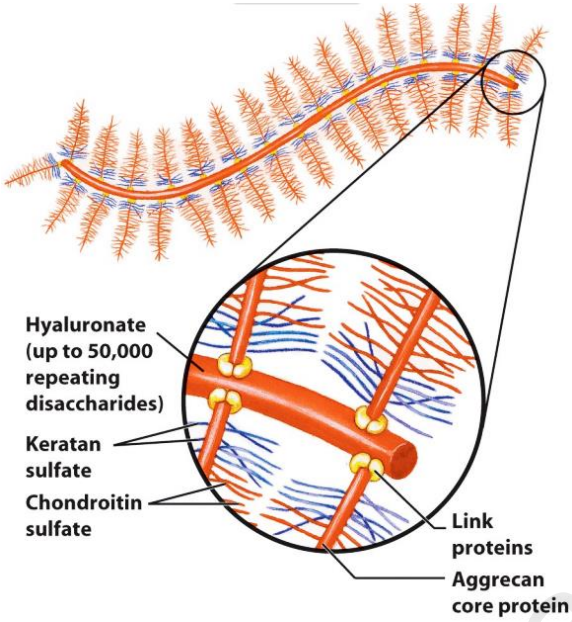

Figure 2.10: Structure of a proteoglycan. Schematic representation of a proteoglycan consisting of a core protein molecule to which a large number of chondroitin sulfate and keratan sulfate chains are attached (Lehninger, Nelson, & Cox, 2004).

Aggrecan is the major proteoglycan found in cartilage and exists as a proteoglycan aggregate in association with HA and link proteins. Cartilage also contain a variety of small leucine-rich repeat proteoglycans (SLRPs), like decorin, biglycan, fibromodulin and lumican, which help in maintaining the integrity of the tissue and modulate its metabolism (Table 2.7) (Roughley, 2006). At high concentrations, proteoglycans create a large osmotic swelling pressure and draw water into the tissue. This occurs because the negatively charged anionic groups on the GAG chains of aggrecan attract mobile counter ions such as Na^+ that creates a large difference in ion concentration between the cartilage and surrounding tissue. Water is drawn into cartilage because of this osmotic imbalance and the addition of water causes the aggrecan-rich matrix network to swell and expand. This water-swollen matrix and the stiff collagen/aggrecan network offers resistance to deformation and is critical to the biomechanical properties of the cartilage. Hence, cartilage is referred to as a visco-elastic tissue as it is stiff enough to resist sudden impact

loading and also shows some slow inelastic deformation with sustained loads (Kiani, Chen, Wu, Yee, & Yang, 2002).

Table 2.7: Summary of the components present in articular cartilage (Mow VC, 2005).

% Wet Weight	Molecule
60-85	Water
15-22	Collagen Type II
4-7	Aggrecan Hyaluronan Link Protein Type VI collagen Type IX collagen Type XI collagen COMP Decorin Biglycan Fibromodulin Fibronectin Thrombospondins

COMP: cartilage oligomeric matrix protein.

2.4.1.3 Water

Water is the most abundant component of the articular cartilage and its concentration decreases from about 80% at the superficial zone to 65% in the deep zone (Buckwalter & Mankin, 1997). Water takes up most of the compression pressure and facilitates the lubrication of the joint. In response to pressure, water from the cartilage squeezes out into the joint space where it comes in contact with the synovial fluid. After the pressure is removed, cartilage reabsorbs the water and is hydrated again. During this process, the water transfers nutrients from the synovial fluid and nourishes the chondrocytes (Khabarov, Boykov, Selyanin, & Polyak, 2014).

2.4.1.4 Chondrocytes

Chondrocytes make up to 1% of the normal articular cartilage (Lewis et al., 2006). They receive nutrition via diffusion from the synovial fluid considering the avascular nature of articular cartilage (Sophia Fox et al., 2009). They secrete ECM to maintain and sustain the cartilage and also respond to outside stimuli and tissue damage. Moreover, these cells function in a low oxygen environment and have a low metabolic turnover (Akkiraju & Nohe, 2015; Buckwalter et al., 2005).

2.4.2 Articular cartilage injury and spontaneous repair

Articular cartilage damage is usually caused by sports injuries or accidental trauma and aging. It regularly progresses to more serious joint disorders such as osteoarthritis (OA), necrosis of subchondral bone tissue or arthritis (Zhu et al., 2013). There are two distinct types of chondral injury based on the attributing factors: focal lesions and degenerative lesions. Focal lesions are well delineated defects, usually caused by trauma, osteochondritis dissecans or osteonecrosis. Degenerative defects are typically poorly demarcated and usually occurs as a result of ligament instability, meniscal injuries, malalignment or OA (Willers, Wood, & Zheng, 2003). The injuries can be further classified into three distinct types: (1) microdamage to the cells and matrix without visible or mild disruption of the articular surface (partial thickness), (2) macrodisruption of the articular cartilage alone (chondral fractures) (full thickness), and (3) fracture of the articular cartilage and the subchondral bone (osteochondral fractures) (Buckwalter et al., 1994; Falah, Nierenberg, Soudry, Hayden, & Volpin, 2010).

A single moderately severe impact or less severe repetitive trauma can damage cartilage. Compressive forces acting on an articular surface will produce a variety of stresses (tension, compression, shear, and hydrostatic pressure) within the cartilage. These stresses, if sufficiently high, can cause chondral fissures, flaps, and fractures, as well as chondrocyte damage (Buckwalter & Mow, 1992). Localized fibrillation or disruption of the most superficial articular cartilage layers represent the earliest sign of tissue degeneration (Buckwalter et al., 2005; Ehrlich, Armstrong, Treadwell, & Mankin, 1987). The molecular mechanisms underlying the progressive loss of articular cartilage is unknown, however, the process has been divided into three overlapping stages: cartilage matrix damage, chondrocyte response to tissue damage, and the decline of the chondrocytic synthetic response and progressive loss of tissue (Buckwalter, 1995; Mankin, 1974a).

In the first stage, the matrix macromolecular framework is disrupted at the molecular level and the water content increases. The increase in water content is followed by a decrease in proteoglycan aggregation and aggrecan concentration and a decrease in the length of the glycosaminoglycan chains. This is accompanied by an increase in the water permeability and a decrease in the stiffness of the matrix, which subsequently increases the vulnerability of the tissue to further mechanical damage (Buckwalter et al., 2005; Mankin & Thrasher, 1975). The second stage involves the response of the chondrocytes to the tissue damage. At this stage, the chondrocytes proliferate and respond in both an anabolic and catabolic manner. Chondrocytic response to cartilage degeneration is marked by proliferating cell clusters surrounded by new extracellular matrix (Mankin & Lippiello, 1970; Martin & Buckwalter, 1996). Chondrocytes also produce nitric oxide in response to mechanical or chemical stresses, which induce the production of interleukin IL-1. IL-1 stimulates the expression of metalloproteases that degrade the matrix

macromolecules including type IX and type XI collagens. This destabilizes the collagen fibril network allowing diffusion of water into the cartilage tissue (Blanco, Ochs, Schwarz, & Lotz, 1995). The anabolic repair response by the chondrocytes may reduce the catabolic effects, and in some cases, may repair the tissue. This repair response may last for few years and temporarily halt further tissue degeneration. In most cases when the newly synthesized matrix and proliferating cells do not fill the tissue defect, and thereby failing to restore the articular surface, this would lead to the third stage of progressive tissue degeneration. This stage is characterized by a decline in chondrocytic anabolic and proliferative response (Mankin, 1974b; Martin & Buckwalter, 1996).

Acute joint injuries from more severe impact may also result in fractures that extend through the cartilage into subchondral bone. Unlike injuries that are limited to cartilage, fractures that extend into subchondral bone cause haemorrhage and fibrin-clot formation, thereby activating the inflammatory response (Buckwalter, 2002). Platelets within the clot release growth factors and cytokines, which stimulate the repair of the osteochondral defects by initiating vascular invasion and migration of undifferentiated cells, proliferation of these cells, and differentiation into chondrocyte-like cells in the chondral portion of the defect. The cells within the chondral region produce a repair cartilage that usually contains a high concentration of type II collagen and proteoglycans, but often also contains some type I collagen (Buckwalter et al., 1994).The composition of the newly formed cartilage repair tissue rarely resembles the structure of normal articular cartilage (Mankin, Mow, Buckwalter, Iannotti, & Ratcliffe, 1994; Poole, 1997). In most large osteochondral injuries, the chondral repair tissue begin to show evidence of depletion of matrix proteoglycans, fragmentation, fibrillation, and loss of chondrocyte-like cells (Buckwalter & Mow, 1992; Buckwalter, 1998). The remaining cells typically assume the appearance of fibroblasts as the surrounding matrix primarily consist of densely packed

type I collagen fibrils. This fibrous tissue usually fragments and often disintegrates within a year, and may leave areas of exposed bone (Buckwalter et al., 1994).

2.4.3 Surgical treatment of articular cartilage defects

Currently, there are a number of surgical approaches that offer reparative and restorative treatment strategies with a possibility of articular cartilage regeneration (Buckwalter et al., 1994; Lewis et al., 2006). Methods of stimulating cartilage repair that are currently used in clinical practice include microfracture, autografts and allografts transplantation, total and partial joint replacement, autologous cell implantation (ACI), matrix-induced ACI (MACI), implantation of chondrocytes or mesenchymal stromal cells, and the use of chondrogenesis-stimulating factors.

2.4.3.1 Microfracture

Microfracture surgery is a common method to treat small articular cartilage defects. This method creates microfractures in the underlying subchondral bone by drilling, shaving, or abrasion (Laurencin, Ambrosio, Borden, & Cooper, 1999). It enhances migration of MSCs from bone marrow to the site of the cartilage defect (Makris, Gomoll, Malizos, Hu, & Athanasiou, 2015). This treatment is effective especially for defects smaller than 2 cm^2, and is attractive due to its relatively minimally invasive nature, short surgery and recovery time, and low morbidity (Clair, Johnson, & Howard, 2009). However, the results of articular cartilage repair using microfracture is subjected to high inter-patient variability. Young and active patients and early treatment of the defects produce better cartilage repair (Clair et al., 2009). Microfracture surgery also often results in the formation of fibrocartilage that is biochemically and biomechanically inferior to hyaline articular

cartilage (Bae, Yoon, & Song, 2006; Kreuz et al., 2006), thus resulting in high failure rates and limited effectiveness of this treatment approach (Temenoff & Mikos, 2000).

2.4.3.2 Autografts and Allograft transplantation

Autografts and allograft transplantation are also used for the repair of small cartilage lesions. In autograft transplantation, healthy, cylindrical cartilage tissue plugs are harvested from the patient's low weight-bearing area and then implanted into the defect site (Clair et al., 2009). Although encouraging clinical results has been reported, there are some limitations associated with the use of autografts. These include insufficient donor tissues (both in quantity and quality), donor site morbidity, surface mismatch of the graft and implant sites, graft instability, and failure of long-term survival of the implant at its new high weight-bearing location (Laurencin et al., 1999; Zhang, Hu, & Athanasiou, 2009). For allograft transplantation, cartilage tissues are obtained from tissue banks. This avoids donor site morbidity, insufficient donor tissue supply and the multiple step surgeries required in autograft transplantations. However, it has similar limitations to autografts, such as surface mismatch and uncertain load bearing capacity. Apart from these, the use of allografts may also induce immune reactions such as inflammation at the defect site and tissue rejection (Zhang et al., 2009).

2.4.3.3 Total and Partial Joint Replacements

Total or partial joint replacements are performed in cases involving severe joint injuries or advanced osteoarthritis. The damaged osteochondral tissue is partially or completely removed and resurfaced. An artificial implant made of metal (titanium, stainless steel or alloys), a polymer piece (polyethylene for smooth gliding) and a metal stem is implanted to replace the damaged joint (Zhang et al., 2009). However, due to frequently reported complications including infection, implant loosening, wear debris and osteolysis, revision surgeries are often required, which burdens the patient with increased pain and costs (Fritz, Lurie, & Potter, 2015).

2.4.3.4 Autologous chondrocyte implantation

Autologous chondrocyte implantation (ACI) is the first generation of cell transplantation techniques for cartilage repair (Brittberg et al., 1994). It has been recommended for patients who have cartilage lesions between 1 cm^2 - 12 cm^2, or have had previously failed microfracture surgeries (Gikas, Bayliss, Bentley, & Briggs, 2009). This technique involves two surgeries. Cartilage sample from a low-weight-bearing region of the joint is collected by biopsy punch during the first arthroscopic operation to provide a chondrocyte population that is then expanded *in vitro*, yielding approximately 12 - 48 million cells. During the second operation, the chondrocytes are implanted into the debrided cartilage defect and covered by a membrane. The two major advantages of this technique is that it avoids immune complications or viral infections potentially acquired from allogeneic or foreign materials since it uses patient's own cells, and it minimises donor site morbidity compared to autograft transplantation since only a small biopsy is performed (Makris et al., 2015; Saris et al., 2009; Saris et al., 2008). However, this technique still has some

limitations. For example, the invasive ACI procedure has a long recovery time (6–12 months) and it requires multiple surgeries to harvest healthy cartilage and periosteal patch, and to re-implant the healthy cells (Chiang & Jiang, 2009). In addition, the possibility of periosteal hypertrophy, dedifferentiation of patients' chondrocytes during *in vitro* culture, and decreased number of human chondrocyte associated with aging may impair or even result in failure of repair using ACI (Bobacz, Erlacher, Smolen, Soleiman, & Graninger, 2004; Clair et al., 2009; Stockwell, 1967).

2.4.3.5 Matrix-induced ACI (MACI)

As a result of the technical difficulty and associated complications, the second generation of ACI was developed using a collagen scaffold instead of a periosteal patch (Bartlett et al., 2005; Gooding et al., 2006; Haddo et al., 2004). However, this technique still requires an arthrotomy (Dunkin & Lattermann, 2013). Further technological advances have led to the development of third-generation ACI, where chondrocytes are embedded into three-dimensionally constructed scaffolds (i.e. 3D environment) for cell growth (Marcacci et al., 2005). Specifically, culturing the cells on to a biodegradable type I or III collagen membrane is commonly referred to as matrix-induced ACI (Jacobi, Villa, Magnussen, & Neyret, 2011). The benefits of MACI include no periosteal harvesting, good stability of implant, being less invasive and allowing early mobilisation of the joint (Falah et al., 2010). However, MACI still requires two surgical procedures and is an expensive solution (Gobbi et al., 2009). The superiority of MACI technique over the existing treatments also remains unproven (Makris et al., 2015).

2.4.3.6 Implantation of Chondrocytes or MSCs

As mature chondrocytes have limited ability to repair cartilage defects, one of the constructive approaches to repair cartilage defect is by introducing a new cell population into the chondral defect. These cells may be obtained from populations grown in culture supplemented with chondrogenesis factors and used with artificial matrices to enhance the formation of new cartilage (Chiang & Jiang, 2009; Yu, Han, & Kim, 2012). Experimental evidence of this approach indicated that the transplanted cells can survive and synthesize a cartilaginous matrix that appears to more closely resemble normal cartilage than the fibrous tissue that forms in similar defects without cell transplants (Buckwalter et al., 1994; Deng et al., 2014). One possible method of applying this approach in humans would be to harvest mesenchymal stromal cells or chondrocytes, expand them in culture, implant them in an artificial matrix, and then implant the matrix and the cells into a cartilage defect (Buckwalter et al., 1994).

2.4.3.7 Chondrogenesis-Stimulating Factors

A variety of polypeptide growth factors (e.g., TGF-β, bone morphogenic proteins, insulin-like growth factor, fibroblast growth factor, and platelet-derived growth factor) influence chondrocyte and other mesenchymal cell functions, such as cell migration, proliferation, matrix synthesis, and cellular differentiation (Fortier, Hackett, & Cole, 2011). The effects of these growth factors on chondrocytes are mediated by cell-surface receptors (integrins). These factors may also directly modify the extracellular matrix and thus modulate the signals (e.g., stresses, strains, and fluid pressure and flow) transmitted to the cells from the surrounding extracellular matrix (Xie, Zhang, & Tuan, 2014). Experimental work has shown that selected growth factors can stimulate formation of cartilaginous

tissue *in vitro* and *in vivo*. Most of these growth factors have demonstrated mitogenic activity on chondrocytes *in vitro*, while basic fibroblast growth factor, insulin-like growth factor I, and TGF-β have also been shown to stimulate matrix synthesis *in vivo* (Xie et al., 2014). In addition, some growth factors potentiate the metabolic effects of other growth factors. For example, TGF-β can potentiate the mitogenic effects of basic fibroblast growth factor or insulin-like growth factor I, and insulin-like growth factor I and basic fibroblast growth factors act synergistically to increase the matrix synthesis (Buckwalter et al., 1994; Vinatier et al., 2009). These growth factors were also transfected with a viral vector into MSCs or chondrocytes and used *in vivo* or clinical settings for the repair of cartilage defects (Diao et al., 2009; Ha, Noh, Choi, & Lee, 2012). In rabbits with full-thickness cartilage defects, the implantation of MSCs seeded in plasmid transforming growth factor beta1 (pTGF-beta1) encapsulated in three-dimensional scaffolds was found to stimulate hyaline-like cartilage matrix synthesis (Diao et al., 2009; Guo et al., 2006). Phase I clinical trial using retrovirally transduced human chondrocytes expressing TGF-β1 in degenerative OA patients showed symptomatic improvements; however, no significant differences in the symptomatic scores were observed after 12 months of follow up (Ha et al., 2012). These studies used only a single growth factor to promote chondrogenesis. A combination of multiple growth factors would be ideal for a more effective chondrogenesis and for the formation of hyaline cartilage tissue. It has been previously shown that the synergistic actions of a combination of growth factors such as BMP-7 and IGF-I (Loeser, Pacione, & Chubinskaya, 2003), and IGF-I, FGF-2, and TGF-β (Shi, Mercer, Eckert, & Trippel, 2009) further enhanced cartilage matrix synthesis. The notion that a combination of bioactive growth factors would probably be necessary to enhance cartilage repair has led to an increasing interest in recent years to explore the role of natural growth factors reservoir such as PRP in articular cartilage repair (Fortier, Barker, Strauss, McCarrel, & Cole, 2011).

2.4.4 Application of PRP in articular cartilage repair

PRP has recently emerged as a non-operative modality for treatment of cartilage injuries (Andia, Sanchez, & Maffulli, 2012; Fortier, Hackett, et al., 2011). There are numerous growth factors in PRP that stimulate cartilage matrix synthesis and mitigate the effects of catabolic cytokines such as interleukin (IL)-1 and tumour necrosis factor-α (TNF-α) (Fortier, Hackett, et al., 2011). In a previous study, large osteochondral defects (5-mm diameter × 4 mm deep) created in rabbits were treated with either autogenous PRP in a poly-lactic-glycolic acid (PLGA) carrier, PLGA alone, or left untreated. At 4 and 12 weeks post-treatment, the PRP group showed a higher extent of osteochondral formation and increased production of glycosaminoglycans in the extracellular matrix compared to other groups. Only PLGA resulted in the formation of fibrocartilage. The authors postulated that when PRP incorporated PLGA scaffolds are used, the growth factors are released gradually as the PLGA biodegrades. In general, most growth factors undergo rapid degradation when injected directly in soluble form to the injury site, so they could not sustain biological activity *in vivo* (Sun, Feng, Zhang, Chen, & Cheng, 2010). Another study compared the effect of microfracture alone, microfracture followed by transplantation of PRP gel, and microfracture followed by intra-articular injection of unclotted PRP in a sheep model of full-thickness cartilage defects. The data at 6 months post-treatment indicated that the use of PRP had a positive effect and the procedure was more effective when PRP was transplanted as a gel compared to liquid intra-articular injection. However, the tissue formed had great amount of cell clusters and small but deep clefts, and lateral integration with surrounding cartilage reported at macroscopic evaluation was not confirmed at histology indicating short-term durability of the repaired tissue. Furthermore, presence of poorly stained areas at Safranin-O staining was indicative of limited metabolic cell activity and poor-quality extra-cellular matrix. The

procedure adopted in this study can be used as a one-stage treatment as they did not incorporate cultured cells (Milano, Sanna Passino, et al., 2010). Apart from the use of PRP alone, the concomitant use of PRP with MSC has also been considered as an alternative treatment strategy as the advantages of each method could be combined in partnership to further enhance the repair of cartilage defects. Previous studies have shown that a combination of PRP and MSCs yielded better repair compared to only PRP and only FBS (control) in treating defects extending into the sub-chondral bone (Van Pham et al., 2013; Xie et al., 2012). However, their effect in treating defects limited to the chondral surface is still not known. *In vivo* studies involving PRP application in cartilage repair are summarized in Table 2.8.

Table 2.8 Summary of *in vivo* studies on PRP application in articular cartilage repair.

No.	Animal Model	Defect	Treatment	Activator	Outcome	Reference
1	Rabbit	ACLT	PRP with biodegradable gelatin hydrogel microspheres	$CaCl_2$	PRP with biodegradable gelatin hydrogel induced better healing than intra articular PRP injection	(Saito et al., 2009)
2	Rabbit	Osteochondral	Bilayer collagen matrix+ PRP	-	PRP in scaffold improved healing	(Qi et al., 2009)
3	Rabbit	Osteochondral	PRP in a poly-lactic-glycolic acid (PLGA)	$CaCl_2$	PRP in scaffold improved healing	(Sun et al., 2010)
4	Sheep	Chondral	Microfracture and PRP in fibrin glue	Ca-gluconate solution	PRP in fibrin glue induced better healing than intra articular PRP injection	(Milano, Passino, et al., 2010)
5	Sheep	Osteochondral	Hydroxyapatite-collagen scaffold+PRP	$CaCl_2$	PRP+scaffold had a negative effect	(Kon et al., 2010)
6	Rabbit	Osteochondral	Bone marrow-derived rabbit MSCs in PRP gel	$CaCl_2$	MSC+PRP gel induced better healing compared to PRP gel alone	(Xie et al., 2012)
7	Mouse	Hind-limb joint destruction	ADSC+PRP injection	$CaCl_2$	ADSC+PRP induced better healing compared to ADSC treated with FBS	(Van Pham et al., 2013)
8	Rabbit	Chondral	PRP injection +Microfracture	-	PRP+Microfracture induced better healing compared to microfracture alone	(Huh et al., 2014)
9	Rabbit	Osteochondral	PRP injection	$CaCl_2$	PRP induced better healing than HA and joint fluid had low IL-1 concentration	(Liu et al., 2014)

ACLT: anterior cruciate ligament transection; ADSC: adipose derived stem cells; IL-1: interleukin 1; PRP: Platelet rich plasma; FBS: Fetal bovine serum

Evidence from clinical trials also suggest that PRP is effective as a therapeutic application for patients with knee OA (Sermer, Devitt, Chahal, Kandel, & Theodoropoulos, 2015). Clinical studies involving PRP application in cartilage repair are summarized in Table 2.9. In general, all studies showed that PRP is safe and has the potential to reduce pain and improve function and quality of life. In comparison with HA, most studies reported that PRP was better than HA (Cerza et al., 2012; Kon et al., 2011; Spakova, Rosocha, Lacko, Harvanova, & Gharaibeh, 2012), however, Filardo et al. reported no significant difference between PRP and HA treatment (Filardo et al., 2015). The discrepancy in PRP effectiveness in this case could be attributed to an older average age of patients included in the study compared to other studies. The authors postulated that PRP has more biological and clinical therapeutic potential in younger patients. This was supported by Kon et al, who showed that PRP produced better results in patients aged 50 years or younger with low degree of cartilage degeneration (Kon et al., 2011).

The presence of leukocytes in PRP did not seem to affect the outcome of treatment. The use of both leukocyte-rich PRP (Spakova et al., 2012) and leukocyte-filtered PRP (Patel, Dhillon, Aggarwal, Marwaha, & Jain, 2013) showed improvement in WOMAC score until 6 months. The effect of leukocyte has been controversial, as few studies have indicated that leukocyte content does not induce or impair the potential benefit of PRP when used in joints (Everts et al., 2008), while others claimed that it might cause deleterious effects due to the release of proteases and reactive oxygen species from the white cells (Kon, Filardo, Di Matteo, & Marcacci, 2013). To date, no consensus could be reached on the benefits of using leukocyte-rich or leukocyte-poor PRP as there is no study comparing the effects of both.

Unlike other studies, Haleem et al, Patel et al. and Spakova et al. showed that the improvement in the clinical scores lasted only till 6 months, indicating no sustained long-term effects and the beginning of diminishing therapeutic benefits (Haleem et al., 2010; Patel et al., 2013; Spakova et al., 2012). Filardo et al. reported that the median duration of the beneficial effects of PRP was 9 months (Filardo et al., 2011) Interestingly, the study by Patel et al., also showed no significant difference in the WOMAC score between the group that received one PRP injection and two PRP injections indicating no difference in the number of PRP injections used for the treatment (Patel et al., 2013). However, this could not be confirmed as there is currently lack of studies investigating the effect of frequency of PRP injections on clinical outcome of the recipients.

Although there is a relatively large body of evidence on the effect of PRP injections administered as a sole therapeutic agent, there are only very few studies that have investigated the effects of PRP in combination with MSCs. Haleem et al. showed improvement in the symptoms in patients treated with MSCs seeded on platelet rich-fibrin glue (PR-FG) implanted after sub-chondral drilling (Haleem et al., 2010). However, as this study involved the drilling of sub-chondral bone, the observed effect could also be attributed to stimulation of cells from the bone marrow. Giannini et al, showed complete integration of the regenerated tissue with the surrounding cartilage in 76% of the cases treated with bone marrow concentrate and platelet-rich fibrin gel with stable results observed for about 3 years (Giannini et al., 2010). These studies show the potential of enhancing cartilage repair using a combination of MSC and PRP. However, further studies are necessary to verify this beneficial outcome.

Overall, the studies were diverse in nature with some having a small sample size, some having no controls and some having used multiple injections. There is also uncertainty on the optimal dose or the interval of PRP injections required to ensure maximum efficacy. The current literature is complicated due to a lack of standardization of study protocols, platelet separation techniques, and outcome measures.

Table 2.9 Summary of clinical studies on PRP application in cartilage repair.

No.	Sample size	Study Design	Level of Evidence	Condition	Type of PRP	Procedure	Clinical Outcome	Reference
1	60	Retrospective cohort study	III	Idiopathic and secondary post-traumatic and mechanical OA	PRGF ($CaCl_2$ activated)	3 PRGF injections at 1-week interval; control group: HMW-HA	Significant improvement in patients injected with PRGF compared to HA at 2 months in WOMAC pain index.	(Sanchez, Anitua, Azofra, Aguirre, & Andia, 2008)
2	5	Case Series	IV	full-thickness cartilage lesion	BM-MSCs +PR-FG mixture	Sub-chondral bone drilling followed by implanting MSC seeded in a platelet rich fibrin glue.	Improvement at 6 and 12 months postoperatively in Lysholm and RHSSK score. MRI of 3 patients at 12 months postoperatively revealed complete defect fill.	(Haleem et al., 2010)
3	81	Therapeutic study	IV	focal osteochondral monolateral lesions	Bone marrow concentrate + PRF	Hyaluronic acid membrane loaded with 2 mL of bone marrow concentrate and with 1 mL of PRF	Improvement in AOFAS score from preoperatively to 12 months and from 12 to 36 months.	(Giannini et al., 2010)
4	50	Case series	-	Knee OA	Non-activated PRP	2 intra-articular injections of autologous PRP (monthly once)	All patients showed significant improvement in IKDC score at 6 and 12 months.	(Gobbi, Karnatzikos, Mahajan, & Malchira, 2012)

Table 2.9 Summary of clinical studies on PRP application in cartilage repair (continued)

No.	Sample size	Study Design	Level of Evidence	Condition	Type of PRP	Procedure	Clinical Results	Reference
5	120	-	-	OA	Leukocyte rich PRP	3 intra-articular injections – once every week of PRP or HA	PRP injections better than HA. Results of WOMAC Index decreased by six months in both PRP and HA group.	(Spakova et al., 2012)
6	78	Randomized controlled trial	I	Bilateral knee OA	Leukocyte filtered PRP (activated)	1 or 2 (after 3 weeks) injections of PRP+ $CaCl_2$ into a suprapatellar pouch	WOMAC score showed no significant difference between 1 and 2 injections. Improvements in WOMAC score reduced at 6 month.	(Patel et al., 2013)
7	120	Randomized controlled trial	I	OA (Gonarthrosis)	PRP (Autologous conditioned plasma)	4 intra-articular injections of PRP or HA	By week 24 continuous improvement in WOMAC score noticed in PRP group. Subjects treated with HA showed a sharp worsening.	(Cerza et al., 2012)
8	96	Randomized controlled trial	I	Early OA	leukocyte-rich PRP ($CaCl_2$ activated)	3 weekly intra-articular injections of either PRP or HA	No significant difference between PRP and HA injections after 12 months. Analyzed using IKDC subjective measure, KOOS, EuroQol visual analog scale, and Tegner score.	(Filardo et al., 2015)

Table 2.9 Summary of clinical studies on PRP application in cartilage repair (continued)

No.	Sample size	Study Design	Level of Evidence	Condition	Type of PRP	Procedure	Clinical Results	Reference
9	91	-	-	OA	PRP+ $CaCl_2$	Injections were administered every 21 days	Overall reduction observed at 24-month follow-up compared to the 12-month. Median duration of clinical improvement was 9 months. Analyzed using IKDC.	(Filardo et al., 2011)
10	44	Uncontrolled, prospective clinical trial	-	Early OA and Degenerative chondropathy	PRP	PRP injected twice intra-articularly with an interval of four weeks	Significant improvement in pain by 6 months. Analyzed by VAS, Lysholm Knee Scale, and Cincinnati Knee Rating System	(Lee et al., 2013)
11	150	Prospective comparative study.	II	Early degeneration and OA	PRP+ $CaCl_2$	Injections were administered every 14 days	PRP showed better performance (IKDC score) compared with high and low molecular weight HA by 6 months	(Kon et al., 2011)
12	52	Therapeutic study	IV	OA	PRP	Bone-marrow stimulation followed by implantation of PGA-hyaluronan scaffold immersed with PRP	KOOS score improved by 12 months	(Siclari, Mascaro, Gentili, Cancedda, & Boux, 2012)

Table 2.9 Summary of clinical studies on PRP application in cartilage repair (continued)

No.	Sample size	Study Design	Level of Evidence	Condition	Type of PRP	Procedure	Clinical Results	Reference
13	14	Uncontrolled, prospective preliminary study	-	OA		Intraarticular PRP injections	Improvement in pain and KOOS by 12 months. Outcome measures included the Brittberg-Peterson VAS, and KOOS	(Sampson, Reed, Silvers, Meng, & Mandelbaum, 2010)
14	52	Case series	IV	OA	PRP	Bone-marrow stimulation followed by implantation of PGA-hyaluronan scaffold immersed with PRP.	KOOS showed significant improvement at 1 and 2 year follow-up	(Siclari et al., 2014)
15	176	Randomized controlled multicenter trial	I	OA	PRGF	3 PRGF injections at 1-week interval; control group: HMW-HA	WOMAC secondary outcome score showed no significant difference between PRP and HA at 6 months	(Sanchez et al., 2012)

AOFAS: American Orthopedic Foot and Ankle Society Score; HA: Hyaluronic Acid/Hyaluronan; HMW: high molecular weight; IKDC: International Knee Documentation Committee; KOOS: Knee Injury and Osteoarthritis Outcome Scores; PRGF: Preparation rich in growth factors; PRF: platelet-rich fibrin gel; PR-FG: platelet-rich fibrin glue; RHSSK: Revised Hospital for Special Surgery Knee; VAS: Visual Analogue Scale; WOMAC: Western Ontario & McMaster Universities Osteoarthritis Index;

Considering the available literature, PRP has vast potential when used both *in vitro* and *in vivo*. Nevertheless, no clear evidence is yet available on the contribution of PRP alone in the *in vitro* proliferation and differentiation of MSCs. Moreover, the potential of PRP in inducing differentiation to various lineages in comparison to the commercially available differentiation inducing medium is unknown. The literature also shows that PRP has been used for cartilage repair both in *in vivo* and clinical studies. However, their therapeutic effect when used in combination with MSCs for the repair of defects limited to the chondral surface is not known. These defects are usually difficult to heal and the current repair strategies often result in the formation of fibrocartilage, with inferior mechanical properties to the hyaline cartilage resulting in only short term benefits. The use of PRP and MSC combination might provide a better sustained long term therapeutic effect. Indeed, the association of PRP with other biological treatments such as microfracture or the use of differentiation inducing scaffolds, and the lack of comparative studies have hampered the possibility to define the potential effect of PRP alone in improving cartilage repair outcomes (Marmotti et al., 2015). Hence, this thesis aims to address the above issues to provide a better understanding of the overall potential therapeutic benefit of platelet rich preparation in tissue repair.

CHAPTER 3: OPTIMIZATION OF PRC PREPARATION AND DETERMINATION OF ITS EFFECT ON HUMAN MESENCHYMAL STROMAL CELL PROLIFERATION AND EARLY DIFFERENTIATION IN THE MONOLAYER CULTURE SYSTEM

3.1 INTRODUCTION

Platelet rich concentrate (PRC) is a biological growth promoter that could enhance the regenerative potential of human mesenchymal stromal cells (hMSC). However, it has been previously noted that the effect of platelet rich concentrate on cells vary depending on the method of its preparation and the dosing protocol (Dhurat & Sukesh, 2014). Hence, before proceeding to investigate the effect of platelet rich concentrate on human mesenchymal stromal cells, it was essential to optimise the PRC preparation to get the best outcome. This chapter mainly describes the preliminary work required to optimise PRC preparation for its use in subsequent *in vitro* studies. The investigations carried out in this part of the study is also aimed to determine whether the use of platelet rich concentrate alone (without additional supplementation of other differentiating inducing factors in the culture medium) could promote proliferation and direct early differentiation of human mesenchymal stromal cells (hMSCs) *in vitro* within a very short time frame.

The use of MSCs for regeneration of damaged tissues offers a great advantage owing to their proliferative and multi-lineage differentiation potential. For MSC treatment to be effective, the cells need to be expanded to reach sufficient numbers, usually in the order of millions, in addition to having the ability to undergo differentiation to a specific lineage. Previous studies have demonstrated that platelet concentrates increased proliferation of MSCs (Iudicone et al., 2014; Rauch et al., 2011; Schallmoser et al., 2007)

and induced their differentiation when used in conjunction with additional lineage-specific differentiation factors (Iudicone et al., 2014; Schallmoser et al., 2007). The use of fetal bovine serum (FBS) and/or other mitogenic and differentiation inducing growth factors in conjunction with platelet rich preparation presents two major issues. Firstly, the presence of xenogenic and allogeneic components in the media could result in viral transmission and/or trigger immunological reactions following transplantation of the cultured cells in the clinical setting (Pham et al., 2014). Cultured MSCs have been shown to internalize xenogenic proteins in high amounts, thereby posing an additional risk of allergic reactions when transplanted *in vivo* (Spees et al., 2004). Secondly, the extent of contribution of platelet rich concentrate itself to the observed enhancement of MSC proliferation and differentiation remains unclear, since the increase in cell proliferation and differentiation could have been mainly induced by the external growth factors additionally supplemented to the culture media rather than the platelets themselves. Hence, there is a need to determine the effectiveness of platelet rich concentrate alone in inducing proliferation and differentiation of MSCs without other confounding variables. Moreover, it is not known if PRC alone would induce MSC differentiation to only one preferential lineage, or multiple mesenchymal lineages in a single experimental setting. Previous studies have focussed on investigating the effect of platelet rich preparation on hMSC differentiation to only one particular lineage of interest, with the concurrent use of lineage-specific culture media (Parsons et al., 2008; Verrier et al., 2010).

Another issue that would influence the effect of PRC on the regenerative potential of MSC is related to its preparation technique. There appears to be a wide variation in PRP preparation protocols reported in the literature. Prior to the platelet activation step, the variables that might influence the composition and effectiveness of platelet rich plasma include the number of spins, centrifugal acceleration and duration, lack of control over

blood coagulation and platelet aggregation during the preparation process, and variability in the blood components between blood samples (Araki et al., 2012; S. Harrison et al., 2011). These variations in the platelet rich preparation protocols lead to variability in the yield of platelets, which would result in variable composition of the biologically active molecules in the formulation, thereby producing different biological responses and efficacy of treatment (Dhurat & Sukesh, 2014; Nagata et al., 2010; Perez et al., 2014). There is presently no consensus regarding the most optimal concentration of platelet rich plasma for clinical use. Most studies only reported the platelet number and growth factor concentration in their platelet rich preparation while the amount of platelets being activated during the preparation process was not specified. It has been speculated that mechanical trauma produced during the centrifugation steps might partially activate the platelets even before their application (Dhurat & Sukesh, 2014; Weiss, Evanson, McClenahan, Fagliari, & Walcheck, 1998).

In addition to the lack of accurate characterization of platelets in the preparation, consensus regarding the necessity for platelet activation before its use has also not been established. Despite the common notion that PRP needs to be activated prior to its use (Eppley et al., 2004), Mishra et al. advocated the use of non-activated PRP for the treatment of musculoskeletal tissue injury, as it has been suggested that platelets would be activated when exposed to collagen *in vivo* (Mishra et al., 2009). While platelet activation results in an immediate release of growth factors within ten minutes (Everts et al., 2006; Marx, 2001), more than 95% of the growth factors would have been depleted within 60 minutes (Marx, 2004). Hence, prior activation of platelets before use may be unnecessary as the platelets will inevitably be activated when in contact with collagen present in the musculoskeletal tissues. As a consequence, there is a more sustained release of growth factors, which would maintain a longer therapeutic effect (Mishra et al., 2009).

Besides the ease of preparation, the use of non-activated PRP would also eliminate the need to add bovine thrombin, which has potential immunological and haematological side effects (Lawson, 2006).

In addition to the issues mentioned above, it has been noted that in previous studies, PRP was supplemented to the culture medium at frequent intervals i.e. every 3 days (Gruber et al., 2004; Verrier et al., 2010). This requires a large quantity of blood, which sometimes might be impractical in clinical situations. It would be of interest to investigate whether infrequent supplementation of PRC would still support MSC proliferation and differentiation. This has an implication of being able to discern the potency of the growth factors released by platelets in producing sustained effects on the cells, apart from being a more practical and economical approach for *in vitro* cell expansion.

Therefore, there are a number of unexplored questions in the literature regarding the effect of PRC in enhancing the regenerative potential of hMSC, specifically: 1) To what extent does PRC alone induce hMSC proliferation without the aid of external mitogenic factors concurrently supplemented in the culture media?; 2) What is the optimal concentration of PRC required to induce the highest proliferation rate of the hMSC?; 3) Is there a need to activate the platelets prior to its use in the *in vitro* culture system?; and, 4) Would a single application of PRC supplemented within an 8-day duration without addition of lineage-specific differentiation inducing factors be sufficient to direct hMSC differentiation to any particular lineage(s)?

Therefore, the work presented in this chapter aims to clarify the above issues in the process of appropriate development of the experimental protocol for obtaining an optimised PRC preparation. The main objective of this chapter is to investigate the effect

of a single application of PRC in serum-free medium on proliferation and early differentiation (within 8 days) of hMSCs without the aid of external lineage-specific growth factors in the culture medium.

3.2 METHODS

3.2.1 Isolation, expansion and verification of human mesenchymal stromal cells (hMSCs)

3.2.1.1 Isolation of hMSCs

Bone marrow was aspirated from patients undergoing total knee/hip arthroplasty in the University of Malaya Medical Centre. The study was approved by the Medical Ethics Committee of the institution (UMMC, reference number 967.10) and written informed consent was obtained from each participant. Aspirated bone marrow was added to equal volume of phosphate-buffered saline (PBS, Invitrogen-Gibco, USA; pH 7.2) and layered onto Ficoll-Paque Premium of density 1.073 g/mL (GE Healthcare, Sweden) and centrifuged at 960x g for 25 minutes. The mononuclear cells (the cloudy layer) were then isolated carefully and resuspended in 10 mL of low glucose Dulbecco's modified eagle medium (L-DMEM) and centrifuged again at 645x g for 5 minutes. The supernatant was discarded and the cell pellet obtained was cultured in growth medium (L-DMEM supplemented with 10% FBS, 1% Penicillin/Streptomycin (100 U/mL, Invitrogen-Gibco) and 1% Glutamax-1 (Invitrogen-Gibco) in T-25 tissue culture flasks (Nunc, MA, USA). Medium was changed every 3 days until the cultures were 80% confluent and then the cells were serially passaged.

3.2.1.2 Cell Culture and Passaging

The isolated mononuclear cells were cultured in a T-75 culture flask (Nunc, MA, USA) containing growth medium, which was replaced every three days. With the first change in medium, the non-adherent cells were washed out. The adherent cells were further passaged after the cells were 80% confluent. Each time, the cells were cultured until they reached 70-80% confluence. After decanting the medium, the flask was washed with 1X PBS to remove any remaining traces of FBS and then 3 mL TrypLE™ (Invitrogen) was added (3 mL for T-75 flask and 1.5 mL for T-25 flask). After 3 minutes of incubation with TrypLE at 37ºC, the cells were monitored under a phase contrast microscope to verify detachment of the cells (about 90%), before adding 6 mL DMEM (6 mL for T-75 flask and 3 mL for T-25 flask) containing 10% FBS to the culture. The remaining cells were then detached completely from the surface using a cell scrapper and centrifuged in 15 mL propylene tubes at 645x g for 5 minutes. The pellet was re-suspended in 1 mL growth medium. Cell count was conducted using a haemocytometer and the cells were then sub-cultured in T-75 culture flasks. The medium was changed every 3 days.

3.2.1.3 Cryopreservation and cell revival

After trypsinization, viable cells were counted and transferred to a cryo-medium containing 10% cryoprotectant dimethyl sulphoxide (DMSO) (Sigma-aldrich, Germany) (100 µL) and 90% fetal bovine serum (FBS) (900 µL). The medium was prepared in 1mL cryovials (Nunc, USA) and the cell pellet was slowly resuspended in the cryo-medium. The cryovials were placed in a cryo freezing container having a rate of cooling close to 1°C per minute (Nalgene®, Mr Frosty, Nunc, USA) at -80ºC overnight before being transferred into liquid nitrogen tanks (-196ºC).

For cell revival, the cryovial was removed from the liquid nitrogen tank and placed directly into a 37°C water bath and shaken until completely thawed. The cryovials were wiped with 70% ethanol (Classic Chemicals, Selangor, Malaysia) before they were transferred to the laminar flow cabinet. The cell suspension from the cryovial was then immediately resuspended in 10 mL growth medium and cultured in T-75 flasks.

3.2.1.4 Verification of human mesenchymal stromal cells

Cells isolated from human bone marrow were verified as mesenchymal stromal cells based on immunocytochemical staining for various surface markers, flow cytometry analysis to determine the percentage of expression of relevant surface markers, and cytochemical staining to determine their tri-lineage potential.

Immmunocytochemical staining for cell surface markers

Cells isolated from human bone marrow were seeded at a density of 10,000 cells per chamber in four-well chamber slides (Nunc, MA, USA) and staining was performed according to the manufacturer's recommendation (Dako, Glostrup, Denmark). After the cells were confluent, they were fixed in 4% formaldehyde in PBS for 15 minutes, and then blocked for 30 minutes using hydrogen peroxidase (H_2O_2) containing sodium azide to prevent endogenous activity. Human MSCs were then incubated with primary mouse anti-human monoclonal antibody against $CD44^+/CD105^+$ and $CD45^-/CD34^-$, (1:100 dilution, Abcam, Cambridge, UK) at room temperature for 30 minutes. After washing with PBS, the cells were incubated with secondary antibody (goat anti-mouse IgG, Dako) at 1:200 dilution for 30 minutes. The cells were then washed with PBS, stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen substrate in the dark and

examined under light microscopy (Nikon Eclipse TE2000-S; Nikon Corporation, Tokyo, Japan).

Flow cytometry analysis of cell surface markers

The expressions of surface markers on cells isolated from the human bone marrow were also confirmed by flow cytometry (FACSCanto II flow cytometer, BD Biosciences, CA, USA) analysis. Each antibody (Mouse anti-Human) was conjugated with a fluorochrom: PerCP-Cy5.5 CD105, APC CD44, FITC CD73, PE-Cy7 CD90, PE CD34, and APC-H7 CD45 (BD Pharmingen, CA, USA). Unstained samples were used as a negative control. The samples were gated based on forward scatter (FSC) and side scatter (SSC) parameters.

MSCs were trypsinized at passage 3 and the cells ($50x10^3$) were resuspended in ice cold 100 µL stain buffer (BD Pharmingen). The monoclonal antibodies were added in the following concentrations: PerCP-Cy5.5 CD105: 5 µL, APC CD44: 20 µL, FITC CD73: 5 µL, PE-Cy7 CD90: 5 µL, PE CD34: 20 µL and APC-H7 CD45: 5 µL. An isotype control was also used at the same concentration as the antibodies. The samples were incubated on ice in the dark for 20 minutes. The samples were washed twice with ice cold stain buffer. Stain buffer was added and centrifuged at 300x g for 5 minutes. The supernatant was discarded and 100µl of stain buffer was added and vortexed. Samples were kept in dark on ice before flow cytometry analysis. Gating was performed using forward and side scatter to exclude cellular aggregates and debris. At least 10000 events per sample were analysed by the BD FACSDiva software (BD Biosciences).

Tri-lineage differentiation

Adipogenic and osteogenic differentiation of hMSCs were induced in the monolayer culture system. A total of 1 x 10^4 cells were seeded in each well of a 4-well chamber slide (Lab-Tek, Permanox, Nunc, Thermo Fisher Scientific, USA). Cells were cultured in 1 mL of growth medium containing 10% FBS until they were confluent. After the cells were confluent, they were cultured in specific differentiation inducing media. For adipogenic differentiation, cells were cultured in StemPro® adipogenic induction medium (Invitrogen) for 14 days. Medium was changed every 3 days, and after 14 days, the cells were washed with 1x PBS and fixed with 4% buffered formaldehyde. The cells were then incubated with oil red O stain (Sigma-Aldrich, St. Louis, USA) for 10 minutes.

To induce osteogenic differentiation, the confluent cells were cultured in StemPro® osteogenic induction medium (Invitrogen) for 21 days. Medium was changed every 3 days and after 21 days, the cells were fixed with 4% buffered formaldehyde and incubated with alizarin red S stain (Ricca chemical company, TX, USA) for 10 minutes. After 10 minutes, both the stains were removed and the slides were rinsed with tap water until the rinsed off water was clear. Finally, 1 mL of water was added and the stained slides were viewed under the phase contrast microscope (Nikon Eclipse TE2000-S, Nikon Corporation, Japan).

Differentiation of cells to chondrogenic lineage was induced using pellet culture. A total of 250 x 10^3 cells were centrifuged at 450x g for 5 minutes, the supernatant discarded and the pellet was supplemented with 2 mL of StemPro® chondrogenic induction medium (Invitrogen) for 21 days. Medium was changed every 3 days. The pellet was embedded in 4% agarose and fixed with 4% buffered formaldehyde and then processed for routine

histology. Paraffin sections (5-µm thick) were deparaffinised in xylene and dehydrated in ethanol in a stepwise manner from 70% up to 100%. The sections were then stained with safranin O and Fast green and viewed under the phase contrast microscope. All experiments were performed three times.

3.2.2 Preparation, activation and characterization of platelet rich concentrate (PRC)

3.2.2.1 Preparation of PRC

Blood (25 mL) was collected from six healthy volunteers after obtaining written informed consent from each participant. Blood samples were collected in vaccutainers containing ACD-A anticoagulant. PRC was prepared using double centrifugation method as described earlier with slight modifications modifications (Cho et al., 2011; H. R. Lee, Park, Joung, Park, & Do, 2012; Samuel et al., 2016; Shani et al., 2014). Briefly, the anticoagulated blood was centrifuged at 450x g for 10 minutes to initially separate the plasma from the red blood cells (Figure 3.1). To minimise inter-individual variations, the plasma containing platelets were pooled in a 50 mL falcon tube and centrifuged again at 1740x g for 10 minutes. The supernatant portion of the plasma was discarded and only the platelet pellets were isolated and resuspended in sterile phosphate buffered saline (PBS, pH 7.2) at 1/10th the initial blood volume (Verrier et al., 2010). This constitutes the platelet-rich concentrate (PRC). Prostaglandin (PGI2) (0.5 µL in 1 mL of platelet suspension) was added to prevent the transitory activation of platelets during the centrifugation and re-suspension steps (Cazenave et al., 2004). The amount of platelets in PRC and whole blood were then determined using the haematology analyser (Sysmex XE 5000).

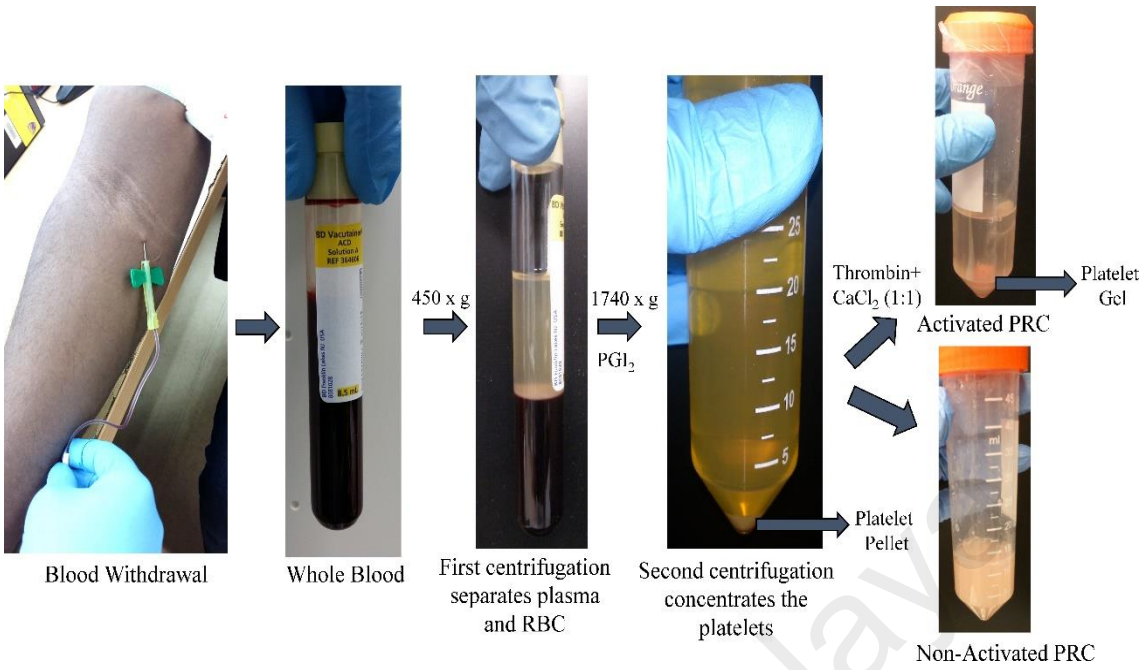

Figure 3.1: Preparation of PRC from human blood. Whole blood was double centrifuged to get the platelet pellet. The pellet was subsequently resuspended in PBS, and was either activated using thrombin and calcium chloride (activated PRC), or, used without activation (non-activated PRC).

3.2.2.2 Platelet Activation

The isolated PRC was then divided into two equal portions. One portion was activated using 10% calcium chloride (Sigma-Aldrich, St. Louis, USA) and lyophilised human thrombin (1:1 (v/v)) (Sigma-Aldrich, St. Louis, USA). The activator was added at a ratio of 1:10 (v/v) to one portion of the PRC, and following that, the tubes were incubated at room temperature. After a firm clot was obtained, the tubes were centrifuged at 1980x g for 5 minutes (Eppley et al., 2004). The supernatant that presumably contained most of the platelet contents was referred to as the activated PRC. The other portion of platelet suspension (i.e. whole platelet pellets in PBS) was used directly without any activation, and was referred to as the non-activated PRC.

3.2.2.3 Characterization of platelets in PRC

The PRC was further characterized in order to confirm that the platelets were not inadvertently activated during the preparation process, and verify the presence of an abundant amount of growth factors, which would qualify the substance as a platelet rich preparation. The characterization process included analysis from scanning electron microscopy, flow cytometry, and enzyme linked immunosorbent assays (ELISA).

Scanning Electron Microscopy

The platelets were fixed overnight in 4% glutaraldehyde in 0.1M calcodylate and post-fixed for 1 hour in 1% osmium tetraoxide. It was then washed three times in distilled water before being dehydrated through a graded ethanol series (50, 75, 95 and 100%). Hexamethyldisilazane (HMDS) was added to the fixed platelets for 10 minutes and then it was left to dry in a desiccator. The dried specimens were mounted on aluminium stubs with adhesive carbon tapes and sputter coated with gold before being examined using a digital scanning electron microscope (model JEOL JSM-6360, Japan).

Flow cytometry analysis to determine the activation of platelets

Activation of platelets was further verified by analysing the expression of CD62 on the surface of the outer membrane of platelets using flow cytometer (Becton Dickinson, San Jose, CA). Anti-CD61-PerCP, anti-CD41a-APC and a PE-anti-P-selectin (CD62P) (Becton Dickinson) (2 µL each) were added to 5 µL of non-activated PRC. The mixtures were incubated in the dark for 20 minutes at room temperature, after which the platelets were fixed by the addition of 1 mL of cold (2° - 8°C) 1% paraformaldehyde. The samples

were analysed after 30 minute of incubation in the dark. Forward scatter (FSC), side scatter (SSC), and fluorescence data were obtained with gain settings in the logarithmic mode. Platelets were selected based on their platelet marker positivity and FSC/SSC characteristics.

Enzyme Linked Immunosorbent Assays (ELISA)

Concentration of the major growth factors present in platelets such as platelet derived growth factor- AA, -BB, -AB (PDGF-AA, PDGF-BB, PDGF-AB), transforming growth factor β1 (TGF-β1), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2) and insulin-like growth factor (IGF-1) (USCN, Cloud-Clone Corp., Houston, USA) in the activated, non-activated PRC and whole blood were determined using the commercially available Enzyme Linked Immunosorbent Assay (ELISA) kits according to the manufacturer's instructions.

3.2.3 Monolayer culture system for hMSC expansion

hMSCs at a density of 1.5 x 10^3 hMSCs/well were seeded in 24-well culture plates (Orange Scientific, Braine-l'Alleud, Belgium) and cultured in DMEM containing 10% FBS for 48 hours. The cells were then serum starved (1% FBS in DMEM) for 24 hours to induce cell synchronization before treatment. The cells were then either cultured in complete DMEM growth medium containing 10% FBS (control), or, activated and non-activated PRC of varying concentrations (5%, 10%, 15%, 20% and 25%) in serum-free media. The cells were analysed after 0, 2, 4, 6 and 8 days to determine their rate of proliferation as well as lineage-specific gene and protein expressions. As platelets have a

lifespan of 8-10 days (Spakova et al., 2012), this study evaluated the effect of PRC on hMSCs for an initial culture period of 8 days.

3.2.3.1 Cell Proliferation assay

Cell proliferation was determined using AlamarBlue® assay kit (Invitrogen-gibco) according to the manufacturer's protocol. After each time point, AlamarBlue® reagent was added (in the dark) to the medium at a concentration of 10% (v/v). The solution was thoroughly mixed using a pipette. The culture plates were protected from light with an aluminium foil and incubated for 5 hours in the incubator. After 5 hours, the supernatant was transferred into a 96-well plate (100µL per well). Because the absorption spectra of the oxidized (blue) and the reduced (pink) forms of AlamarBlue® overlapped, the absorbance was measured at two wavelengths: 570 nm for the reduced form and 600 nm for oxidized forms using a microplate reader (Epoch BIOTEK, VT, USA). Wells containing medium and AlamarBlue® without cells were used as blanks. Cells cultured in medium supplemented with FBS served as control. As cells proliferate, the innate metabolic activity results in a colour change due to the chemical reduction of AlamarBlue®, which gives a quantitative measure of cell proliferation. The rate of cell proliferation was reflected by the percentage of AlamarBlue® reduction using the equation provided in the manufacturers' instructions. (Appendix I). All experiments were performed in triplicate and repeated six times.

3.2.3.2 Analysis of lineage-specific gene expressions

QuantiGenePlex 2.0 (set 12216) Assay Kit (Panomics/Affymetrix, Inc., Fremont, CA) was used to screen the expression of lineage-specific genes in the cells treated with PRC. The expression of osteogenic (alkaline phosphatase (*ALP*), runt-related transcription factor 2 (*RUNX2*)), chondrogenic (sex-determining region box 9 (*SOX9*), collagen type II (*COL2A1*)), and adipogenic (peroxisome proliferator-activated receptor γ (*PPARG*)) markers were analyzed. The cells were trypsinized after 0, 2, 4, 6 and 8 days and lysed to release the RNAs, and incubated overnight with target specific probe sets. Beads and bound target RNA were then washed and sequentially hybridized with preamplifier, amplifier and label probe (biotin) and incubated with streptavidin-conjugated R-phycoerythrin (SAPE), which binds to the biotinylated probes. The resulting fluorescence signal associated with individual capture beads was read on a Luminex® flow cytometer. The signal, reported as median fluorescence intensity (MFI), is proportional to the number of target RNA molecules present in the sample. Signals from three housekeeping genes, namely Hypoxanthine-guanine phosphoribosyltransferase 1 (*HPRT1*), Phosphoglycerate kinase 1(*PGK1*) and TATA-box binding protein (*TBP*) were used to normalize the gene expression data for the test and control samples.

3.2.3.3 Cytochemical staining

After each time point, the cells cultured in the monolayer were fixed with 4% buffered formaldehyde and stained with either oil red O, alizarin red S, or safranin O stain to determine the presence of lipid droplets, calcium deposits and proteoglycan synthesis, indicating their differentiation to the adipogenic, osteogenic and chondrogenic lineages, respectively. The cells were incubated with the stain for 10 minutes and washed with double distilled water until the water was clear. The cells were then observed under the microscope (Nikon Eclipse TE2000-S; Japan).

3.2.3.4 Protein assays

The intracellular lipid stained by the oil red O was quantified using adipogenesis assay kit (Cayman Chemical, Ann Arbor, MI). The oil red O stain was eluted from the lipid droplets by adding dye extraction solution for 10 minutes and the optical density (OD) was measured at 490 nm.

Concentration of glycosaminoglycan (GAG) released in the supernatant was normalized to the total protein. Protein and GAG were determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, CA) and Blyscan sulfated glycosaminoglycan assay kit (Biocolor Ltd, County Antrim). Spectrophotometer absorbance measurements were performed at 750 nm and 656 nm for protein and GAG assay, respectively. GAG content was normalized with the total protein content (GAG μg/protein mg).

The alkaline phosphatase assay kit (BioVision, CA, USA) was used to measure ALP activity in the supernatant of the cell cultures according to the manufacturer's instructions. The ALP activity was reflected by the absorbance of the dephosphorylated pNPP (p-nitrophenyl phosphate) by ALP to a yellow coloured *p*NP (*p*-nitrophenol) measured at 405 nm. All experiments were repeated three times with three replicates.

3.2.4 Statistical Analysis

Values are expressed as mean ± standard deviation. The differences between groups were analyzed using a non-parametric test (Kruskal-Wallis). If values were significant, Mann Whitney U tests were performed to evaluate the level of significance between the groups. Differences were considered to be significant at $p < 0.05$. Data were analysed with SPSS software version 17.0 (IBM Corp., Armonk, NY, USA).

3.3 RESULTS

3.3.1 Verification of hMSC characteristics

Immunocytochemistry analysis for the cell surface markers showed positive staining for CD44 and CD105 (Figure 3.2), which are the generally accepted markers of MSCs. No expression of the hematopoietic lineage markers CD45 and CD 34 was observed in the cultured cells.

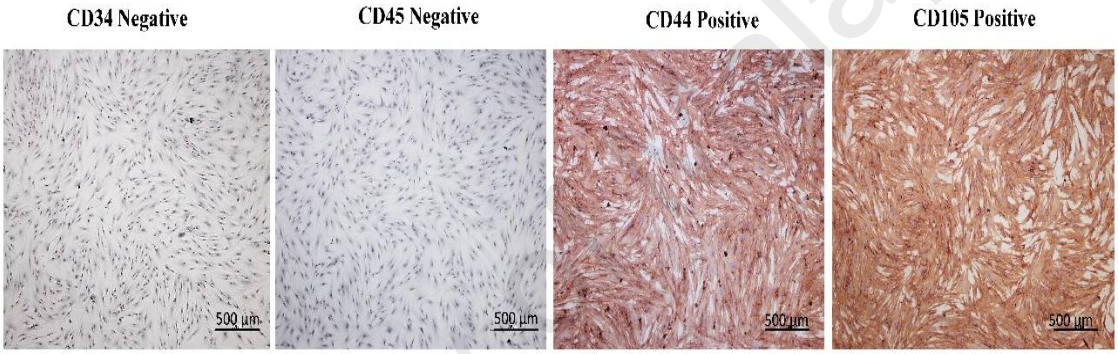

Figure 3.2: Immunocytochemistry analysis of MSC cell surface markers. Brown colour indicates positive staining of the specific CD markers. The cells were also counterstained with haematoxylin. Scale bar: 500 µm.

The results of the flow cytometry analysis (Figure 3.3) showed that the cells isolated from the bone marrow were positive for the surface markers CD73, CD105, CD90, CD44, and were negative for the markers CD34 and CD45, further confirming that the cells were MSCs without the presence of hematopoietic stem cells in significant amounts. At least 90% of the cells expressed double positive, double negative or co-expressed positive and negative markers. This met the minimal criteria set by the International Society for Cellular Therapy (ISCT) for defining mesenchymal stromal cells (Dominici et al., 2006). The results of this analysis are summarized in Table 3.1.

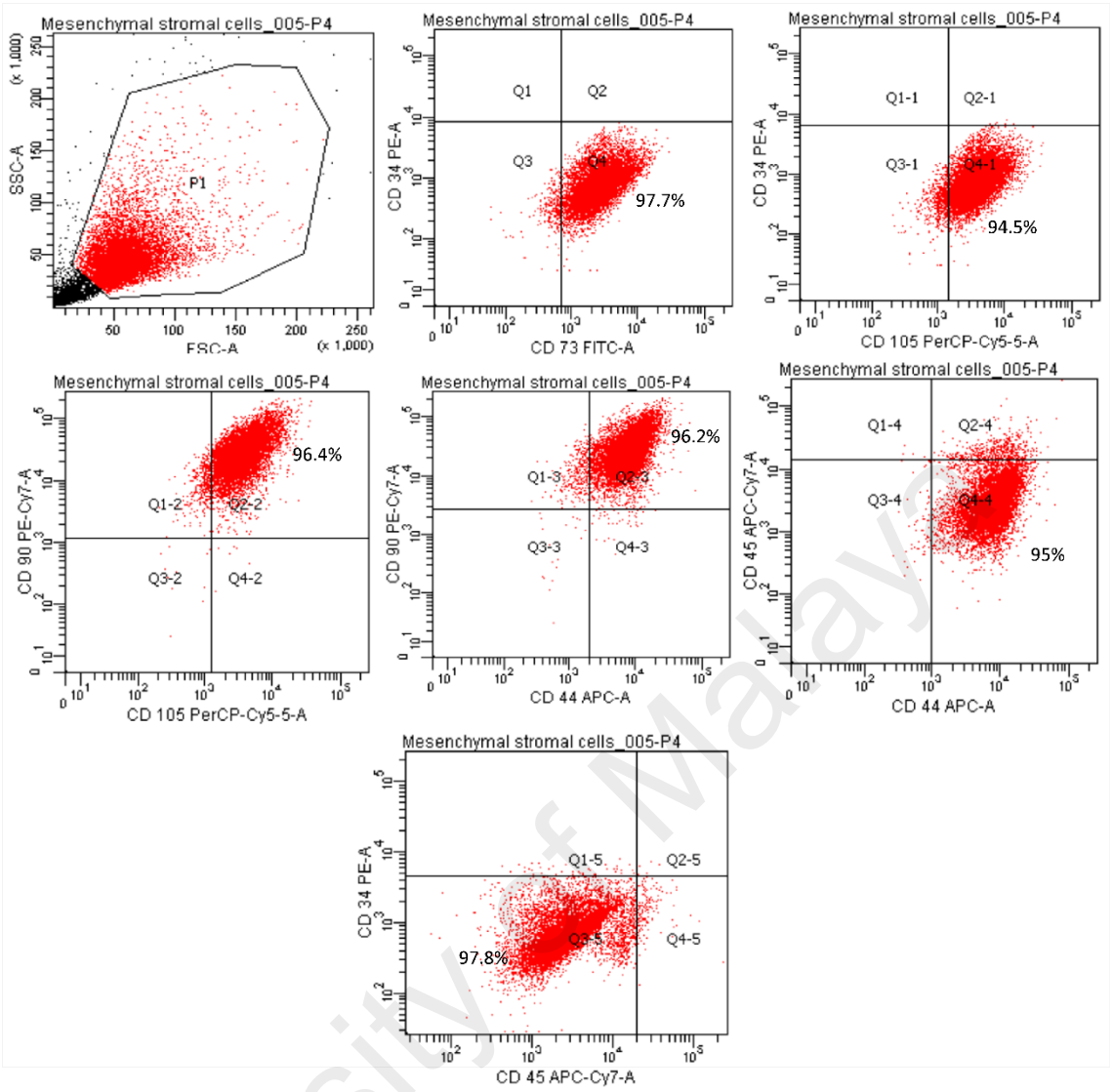

Figure 3.3: Analysis of the surface CD markers co-expressed by the cells isolated from bone marrow. Cells were stained with antibodies against the indicated antigens labelled on each axis. Representative dot plots were derived from gated events and displayed as combination of forward and side light scatters (FSC and SSC) on a log scale. FSC correlates with particle size, while SSC indicates granularity or internal morphology of cells or particles. Cells/events in Q2 are positive and in Q3 are negative for both axes. Q1 is positive for Y and negative for X, Q4 is positive for X and negative for Y.

Table 3.1: Percentage of co-expression of the cell surface CD markers determined by flow cytometry analysis.

Antigen	% Expression
$CD73^+$ and $CD34^-$	97.7
$CD105^+$ and $CD34^-$	94.5
$CD105^+$ and $CD90^+$	96.4
$CD44^+$ and $CD90^+$	96.2
$CD44^+$ and $CD45^-$	95
$CD45^-$ and $CD34^-$	97.8

Finally, the isolated cells were confirmed to be MSCs based on their potential to differentiate into typical mesenchymal lineages, namely adipogenic, osteogenic and chondrogenic lineages. The differentiation assay and their characterisation using confirmatory staining indicated that the cells isolated from the bone marrow had the potential to differentiate to all these lineages. Adipogenesis was confirmed by the presence of fat accumulated in small droplets that were stained red with the oil red O stain (Figure 3.4A). Cells cultured in the osteogenic differentiation medium showed the presence of calcium mineralization that was stained red with alizarin red S stain, confirming differentiation to osteogenic lineage (Figure 3.4B). Cells cultured in the pellet produced an extracellular matrix rich in sulphated glycosaminoglycan, typically found in cartilage tissues, which was stained red with the safranin O stain (Figure 3.4C).

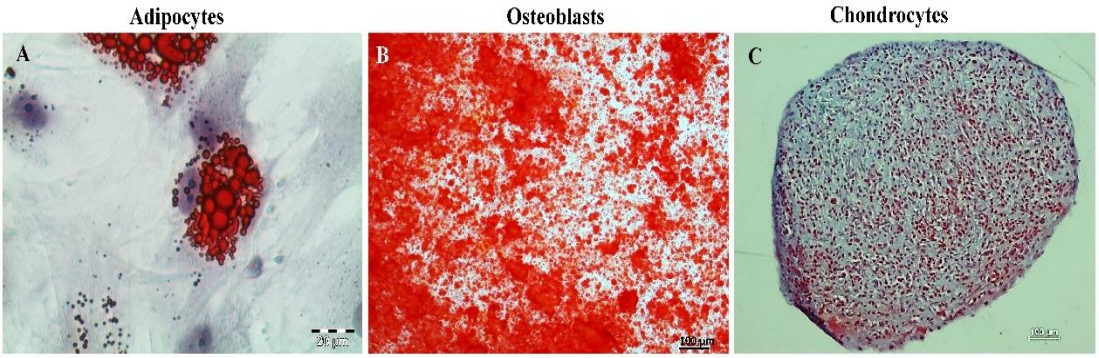

Figure 3.4: Tri-lineage differentiation potential of cells isolated from the bone marrow. (A) Oil red O staining, counter stained with haematoxylin. Scale bar: 20 µm. (B) Alizarin red S staining differentiated human MSCs to osteoblasts. Scale bar: 100 µm. (C) Chondrogenic differentiation of human MSCs stained with safranin O and Fast Green. Scale bar: 100 µm.

3.3.2 Yield of platelets from PRC

The total number of platelets in the PRC is shown in Table 3.2. The platelet number in PRC was about four times higher compared to whole blood (p=0.008). The leukocyte count was significantly low in the PRC preparation (p=0.008).

Table 3.2: Concentration of various cell types in human whole blood and PRC.

Blood cells	Whole blood	PRC
Platelets $\times 10^3/\mu L$	267.6 ± 16.9	1133 ± 23.2*
Leukocytes $\times 10^3/\mu L$	4.3 ± 0.18	0.3 ± 0.08*
RBCs $\times 10^6/\mu L$	5.4 ± 0.8	0.03 ± 0.02*

Denotes statistically significant differences ($p < 0.05$) between the variables in the same row. Data are represented as mean ± SD of six independent experiments (N=6).

3.3.3 Verification of platelet activation during preparation process

The morphology of non-activated platelet is shown in the image from scanning electron microscopy (Figure 3.5A). The non-activated platelets have a smooth surface and appear as oval disks. Upon activation, the platelets changed their shape to form pseudopodia (Figure 3.5B). The formation of pseudopodia facilitates the release of their granular contents, aggregation and adherence to the damaged tissue, and ultimately, formation of platelet plug.

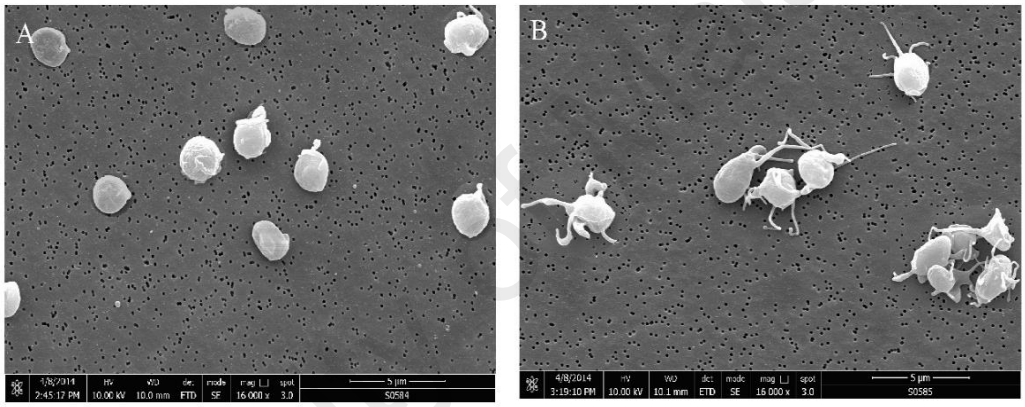

Figure 3.5: Scanning Electron Microscope images showing the structure of platelets in PRC. (A) Non-activated platelets has a smooth surface and (B) activated platelets form pseudopodia.

The region corresponding to platelet population was defined according to their size and granularity (Figure 3.6A). The flow cytometry analysis revealed that 86.9 % of the cells had surface markers $CD41^+$ and $CD61^+$, which are normally expressed by platelets (Figure 3.6B). Furthermore, only 5.2% of cells in the non-activated PRC were positive to $CD62^+$ (Figure 3.6C), a cell surface marker uniquely expressed only by activated platelets (Figure 3.6A). These results confirmed that the non-activated PRC was subjected to very minimal inadvertent activation during the preparation process.

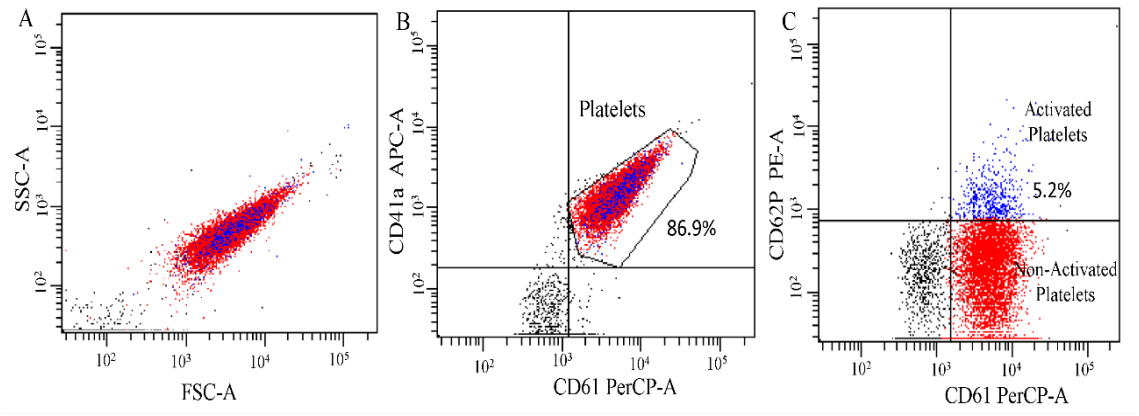

Figure 3.6: Flow cytometry analysis of platelets in PRC. (A) Cells in PRC were gated based on size (forward scatter) and granularity (side scatter). (B) Most of the cells (86.9%) were positive to both platelet surface marker $CD61^+$ and $CD41^+$. (C) 94.8% of cells were positive to $CD61^+$ and only 5.2% were positive for surface marker of activated platelet i.e. $CD62^+$.

3.3.4 Concentration of growth factors in PRC

Concentration of growth factors PDGF-AA, PDGF-BB, PDGF-AB, TGF-β1, VEGF, FGF-2 and IGF-1 are presented in in Figure 3.7. The growth factors concentrations were higher in the activated PRC compared to the non-activated PRC ($p \leq 0.05$). Concentration of PDGF-AA in the activated PRC (83.48 ± 3.77 ng/mL) was the highest compared to all other measured growth factors, with a three-fold increase compared to that found in activated whole blood (25.969 ± 2.61 ng/ml). The concentration of IGF was the lowest of the measured growth factors (0.036 ± 0.009 ng/ml in the activated PRC).

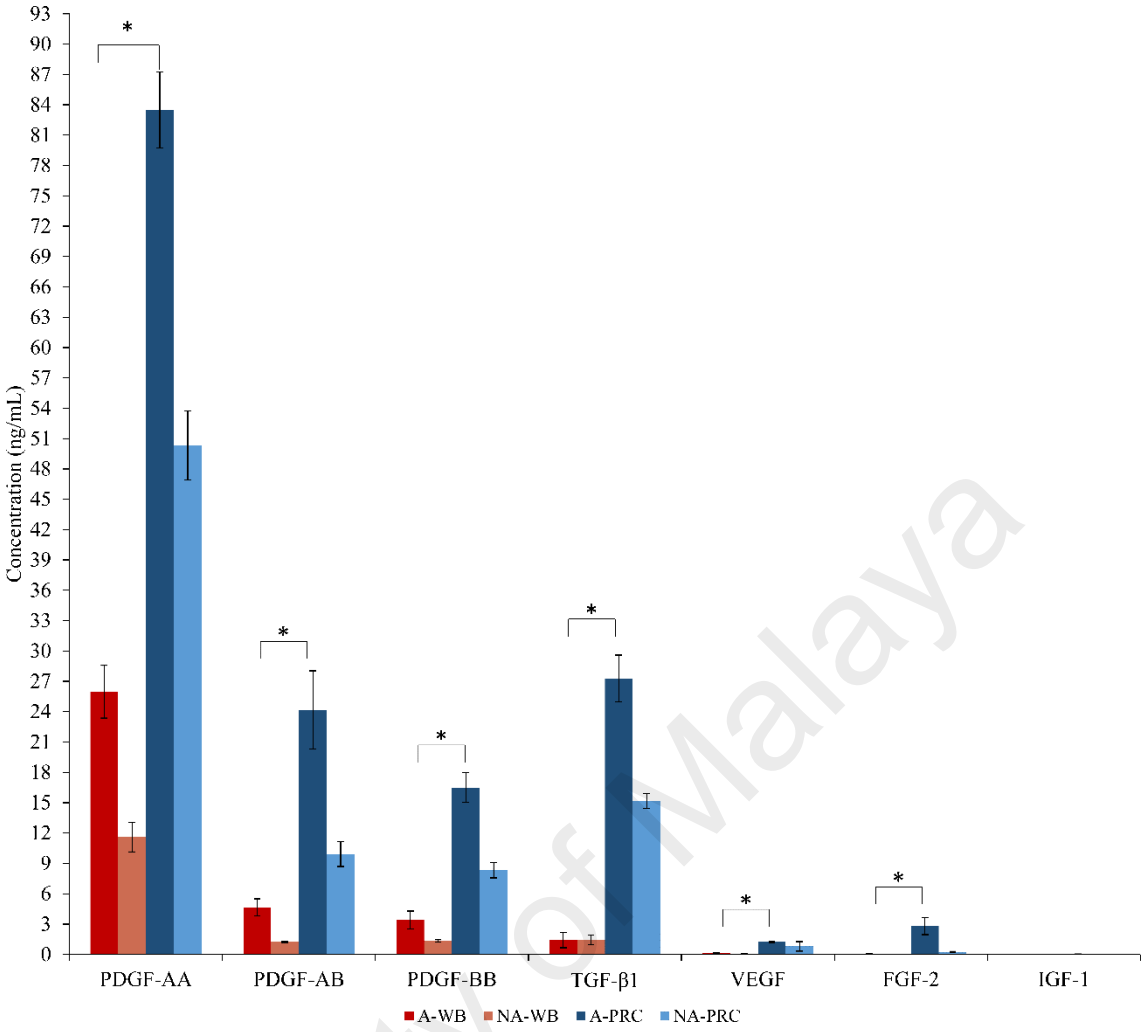

Figure 3.7: Concentration of growth factors in PRC and whole blood (WB). Activated whole blood (A-WB) and PRC (A-PRC) had higher concentration of growth factors compared to non-activated whole blood (NA-WB) and PRC (NA-PRC) (n=3). Data are represented as mean ± SD. *$p \leq 0.05$ denotes statistical significance between A-WB and A-PRC.

3.3.5 Effect of PRC on hMSC proliferation

The effects of different concentrations of activated and non-activated PRC on hMSC proliferation are shown in Figure 3.8. Proliferation gradually increased in a dose-dependent manner at PRC concentrations between 5-20% in both the activated and non-activated PRC groups. However, there was no significant increase in cell proliferation at a higher concentration of PRC (25%) beyond 6 days of culture. The highest cell proliferation was observed at PRC concentration of 15 and 20 %, with no significant

difference observed between the two concentrations in both activated and non-activated PRC groups (at day 8: p=0.637 and 0.658, respectively). Hence, 15% PRC was used in subsequent experiments. At a concentration level of 15%, there was no significant difference in the extent of cellular proliferation between activated and non-activated PRC groups (at day 8: p=0.121).

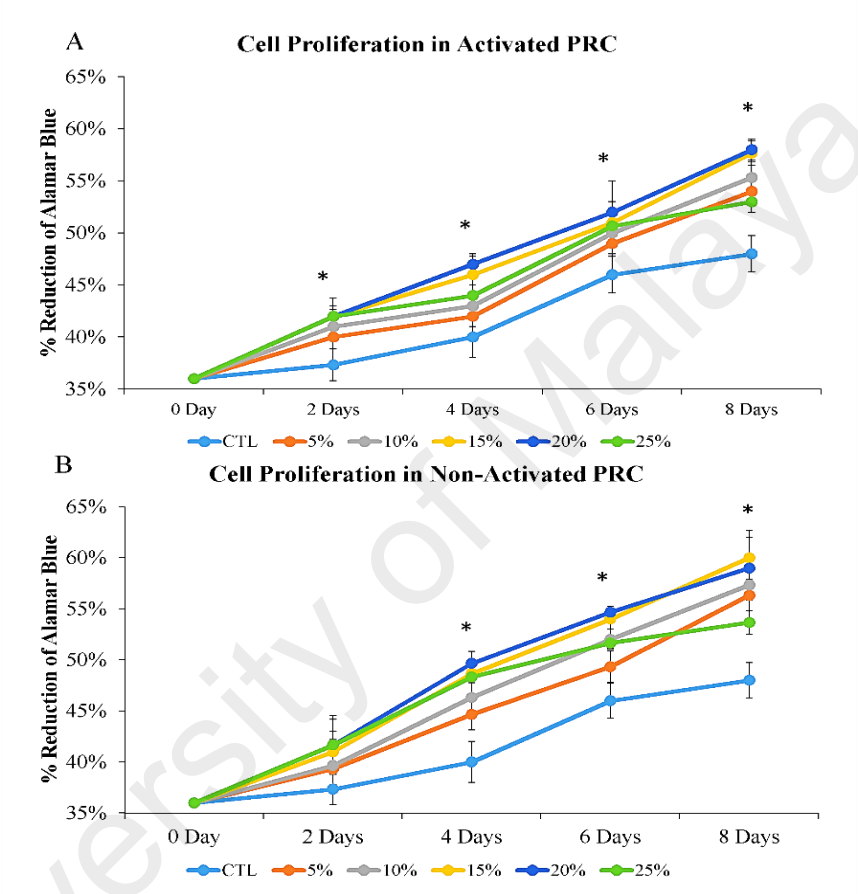

Figure 3.8: Proliferation of cells cultured in different concentrations of activated and non-activated PRC. (A) 15% and 20% activated PRC resulted in significantly higher cell proliferation compared to the control group at all time points (p<0.05). (B) Non-activated PRC showed a significant increase in the cell numbers only from day 4. Values are expressed as means ± SD (n=6).

3.3.6 Effect of 15% PRC on early differentiation of hMSC

The expression of various lineage-specific markers expressed by hMSCs treated with a single supplementation of 15% PRC within 8-day duration of culture is shown in Figure 3.9. The results are expressed as a fold change of the level of markers in the treatment groups relative to the control group i.e. cells cultured in FBS media. In the non-activated PRC group, the expression of chondrogenic marker *SOX9* was significantly higher (1.5 fold) ($p \leq 0.05$) by day 2, while *COL2A1* was down regulated at all time points (Figure 3.9A). The expression of osteogenic marker *ALP* was mostly downregulated. *RUNX2* expression was transiently upregulated at day 4 and 6 (Figure 3.9B). Adipogenic marker *PPARG* was down regulated throughout the 8 days (Figure 3.9C).

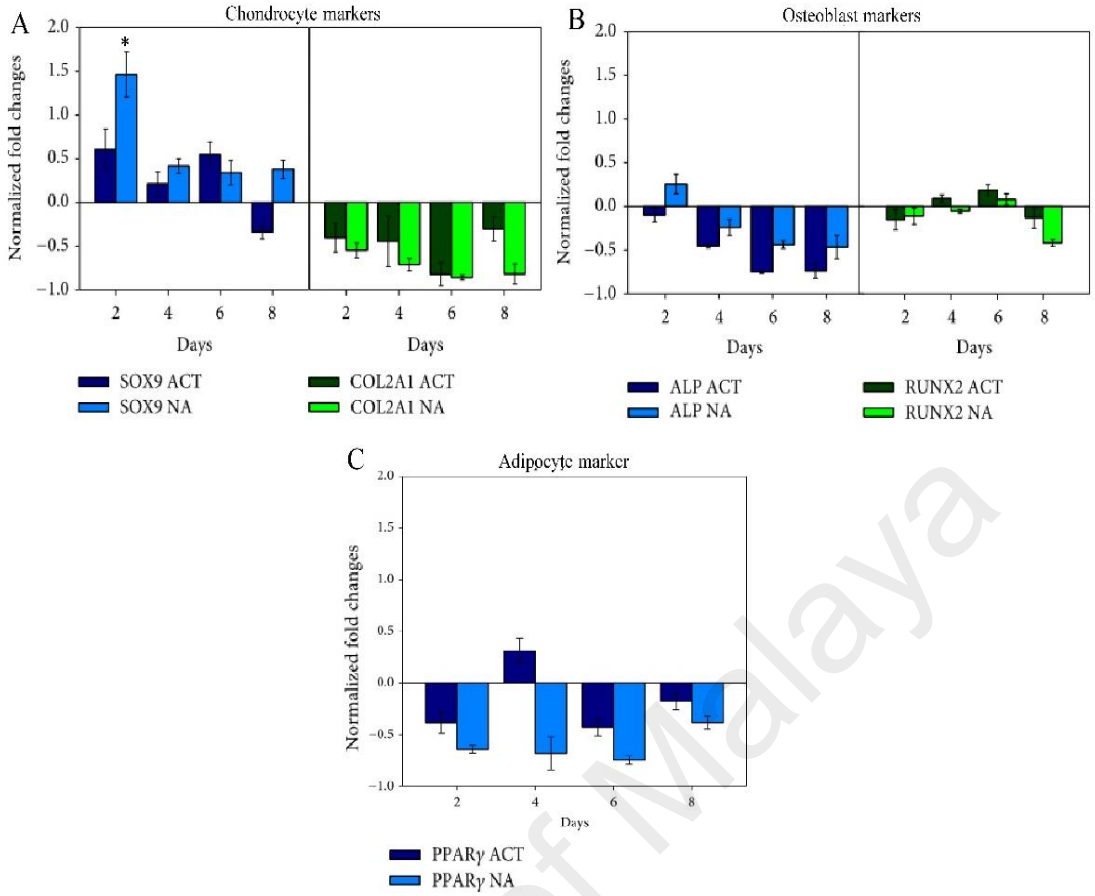

Figure 3.9: Gene expression of lineage-specific markers in cells cultured in activated and non-activated PRC. Expression of the chondrogenic marker *SOX9* was significantly higher in activated PRC group compared to non-activated PRC group at day 2 ($p \leq 0.05$). There was no significant difference in the expression of other markers between the activated and non-activated PRC group. Values are expressed as mean ± SD (n=3). (ACT: activated, NA: non-activated).

3.3.7 Effect of PRC on early tri-lineage differentiation of hMSC: verification by cytochemical staining

Cells in the control group did not show positive staining for oil red O, alizarin red S and safranin O stain (Figure 3.10A). However, cells in the activated and non-activated PRC groups showed formation of lipid droplets (Figure 3.10B) that were stained red with the oil red O stain. Similarly, mild calcium deposition was observed as reflected by the alizarin red S staining (Figure 3.10C). Glycosaminoglycan accumulation was also observed in cells stained with safranin O (Figure 3.10D) stain. Compared to the activated PRC group, cells in the non-activated PRC group showed stronger staining.

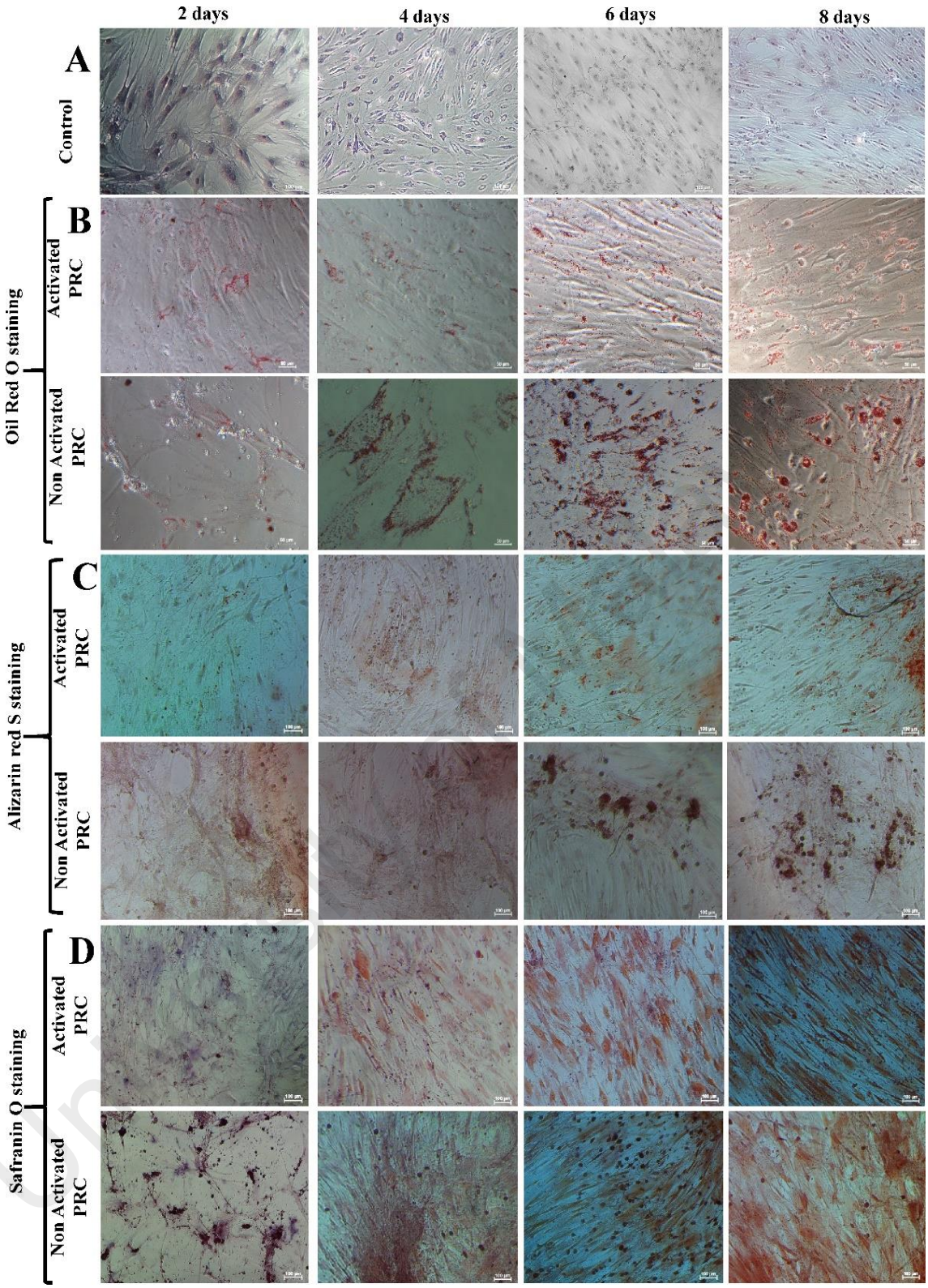

Figure 3.10: Cytochemical staining of cells cultured in activated and non-activated PRC. Staining was not observed in the control group, which was counterstained with haematoxylin (A), while cells treated with activated and non-activated PRC stained positive for lipid droplets (oil red O stain) (B), calcium deposits (alizarin red S stain) (C) and glycosaminoglycans (safrannin O stain) (D).

3.3.8 Effect of PRC on early tri-lineage differentiation of hMSC: verification by biochemical assay

Absorbance of the eluted oil red O stain was significantly higher in the non-activated PRC group compared to the control (p=0.05, at day 8) and activated PRC group (p=0.046, at day 8) (Figure 3.11A), confirming formation of more lipid droplets in this group. ALP concentration was significantly higher in the PRC group compared to the control group, from day 6 onwards (p=0.05) (Figure 3.11B). However, no significant difference in ALP production was observed between the activated and non-activated PRC group (p=0.127, at day 8). Glycosaminoglycan synthesis was also significantly high in the non-activated PRC group compared to the control (p=0.05, at day 8) and activated PRC group (p=0.046, at day 8) (Figure 3.11C).

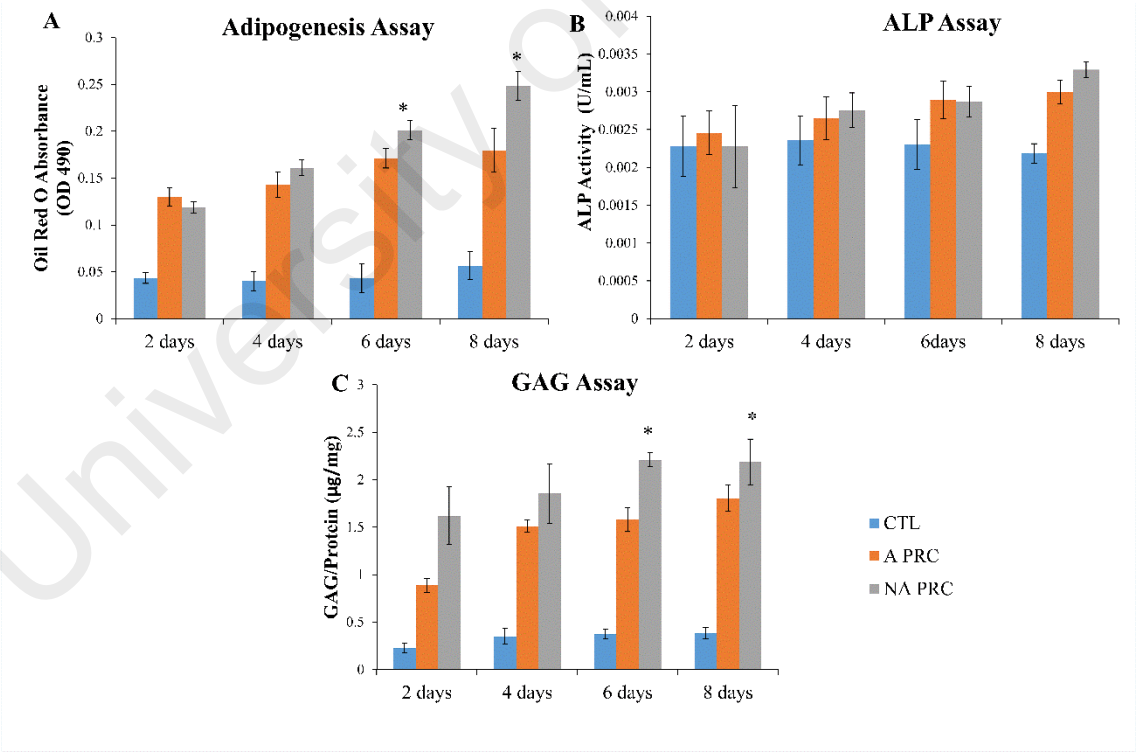

Figure 3.11: Early tri-lineage differentiation of hMSC shown by biochemical assays. Cells cultured in non-activated PRC showed a significantly higher lipid accumulation (A) and glycosaminoglycan synthesis (C). No significant difference in ALP synthesis (B) was observed between the two groups. Values are expressed as mean ± SD (n=3, *, $p \leq 0.05$ between the NA PRC and both A PRC and CTL). (CTL: control, A PRC: activated PRC group, NA PRC: non-activated PRC group).

3.4 DISCUSSION

The results in this chapter demonstrate that PRC alone without any supplementation of external mitogenic factors in the culture media was able to significantly increase hMSC proliferation compared to the FBS supplemented medium. Among the tested concentrations, 15% PRC was the least amount required that resulted in the highest rate of cell proliferation; hence, it was chosen as the optimal concentration for subsequent experiments. There appeared to be no significant difference in cell proliferation and gene expression of certain lineage-specific markers in the cells cultured in activated and non-activated PRC, indicating that activation of platelets did not seem to be a pre-requisite to produce the mitogenic and differentiation inducing effects on hMSC. A single supplementation of PRC was also found to stimulate differentiation to adipogenic, osteogenic and chondrogenic lineages, as shown by the qualitative and quantitative data on protein expressions.

The observation that PRC without supplementation of serum and additional growth factors in the culture media was able to significantly increase cell proliferation implies that the growth factors in PRC alone had a potent mitogenic effect on hMSCs. Platelets contain numerous growth factors known to trigger various cellular activities and functions (Eppley et al., 2004). Growth factors such as TGF-β, PDGF-AA, PDGF-BB, PDGF-AB, BMP and FGF found abundantly in platelets are known to be mitogenic (Eppley et al., 2004; Kruger et al., 2013). The concentration of TGF-β1 in the activated PRC as determined by ELISA in the present study was found to be much lower (27.2 ng/mL) compared to that reported by Eppley et al. (120 ng/mL) (Eppley et al., 2004) and Han et al. (403 ng/mL) (Han et al., 2007). This difference could be due to the disparity in the platelet yield. PRP isolated by Eppley et al. resulted in an 8-fold increase in platelet count

compared to whole blood, while the protocol by Han et al. resulted in a 13-fold increase. The PRC isolated in this study resulted in only a 4-fold increase in platelet count compared to whole blood. Nevertheless, this small amount of platelets was sufficient to induce hMSC proliferation and differentiation. Even in clinical studies, a platelet increase of three- to four-fold above baseline is considered within the acceptable range for tissue repair (Marx et al., 1998). In addition, PRC isolated in the present study was found to contain very few leukocytes (leukocyte-poor), which is preferable in the clinical settings as the presence of white blood cells could generate a pro-inflammatory environment (Riboh, Saltzman, Yanke, Fortier, & Cole, 2015).

Many studies have reported the effect of platelet preparations on mesenchymal stromal cell proliferation (Mishra et al., 2009; Murphy et al., 2012; Parsons et al., 2008; Xu et al., 2015). The observation that increasing PRP concentrations result in decreased cell numbers was also noted in earlier studies (Mishra et al., 2009; Parsons et al., 2008). This decrease in cell numbers with higher concentration of platelets could be due to the presence of negative regulators such as thrombospondin (TSP-1) found abundantly in the α-granule of platelets. Thrombospondin proteins have been found to inhibit endothelial cell proliferation and suppress angiogenesis (Iruela-Arispe, Lombardo, Krutzsch, Lawler, & Roberts, 1999) and TSP-1 concentrations were found to be inversely related to cell proliferation (Hsu, Yuan, & Tseng, 2009). Although different types of PRP preparations were tested on different cell types in previous studies, the general pattern of cellular proliferation was similar to that observed in this study. Jo et al. showed that activated PRP increased tenocyte numbers throughout the experiment (Jo, Kim, Yoon, & Shin, 2012) while Mishra et al. and Parsons et al. reported that non-activated PRP resulted in an initial slow increase followed by a steeper increase in cell numbers at later time points of the experiment (Mishra et al., 2009; Parsons et al., 2008), which was also observed in the

present study. The results from the use of non-activated platelets suggest that there might have been a slow and a sustained release of growth factors over time from the non-activated PRC. Since growth factors have a short half-life ranging from minutes to hours, activating the platelets immediately before their use might decrease the availability of growth factors to exert long term effect, and this would reduce the therapeutic potential of the platelet preparation (Gobbi et al., 2012; Lee, Silva, & Mooney, 2011). Considering that most of the growth factors would remain within the platelets in the non-activated PRC, its use would be more beneficial as it potentially have a greater long term therapeutic effect due to a more gradual and efficient release of growth factor, apart from being easier to prepare and more cost effective.

The results of this study also showed that supplementing PRC alone once within a period of 8 days could induce early differentiation of hMSCs without showing preference to any particular cell lineage. The growth factors released from a single application of PRC were potent enough to concurrently stimulate early differentiation of hMSC to the adipogenic, osteogenic and chondrogenic lineages, as implied by the protein expressions data. In contrast, the gene expressions of the lineage-specific markers were not consistently unregulated throughout the experiment. The poor correlation between the mRNA expressions and protein levels is not clearly understood, but could be due to the longer half-life of proteins compared to mRNA. Hence, protein levels do not mandatorily reflect the mRNA levels (Dozin, Quarto, Rossi, & Cancedda, 1990; Leyh et al., 2014). Nevertheless, the gene expression data remain useful for identifying potential candidate markers that should be verified at the protein level.

The ability of PRC to induce differentiation of hMSCs to various lineages could be due to the effect of various growth factors present in the platelets. TGF-β is a known potent inducer of chondrogenic differentiation of MSCs (Yu et al., 2012). PDGF-AA has been reported to induce osteogenic differentiation (Li et al., 2014) and it also plays a role in early cartilage development (Ataliotis, 2000). FGF has been implicated in both adipogenic (Neubauer et al., 2004) and osteogenic (Ng et al., 2007) differentiation.

A few limitations can be identified in the present study. It can be argued that the duration of culture was too short to generate conclusive data on hMSC differentiation. Nevertheless, the intention of the study was to perform an initial screening to determine whether a single application of platelet rich concentrate on its own could induce proliferation and differentiation of the hMSC. This objective has been accomplished. Secondly, hMSC differentiation to chondrogenic lineage in the presence of PRC was investigated in the monolayer culture system, which is arguably not an ideal environment for chondrogenesis. Although, a three dimensional (3D) culture system would be preferred to induce chondrogenesis, a monolayer culture system may suffice in a preliminary study. The advantage of using monolayer culture system is that it requires low number of cells (1500 cells/well) compared to the 3D culture system (about 40,000 cells/bead). In this study, a positive result on the ability of PRC to induce early chondrogenic differentiation was already attained even when the cell seeding density was much lower.

In conclusion, the results presented in this chapter fulfilled the first objective of the study, indicating that a single application of PRC in serum free medium has the potential to promote proliferation and induce early tri-lineage differentiation of hMSC *in vitro*, without additional aid from lineage-specific growth media.

CHAPTER 4: EFFECT OF PRC ON PROLIFERATION AND DIFFERENTIATION OF hMSCs IN 2D AND 3D CULTURE SYSTEMS DURING 24 DAYS OF CULTURE

4.1 INTRODUCTION

Having established the potential of a single application of PRC to induce proliferation and differentiation of hMSC to multiple lineages within a short time span of 8 days, it was necessary to investigate whether this effect would be sustained throughout the course of a complete differentiation process, which usually takes 21-28 days. This chapter describes the effects of PRC on hMSC proliferation and differentiation to osteogenic and adipogenic lineages in two-dimensional (2D) monolayer culture system, and chondrogenic lineage in a three-dimensional (3D) culture system during 24 days of culture. The efficacy of PRC was also determined by comparing its differentiation inducing effects to those of the standard commercially available lineage-specific media.

Previous studies have demonstrated the synergistic effect of platelet rich preparations and other growth factors on differentiation of MSCs to both osteogenic (Parsons et al., 2008; Verrier et al., 2010) and chondrogenic lineage (Drengk et al., 2009; Wang et al., 2015). However, these studies varied in the experimental design and did not report the effect of platelet rich preparations on MSCs for a longer duration, approximating the entire duration of cellular differentiation. In the study by Parsons et al., the changes in osteogenic gene expression profile under the influence of activated PRP were monitored for only 48 hours (Parsons et al., 2008). In another study, the gene expression profile of collagen type II, a marker of chondrogenesis, was analysed in MSCs cultured in a medium containing activated PRP after only 14 days of culture (Drengk et al., 2009). Wang et al.

determined the expressions of chondrogenic genes collagen type II, *SOX9* and aggrecan 7 days after culturing MSCs in a medium supplemented with activated PRC (Wang et al., 2015). A short time frame of investigation precludes appropriate analysis of the differentiation potential of MSCs as the cells have not yet undergone complete differentiation. Thus, an experiment that extends through the entire duration of cellular differentiation would be valuable in providing a better insight on the effect of platelets on MSC differentiation. Determination of a more comprehensive lineage-specific markers (i.e. early, mid and late differentiation markers) under the influence of PRC would also be of great value to further deduce whether PRC would alter key elements in the normal differentiation process. This is presently lacking in the literature. Furthermore, the notion as to whether PRC has a superior effect on hMSC differentiation potential compared to the commercially available lineage-specific media has not been determined.

Therefore, the main objective of the work presented in this chapter is to compare the effect of PRC on hMSC differentiation to adipogenic, osteogenic and chondrogenic lineages when compared to cells cultured in the respective standard differentiation media during a culture period of 24 days. A sufficient pool of viable cells is required for differentiation. Hence, another objective of this chapter is to investigate the effect of PRC in maintaining cell proliferation during this culture period.

4.2 METHODS

4.2.1 hMSC isolation and PRC Preparation

MSCs were isolated from human bone marrow as described in chapter 3 (section 3.2.1.1, page 72). PRC was prepared from human blood as described in chapter 3 (section 3.2.2.1, page 77).

4.2.2 Culturing cells in the monolayer system

hMSCs at a density of 1.5 x 10^3 hMSCs/well were seeded in 24-well culture plates (Orange Scientific, Braine-l'Alleud, Belgium) and cultured in various growth media for 48 hours. The cells were either cultured in DMEM containing 10% FBS, osteogenic medium (StemPro®, Invitrogen, CA), adipogenic medium (StemPro®, Invitrogen, CA), or 15% non-activated PRC. The cells were then serum starved (1% FBS in DMEM) for 24 hours to induce cell synchronization before the treatment. The cells were analysed after 0, 8, 16 and 24 days. The media was changed every 8 days. After each time point, the cells were detached using TypLE^{TM} express (Invitrogen) and used for gene expression and protein analyses.

4.2.3 Culturing cells in the alginate (3D) constructs

hMSCs in alginate beads were prepared by resuspending the cells in 1.2% low-viscous alginate (Sigma-Aldrich, St. Louis, USA) in 0.15 M sodium chloride as previously described (Kamarul et al., 2008). Briefly, hMSCs were first detached from the T-75 flask surface using TypLE^{TM} express (Invitrogen) and resuspended in sterile alginate solution at 1x10^{6} cells/mL cell density. The cell suspension was dropped into a 102 mM calcium chloride (Sigma-Aldrich, St. Louis, USA) solution using a micropipette tip. The resulting beads were washed with 0.15 M sodium chloride after 10 minutes of polymerization (Tay et al., 2012). Two hMSC-alginate beads were transferred into each well of a low attachment 24-well plate (Corning® Costar®, USA) and cultured in DMEM containing 10% FBS for 48 hours and serum starved to induce cell synchronization before treatment for 24 hours. After 24 hours, they were either cultured in DMEM containing 10% FBS (control), chondrogenic medium (StemPro®, Invitrogen, CA), or 15% non-activated PRC in serum-free medium. Media was changed more frequently i.e. every 4 days due to the higher cell density compared to the monolayer culture system. After each time point (0, 8, 16 and 24 days), the hMSC- alginate constructs were washed once in PBS and incubated with 1mL of dissolving buffer (55 mM sodium citrate) for 10 minutes. The cells were centrifuged for 2 minutes at 20,000x g, washed with PBS, and further centrifuged for 2 minutes at 40,000x g. These cell pellets were used for gene expression and protein quantification.

Morphological analysis of hMSC-alginate constructs

Scanning electron microscopy (SEM) was used to verify the porosity of the alginate bead alone and hMSC-encapsulated alginate beads. After 24 days of culture in DMEM containing 10% FBS or 15% PRC, beads were first fixed in 4% glutaraldehyde and then dehydrated in an ascending series of ethanol followed by a mixture of ethanol/acetone in the following ratios: 3:1, 2:2 and 1:3 each for 15 minutes. The samples were transferred to the critical point drier and mounted onto aluminium stubs with adhesive carbon tapes and sputter coated with gold and subsequently examined by the scanning electron microscope (model JEOL JSM-6360, Japan).

4.2.4 Cell proliferation assay

Cell viability was observed at different time points (0, 8, 16 and 24 days) using AlamarBlue® assay kit (Invitrogen-gibco) according to the manufacturer's protocol. After each time point, AlamarBlue® reagent was added (in the dark) to the medium at a concentration of 10% (v/v). It was thoroughly mixed with the help of a pipette and the culture plates were protected from light with an aluminium foil and incubated for 5 hours in the incubator. After 5 hours, the supernatant was transferred into a 96 well plate (100µL per well). Absorbance readings at 570 nm and 600 nm were obtained using a microplate reader (Epoch BIOTEK, VT, USA). Cells cultured in medium supplemented with FBS served as control. FBS containing medium and PRC supplemented medium without cells, served as the background controls. All experiments were performed in triplicate and repeated six times.

4.2.5 Gene expression analyses of osteogenic, adipogenic and chondrogenic markers

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis was used to compare the relative expression of osteogenic, adipogenic and chondrogenic genes. Cell pellets were lysed with buffer RLT (Qiagen, Valencia, CA) and total RNA was isolated from the cells at days 0, 8, 16 and 24 using RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations and quantified with a nanophotometer (Implen GmbH, Germany). Total RNA was then isolated from the cells using RNeasy Mini Kit (Qiagen) following the manufacturer's recommendations and quantified with a nanophotometer (Implen GmbH, Germany). The measured 260/280 ratio was consistently 2.0 – 0.1 in all samples. One μg of RNA was used to generate cDNA using QuantiTect Reverse Transcription kit (Qiagen) following the manufacturer's instructions. Real-time PCR analysis (CFX96 Real-time system, Bio-Rad) was performed to assess the mRNA levels using QuantiTect SYBR® Green PCR Kits (Qiagen). Each Q-PCR was performed in triplicate for PCR yield validation. Data were analysed by the $2^{-\Delta\Delta Ct}$ method, with normalization by the Ct of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), which was chosen after verifying its stable expression. Results were expressed relatively to the gene expression level of hMSC cultured in FBS medium. The primers used for Q-PCR are summarized in Table 4.1.

Table 4.1: List of primers used for RT-qPCR (5'- 3')

Gene	Sequence	Length (Base Pair)
Osteogenic Markers		
Collagen I (*COL1A1*) F	CCCGCAGGCTCCTCCCAG	18
Collagen I (*COLIA1*) R	AAGCCCGGATCTGCCCTATTTAT	23
Osteopontin (*OPN*) F	GTCCCCACAGTAGACACATATG	21
Osteopontin (*OPN*) R	TCAACTCCTCGCTTTCCATG	20
Osteocalcin (*OCN*) F	GGAGGGCAGCGAGGTAGTGAAGA	23
Osteocalcin (*OCN*) R	GCCTCCTGAAAGCCGATGTGGT	22
RUNX2 F	CCGCCATGCACCACCACCT	19
RUNX2 R	CTGGGCCACTGCTGAGGAATTT	22
BMP2 F	TGGCCCACTTGGAGGAGAAACA	22
BMP2 R	CGCTGTTTGTGTTTGGCTTGACG	22
ALP F	GATGTGGAGTATGAGAGTGACG	22
ALP R	GGTCAAGGGTCAGGAGTTC	19
Osteonectin (*ON*) F	TTGCAATGGGCCACATACCT	20
Osteonectin (*ON*) R	GGGCCAATCTCTCCTACTGC	20
Adipogenic Marker		
PPARG F	TTCAGAAATGCCTTGCAGTG	20
PPARG R	CCAACAGCTTCTCCTTCTCG	20
Chondrogenic Markers		
Collagen II (*COL2A1*) F	AGCCCTGCCGGATCTGTGTCTGT	23
Collagen II (*COL2A1*) R	TGGCGAGGTCAGTTGGGCAGA	21
Collagen X (*COL10A1*) F	CCACCAGGCATTCCAGGATTCC	22
Collagen X (*COL10A1*) R	CCGGTGGGTCCATTGAGGCC	20
Tenascin C (*TNC*) F	CAGCCAAACCCACCTCCACCAT	22

Table 4.1: List of primers used for RT-qPCR (5'- 3') (continued)

Gene	Sequence	Length (Base Pair)
Tenascin C (*TNC*) R	GCCGGATGACTTTCTTGAGGACC	23
Aggrecan (*ACAN*) F	ATCCCGCTACGACGCCATCTG	21
Aggrecan (*ACAN*) R	GCTCCATGTCAGGCCAGGTCACT	23
COMP F	GCGCCAGTGCCGTAAGGACA	20
COMP R	CGTCCGTGTTGCGCTGGTCT	20
SOX9 F	AGGCGGAGGCAGAGGAGGC	19
SOX9 R	GGAGGAGGAGTGTGGCGAGTCG	22
GAPDH F	GCCCCCTCTGCTGATGCCC	19
GAPDH R	GGGTGGCAGTGATGGCATGGA	21

4.2.6 Immunofluorescence analysis to identify the expression of markers specific to osteogenic and chondrogenic lineages at the protein level

Immunofluorescence staining for osteogenic markers RUNX2, osterix (Osx), osteocalcin (OC) and osteopontin (OPN) was done on cells cultured in the monolayer at days 8, 16 and 24. After each time point, cells were fixed with 4% paraformaldehyde for 15 minutes, and were then blocked for 30 minutes using hydrogen peroxidase (H_2O_2) to prevent endogenous activity. Cells were then incubated in goat serum working solution for 15 minutes to block non-specific binding, followed by addition of primary antibody (mouse anti-human monoclonal antibody, 1:100 dilution, Abcam, Cambridge, UK) at room temperature for 30 minutes. After washing with PBS, cells were incubated with goat anti-mouse secondary antibody tagged with Alexa fluoro (Abcam) (1:200 dilution) for 30 minutes. Cells were then washed with PBS and the nucleus was counterstained with

Hoechst stain (NucBlue® Live ReadyProbes® Reagent, Life technologies) and incubated for 15 minutes. After washing with PBS, it was examined under the fluorescent microscope (Nikon Eclipse TE2000-S, Tokyo, Japan).

Immunofluorescence staining for chondrogenic markers collagen type II (COL II), aggrecan (ACAN) and cartilage oligo matrix protein (COMP) was also done at day 8, 16 and 24. Alginate beads were washed once with PBS and fixed with 4% paraformaldehyde for 30 minutes. They were then washed again with PBS, and stored in PBS containing 10mM $CaCl_2$ to prevent gel dissolution. Hydrogel beads that were frozen on dry ice were embedded in optimum cutting temperature compound (ThermoScientific, Denmark). The samples were then cut into 5µm-thick sections using a cryotome FSE Cryostats (Thermo Scientific, Denmark) and mounted on polysine coated microscope slide (Thermo Scientific, Denmark). The samples were kept in -20 °C until further processing. For immunostaining, the slides were fixed in ethanol and then washed with 10mM $CaCl_2$ in PBS. The samples were incubated with 1% bovine serum albumin and 2% goat serum in PBS for 20 minutes at room temperature to block non-specific binding. Samples were then incubated with primary antibodies specific against COL II (Abcam, England), ACAN (Abcam, England) and COMP (Abcam, England) at 1:100 dilutions in 1% bovine serum albumin in PBS overnight at 4°C. Samples were washed thrice in PBS and incubated with 1:400 Alexa-Fluoro 488 goat anti-mouse secondary antibody (Abcam, England) for 1h at room temperature. Nucleus was counterstained with Hoechst stain (NucBlue® Live ReadyProbes® Reagent, Life technologies) and incubated for 15 minutes. Controls were prepared following the same procedure but without the primary antibody. Images of the samples were captured using fluorescence microscope (Nikon Eclipse Ti-U, Japan).

4.2.7 Cell digestion using RIPA buffer and Papain

Cells cultured in the monolayer were trypsinized after each time point and lysed using radio-immuno precipitation assay buffer (RIPA buffer) (Thermo Fisher Scientific, MA, USA). After each time point, 200 µL of ice cold RIPA buffer was added to the cell pellets and incubated on ice for 10 minutes. The lysed samples were centrifuged at 10,000× *g* for 5 minutes, and the clear supernatants were used (B. Gao et al., 2014) for ALP and osteocalcin assay.

Cells retrieved from the alginate beads were digested overnight in 1 mL papain buffer solution (250 µL of papain (Worthington) suspension containing 5 mg of the enzyme, 40 mg cysteine HCl (Sigma), 0.2 M sodium phosphate buffer (Na_2HPO_4–NaH_2PO_4) (Sigma), 200 mg EDTA disodium salt (Sigma) at pH 6.5 in 50 mL nuclease-free water (Ambion, Austin, TX)) at 65°C for 3 hours. Digested cells were then centrifuged at 450x g for 5 minutes and the supernatant was collected for glycosaminoglycan assay and ELISA. All experiments were performed three times in duplicate.

4.2.8 Biochemical assays to verify the expression of genes at the protein level

Quantification of ALP, Osteocalcin and preparation of cell lysates

Cellular alkaline phosphate enzyme (ALP) activity, an early marker of osteogenic differentiation, was determined colorimetrically by incubating the cell lysates with the substrate *p*NPP (*p*-nitrophenyl phosphate) (BioVision, CA, USA) in a 96-well plate at 37°C for 60 minutes. ALP assay involves dephosphorylation of *p*NPP by ALP to a yellow coloured *p*NP (*p*-nitrophenol). The absorbance was measured at 405 nm and the enzyme activity was normalized to total DNA content and expressed as nmol/ng.

Quantification of osteocalcin in cell lysates was performed according to the manufacturer's instructions using the Gla-type Osteocalcin EIA kit (TAKARA, Shiga, Japan). The absorbance was measured at 450 nm in duplicate and the concentration was normalized to total DNA content and expressed as ng/ng. The amount of osteocalcin was determined by interpolation from a standard curve of known concentrations.

Total DNA was assessed in digested samples using a Quant-iT PicoGreen dsDNA kit according to manufacturer's instructions (Molecular Probes, Invitrogen). The procedure of DNA quantification is described in section 4.2.9. The analyses of ALP and osteocalcin levels were performed at days 8, 16 and 24.

Quantification of glycosaminoglycan

Cell pellets collected after 8, 16 and 24 days of culture in either FBS containing medium (control), 15% PRC, or commercially available chondrogenic medium (positive control) were digested using papain as described in section 4.2.7. Total sulphated glycosaminoglycan (GAG) was then quantified with the 1, 9-dimethylmethylene blue-based Blyscan GAG Assay Kit (Biocolor, Carrickfergus, United Kingdom). The result was normalized with the total amount of DNA, determined separately by the Quanti-iT^{TM} PicoGreen®dsDNA assay kit (Life Technologies, USA) (section 4.2.9) according to the manufacturer's protocol.

4.2.9 DNA quantification

Total DNA was quantified using Quant-iT PicoGreen dsDNA kit according to manufacturer's instructions (Molecular Probes, Invitrogen). The cell lysates were diluted in TE buffer. 1.0 mL of the aqueous working solution of Quant-iT™ PicoGreen® reagent was added to each sample. The samples were incubated for 5 minutes in the dark. After incubation, the sample fluorescence was measured using a fluorescence microplate reader (Tecan, Switzerland) at standard fluorescein wavelengths (excitation ~480 nm, emission ~520 nm).

4.2.10 Enzyme Linked Immunosorbent Assays (ELISA) to quantify the chondrogenic ECM markers at the protein level

The intracellular concentration of cartilage-specific ECM protein COL II, ACAN and COMP was determined using ELISA kits. After 8, 16 and 24 days, the cells retrieved from hMSC-alginate constructs were lysed in papain and the concentrations of COL II (USCN, Cloud-Clone Corp, USA), ACAN (DIAsource Immuno Assays, Belgium) and COMP (R&D system, USA) were measured using ELISA kits according to the manufacturer's instructions.

4.2.11 Cytochemical staining of hMCSs to indicate differentiation to adipogenic and osteogenic lineage

After 8, 16, and 24 days, the cells cultured in the monolayer were fixed with 4% buffered formaldehyde and stained with either oil red O or alizarin red S stain to determine the differentiation to the adipogenic and osteogenic lineages, respectively. The cells were incubated with the stain for 10 minutes and washed with double distilled water until the water was clear. The cells were then observed under the microscope (Nikon Eclipse Ti-U; Japan).

4.2.12 Adipogenesis assay and cetylpyridinium chloride extraction

Adipogenesis assay and cetylpyridinium chloride (CPC) extraction was performed on cells stained with oil red O and alizarin red S, respectively. The intracellular lipid accumulation was quantified by the elution of the oil red O stain from the lipid droplets by adding dye extraction solution for 10 minutes; the optical density was measured at 490 nm (Cayman Chemical, Ann Arbor, MI). Quantification of alizarin red S stain was performed with CPC (Sigma-Aldrich). Stained monolayers were incubated with 1mL of

10% (w/v) CPC prepared in PBS (pH 7.0), and shaked for 15 minutes. An aliquot of the extracted dye (200 µL) was then transferred to 96-well plates, and its absorbance was measured at 570 nm with a microplate reader (Epoch; Biotek, Winooski, VT) (Frazier et al., 2013; Lee, Cho, Bui, & Kang, 2014).

4.2.13 Statistical Analysis

Values are expressed as mean ± standard deviation. The differences between groups were analysed using a non-parametric test (Kruskal-Wallis). If values were significant, Mann Whitney U tests were performed to evaluate the level of significance between the groups. Differences were considered to be significant at $p < 0.05$. Data were analysed with SPSS software version 17.0 (IBM Corp., Armonk, NY, USA).

4.3 RESULTS

4.3.1 hMSC proliferation

Cells in the monolayer culture system (Figure 4.1A) and those encapsulated in alginate (Figure 4.1B) underwent significantly higher proliferation in the PRC group compared to the control group at days 8 and 16 ($p<0.05$). However, by day 24, cell proliferation declined, and there was no significant difference between the two groups both in the monolayer ($p=0.077$) and 3D culture systems ($p=0.056$).

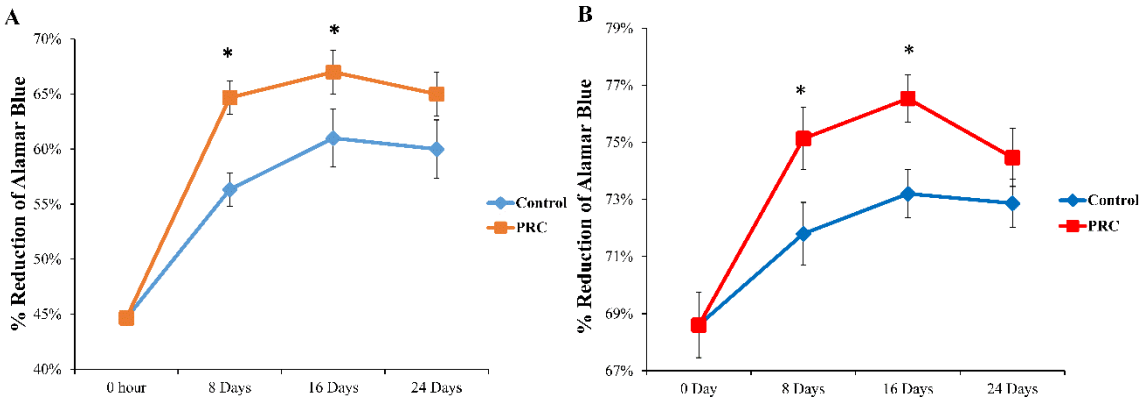

Figure 4.1: Proliferation of cells cultured in the monolayer (A), and cells encapsulated in alginate (B). Proliferation of cells in the PRC group was significantly higher compared to the control group at day 8 and 16. In both culture systems, cell proliferation peaked at day 16. Data are represented as mean ± SD. *$p < 0.05$ denotes statistical significance.

4.3.2 SEM images of cells encapsulated in alginate constructs

The microstructures of the alginate beads with and without hMSCs, captured using SEM, are shown in Figure 4.2. Alginate hydrogel without cells (Figure 4.2A) had a porous structure with submicron sized pores. Cells encapsulated in alginate and cultured in PRC (Figure 4.2C1) and those cultured in FBS medium (Figure 4.2B1) were both spherical in shape similar to that of chondrocytes in a 3D culture system. Cross-sectional view showed that the cells cultured in both the FBS medium (Figure 4.2B2) and PRC (Figure 4.2C2) were distributed within the pores of the alginate beads.

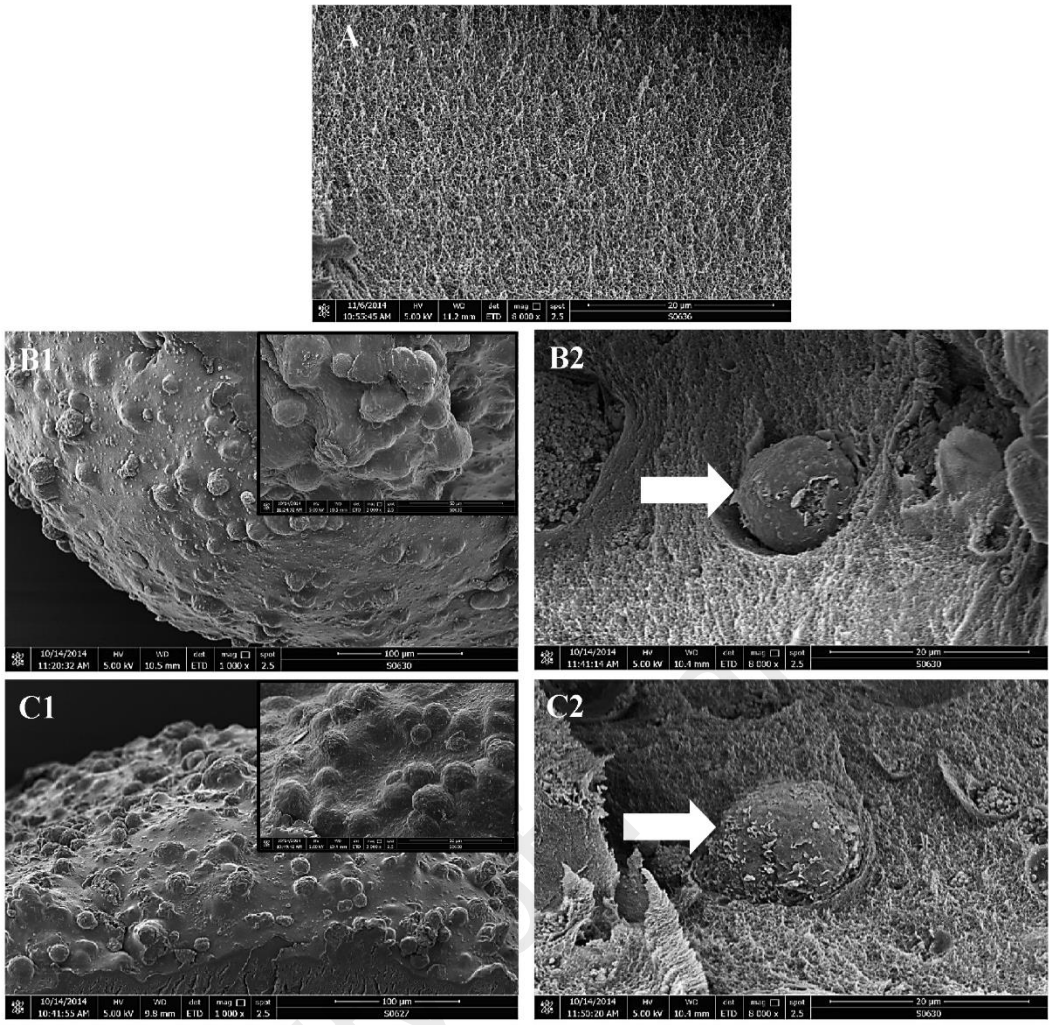

Figure 4.2: **Scanning Electron Microscope Images.** Alginate bead without cells had a porous structure (A).Cells encapsulated in alginate cultured in FBS medium (B1), (B2) and in PRC supplemented medium(C1), (C2) at 24 days post-seeding show a spherical morphology.The arrows point to the cells in the alginate bead. Scale bar of images B1 and C1 is 100 µm and B2 and C2 is 20 µm.

4.3.3 Effect of PRC on the expression of osteogenic markers

Gene expression of osteogenic markers

In general, the temporal pattern of expression of osteogenic gene markers in cells cultured in PRC was similar to that in the osteogenic medium throughout the duration of culture. However, the expressions of certain osteogenic genes were significantly higher in the PRC group compared to osteogenic medium at specific time points (Figure 4.3). At the early time point (day 8), PRC treatment upregulated the expression of the key osteogenic transcription factor, *RUNX2* by 3-fold, and *COL1A1* by 2-fold compared to the cells cultured in osteogenic medium. The expression of *ALP* was 1-fold higher in the PRC group compared to osteogenic medium ($p< 0.05$) at day 16. By day 24, the expression of bone morphogenetic protein 2 (*BMP2*), one of the key growth/differentiation factors involved in osteogenesis, increased by approximately 6-fold in the PRC group compared to osteogenic medium ($p<0.05$). The expression of another ECM marker, *OPN*, produced by osteoblasts was also significantly higher by 5-fold at day 16 and 4-fold at day 24 in the PRC group compared to the osteogenic medium. On the other hand, the expression of *ON*, a late osteogenic marker, was persistently less upregulated in the PRC group compared to osteogenic medium throughout the duration of cell culture ($p<0.05$).

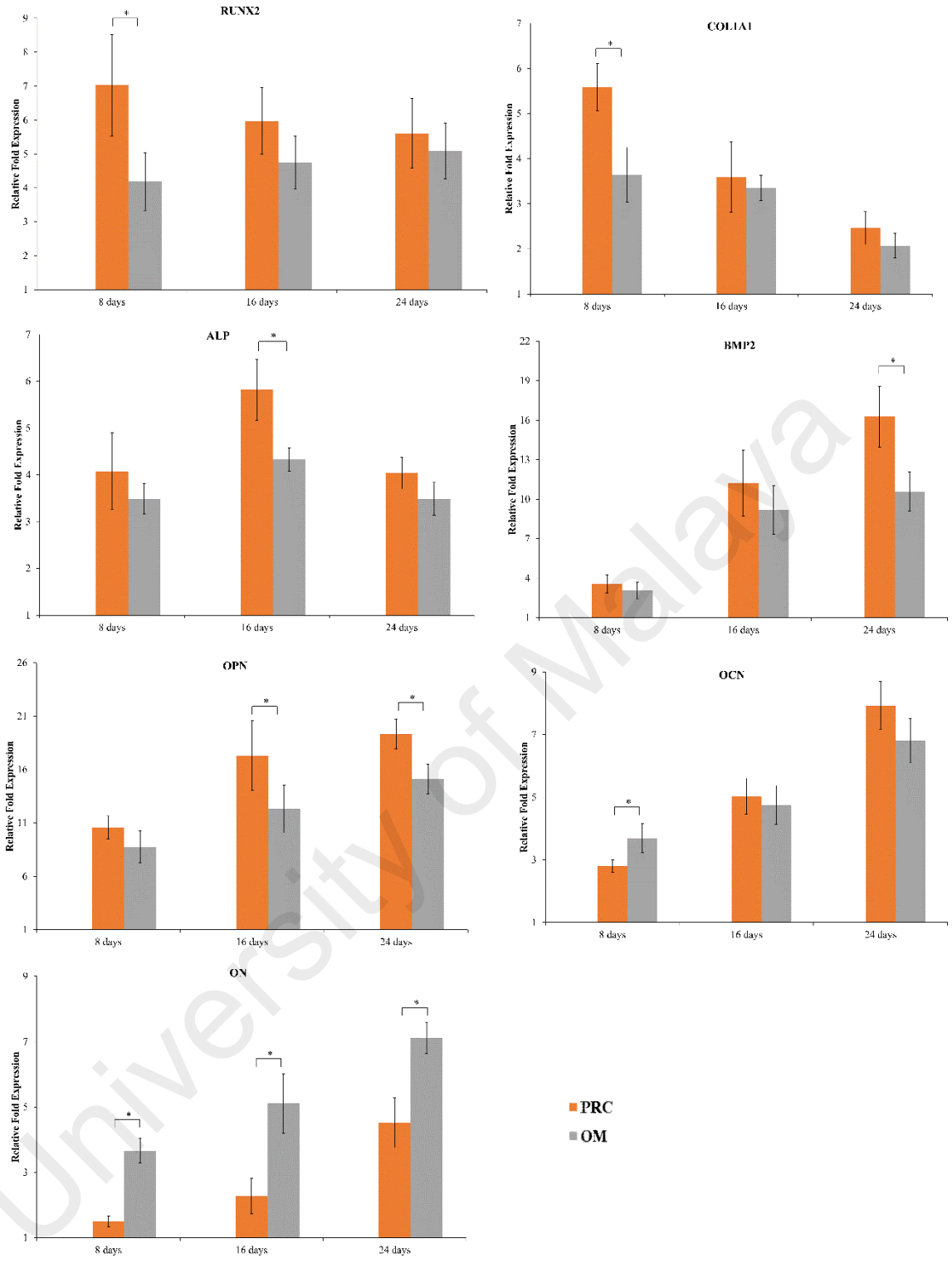

Figure 4.3: Expression of osteogenic genes throughout the experiment. PRC significantly induced greater up regulation of *RUNX2*, *COL1A1*, *ALP*, *OPN* and *BMP2* compared to osteogenic medium (OM) at various specific time points. On the contrary, *ON* level was significantly lower in the PRC group compared to the osteogenic medium throughout the course of study. Results are expressed as fold change relative to the control group (FBS medium). Data are represented as mean ± SD. *$p < 0.05$ denotes statistical significance between the indicated pairs.

Immunofluorescence staining for osteogenic markers

The immunocytochemistry analysis showed that cells treated with PRC had strong staining for RUNX2 (Figure 4.4A) and osterix (Figure 4.4B). Cells in the PRC group were moderately stained for osteocalcin (Figure 4.4C) and osteopontin (Figure 4.4D). Staining for all three markers were not detected in the control group.

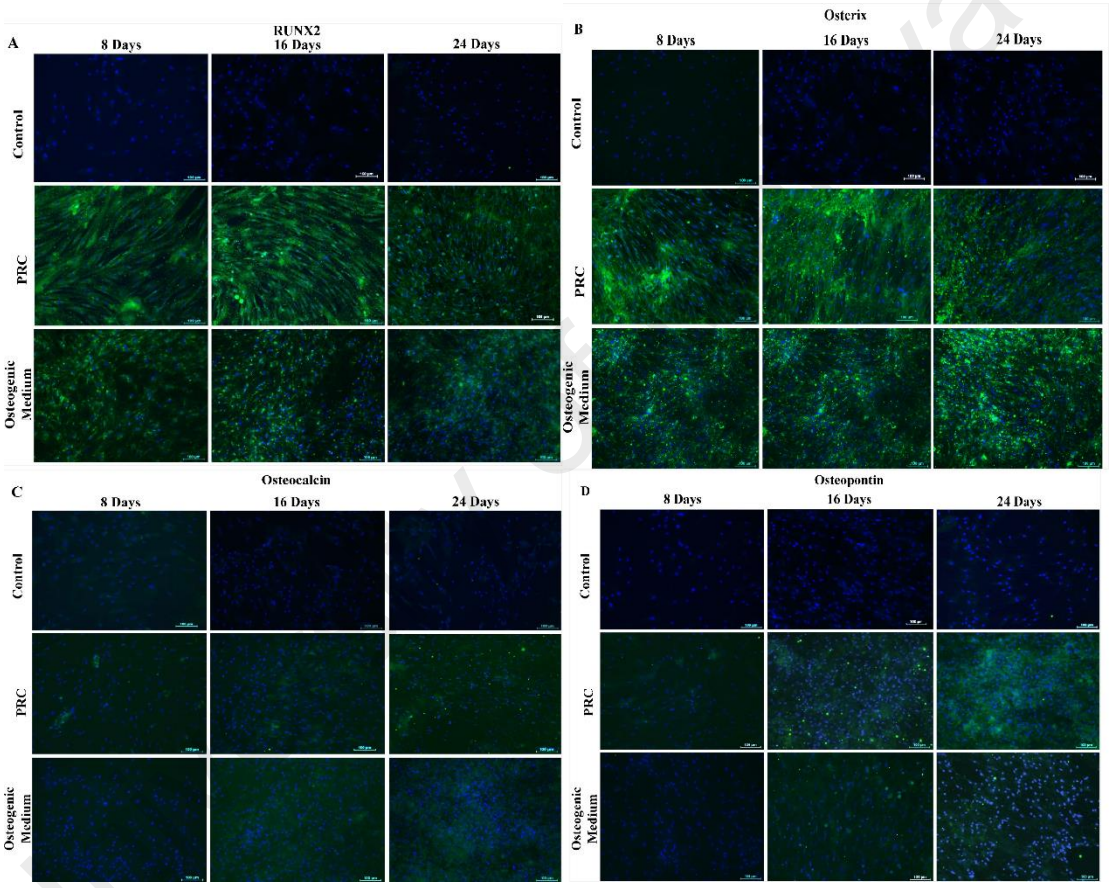

Figure 4.4: Immunofluorescent staining for osteogenic markers. Cells treated with PRC were strongly stained for RUNX2 and osterix as reflected by the green fluorescence. The staining for osteocalcin and osteopontin were of equal intensities in both the PRC-treated cells and those cultured in the osteogenic medium. Nucleus was stained blue by Hoechst. The above are representative images viewed at 10X magnification (scale bar: 100 µM).

ALP and osteocalcin concentration

There was a significant increase in ALP activity of cells cultured in both PRC and osteogenic medium ($p<0.05$), compared to those cultured in FBS medium (Figure 4.5A). The level of activity of this enzyme appeared to be slightly higher in the PRC group compared to that in osteogenic medium at day 8 and 16 ($p<0.05$). There was a gradual increment of osteocalcin protein concentration throughout the duration of cell culture. Similar to ALP activity, osteocalcin level was also significantly higher in cells cultured in PRC and osteogenic medium compared to the control group ($p<0.05$) (Figure 4.5B). However, cells in the PRC group had a higher level of osteocalcin compared to those cultured in osteogenic medium ($p<0.05$).

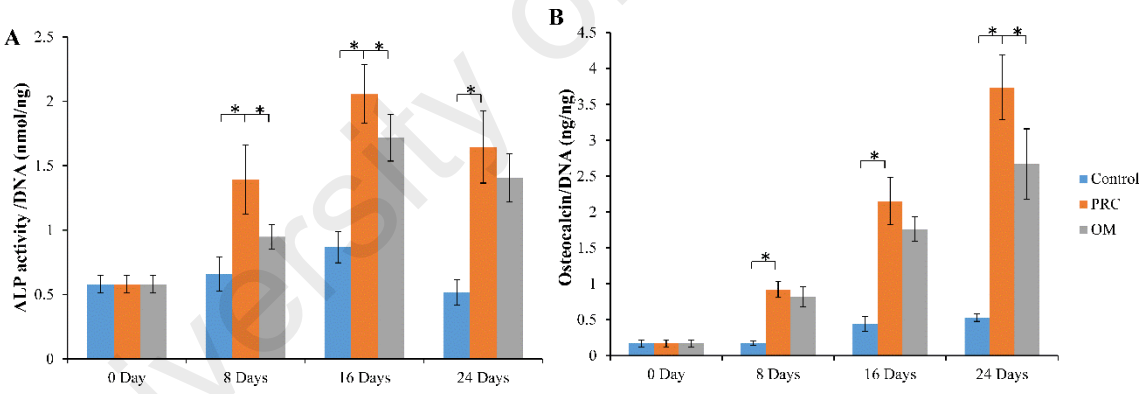

Figure 4.5: ALP activity and osteocalcin protein expression. (A) ALP activity was significantly higher in the PRC group compared to other groups at day 8 and 16; (B) Osteocalcin level gradually increased, peaking at day 24, with PRC group having the highest concentration throughout the study duration. The ALP activity and osteocalcin levels in each sample were normalized to the amount of DNA in the sample. Data are represented as mean ± SD (n=3). *p < 0.05 denotes statistical significance between the indicated pairs. (OM: osteogenic medium).

Alizarin red S staining and absorbance

Cells in the PRC group were intensely stained with alizarin red S stain, indicating high degree of mineralization (Figure 4.6A). The absorbance of alizarin red S stain (quantified by the extraction of the calcified mineral from the stained monolayer) gradually increased in the PRC group and was comparable to that of the osteogenic medium (Figure 4.6B).

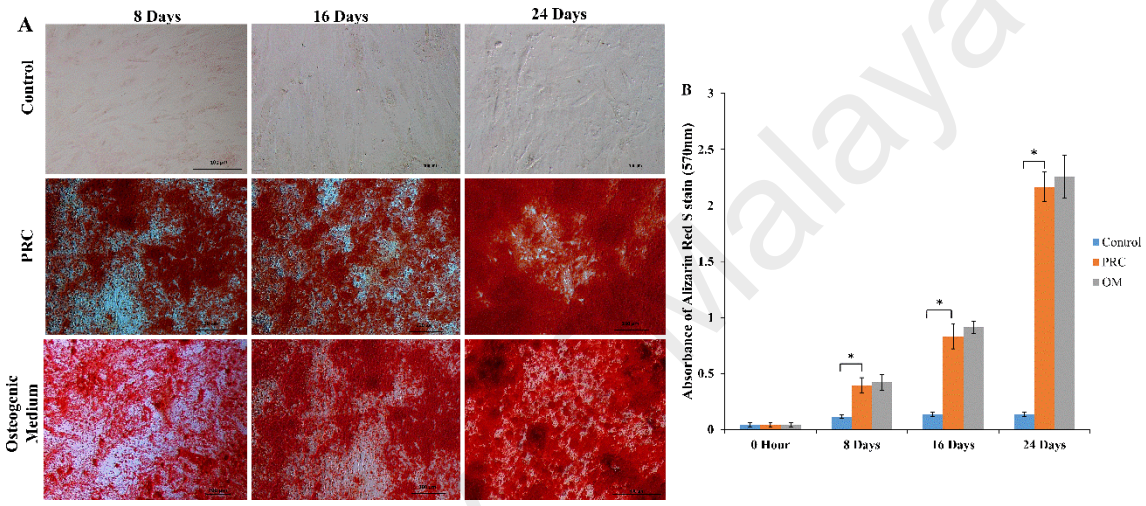

Figure 4.6: Alizarin red S staining and absorbance in cells cultured in PRC and osteogenic medium. (A) Cells treated with PRC were positively stained with alizarin red S stain indicating mineralization. (B) The absorbance level of the stain in the PRC-treated cells was similar to cells cultured in osteogenic medium (OM). Data are represented as mean ± SD (n=4). *$p < 0.05$ denotes statistical significance between the indicated pairs.

4.3.4 Effect of PRC on the expression of adipogenic marker

PPARG gene expression

The expression of peroxisome *PPARG*, a master regulator of adipogenesis, was significantly lower in the PRC group compared to adipogenic medium ($p \leq 0.05$) (Figure 4.7).

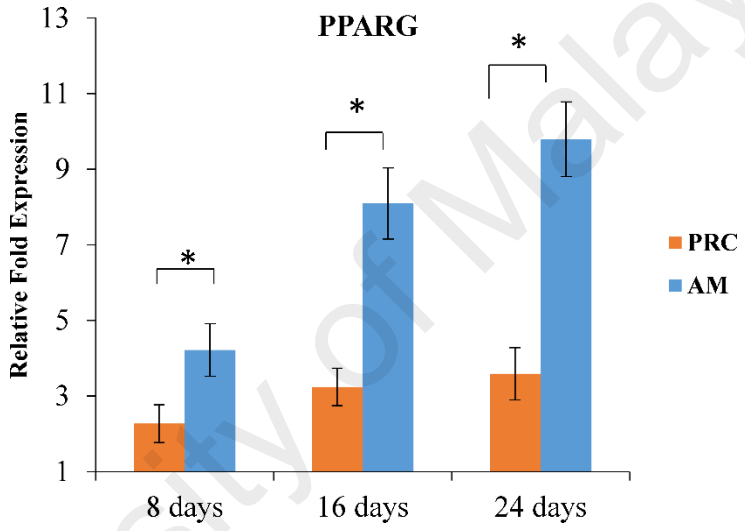

Figure 4.7: Gene expression of the adipocyte marker, *PPARG*. The expression of *PPARG* in the PRC group was significantly lower than the standard adipogenic medium (AM). Results are expressed as fold change relative to the control (FBS medium). Data are represented as the means ± SD. *$p < 0.05$ denotes statistical significance between the indicated pairs.

Oil red O staining and absorbance

Cells in the PRC group were found to contain significantly less lipid droplets with a corresponding lower absorbance value of the solubilized oil red O stain compared to those cultured in adipogenic medium at day 24 ($p \leq 0.05$) (Figure 4.8).

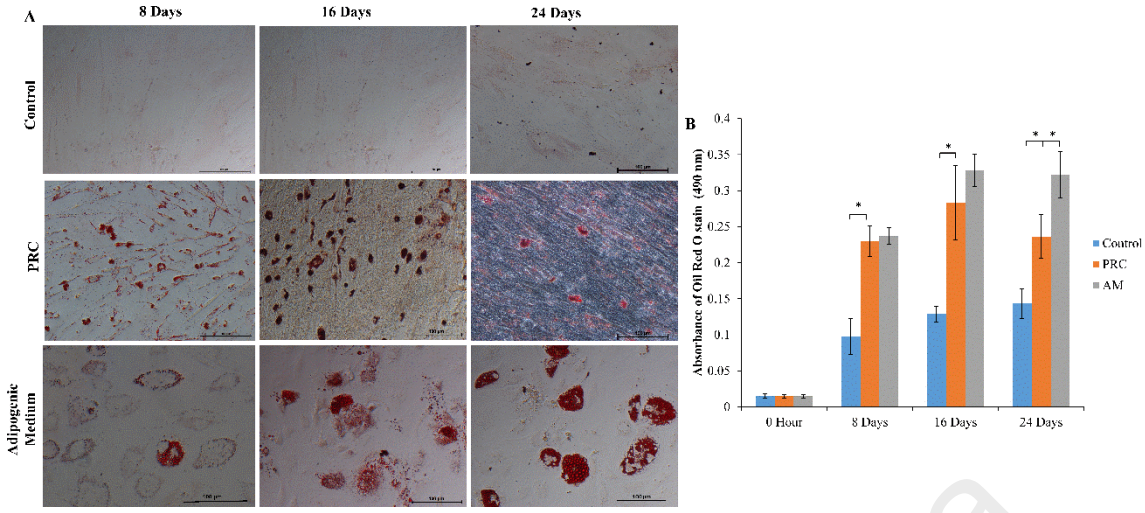

Figure 4.8: Oil red O staining of cells cultured in PRC and adipogenic medium. (A) PRC-treated cells showed positive staining but at significantly lower intensity compared to those in adipogenic medium. (B) The absorbance value of solubilized oil red O was high in cells cultured in adipogenic medium (AM). Data are represented as mean ± SD (n=4). *p < 0.05 denotes statistical significance between the indicated pairs.

4.3.5 Effect of PRC on the expression of chondrogenic markers

Gene expression of chondrogenic markers

The changes in chondrogenic gene expression in the PRC group compared to standard chondrogenic medium are depicted in Figure 4.9. The cells in both groups expressed *SOX9* and *TNC* genes as early as day 8, followed by other markers such as *COL2A1*, *ACAN* and *COMP*. The expression of *SOX9* declined at later time points in both groups; however, at the end of the experiment, cells treated with PRC had significantly higher expression of *SOX9* compared to chondrogenic medium (Figure 4.10A). The gene associated with early stages of chondrogenesis, *TNC*, was expressed about 9-fold higher in the PRC group compared to chondrogenic medium at day 8 (p< 0.05). There was an increasing expression of other chondrogenic markers i.e. *COL2A1*, *COMP* and *ACAN* throughout the experimental duration; the gene expressions of these markers were significantly higher in cells cultured in PRC compared to chondrogenic medium (p< 0.05)

at the end of the experiment. Hypertrophic markers i.e. collagen type X (*COL10A1*) and *RUNX2* were expressed at a significantly lower level in the PRC group compared to chondrogenic medium ($p< 0.05$) at the end of the study (Figure 4.9B).

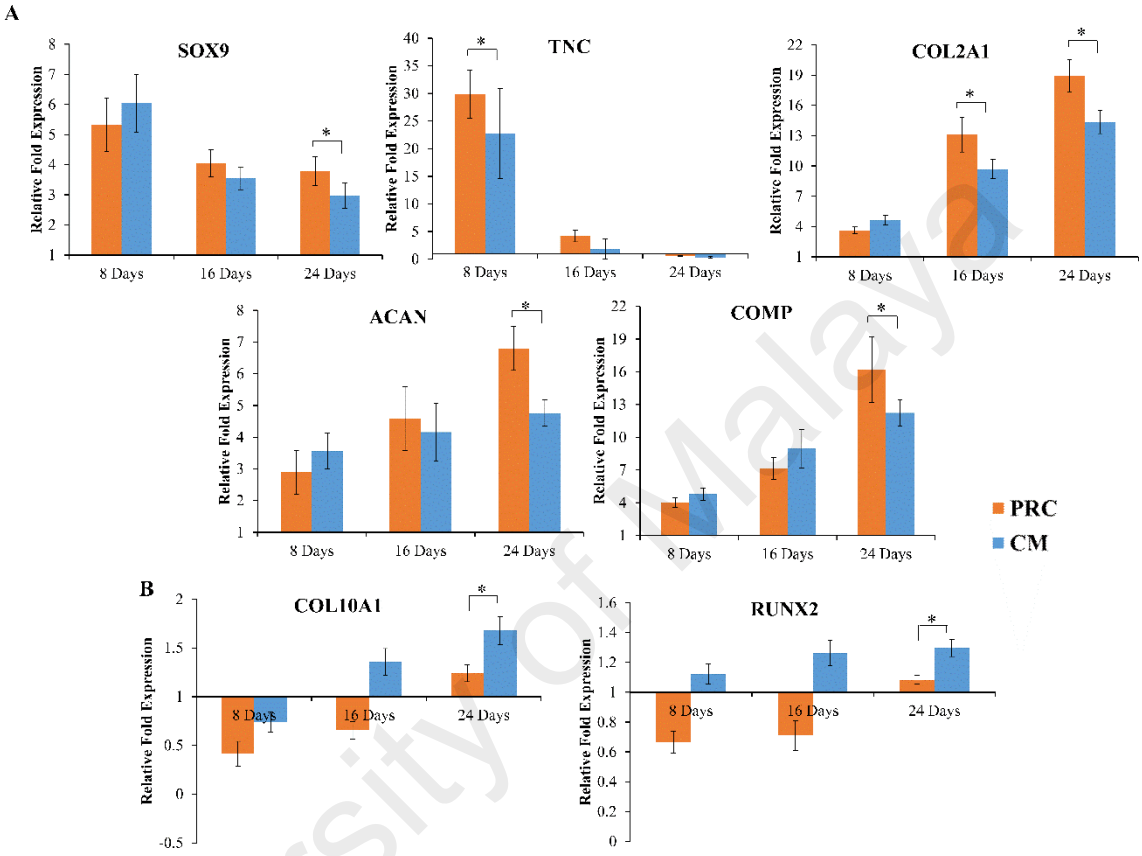

Figure 4.9: Expression of genes by hMSCs cultured in PRC and chondrogenic medium (CM). (A) Gene expression of cartilage extracellular matrix markers showing significantly higher *TNC* level in the PRC group at day 8, while other chondrogenic markers (i.e. *SOX9*, *COL2A1*, *ACAN*, and *COMP*) expressed by hMSC cultured in PRC-supplemented medium were significantly higher compared to the standard chondrogenic medium by day 24. (B) Gene expression of hypertrophic markers, *COL10A1* and *RUNX2*, were expressed at significantly lower levels in the PRC group compared to the chondrogenic medium by day 24. Results are expressed as fold change relative to the control (FBS medium). Data are represented as mean ± SD. *$p< 0.05$ denotes statistical significance between the indicated pairs.

Immunofluorescence staining of cartilage specific proteins

Cells in the PRC group exhibited stronger staining for cartilage specific ECM proteins, namely COL II, COMP and ACAN compared to those cultured in chondrogenic medium, indicating a greater protein deposition in this group (Figure 4.10). Positive staining was not detected in the control group.

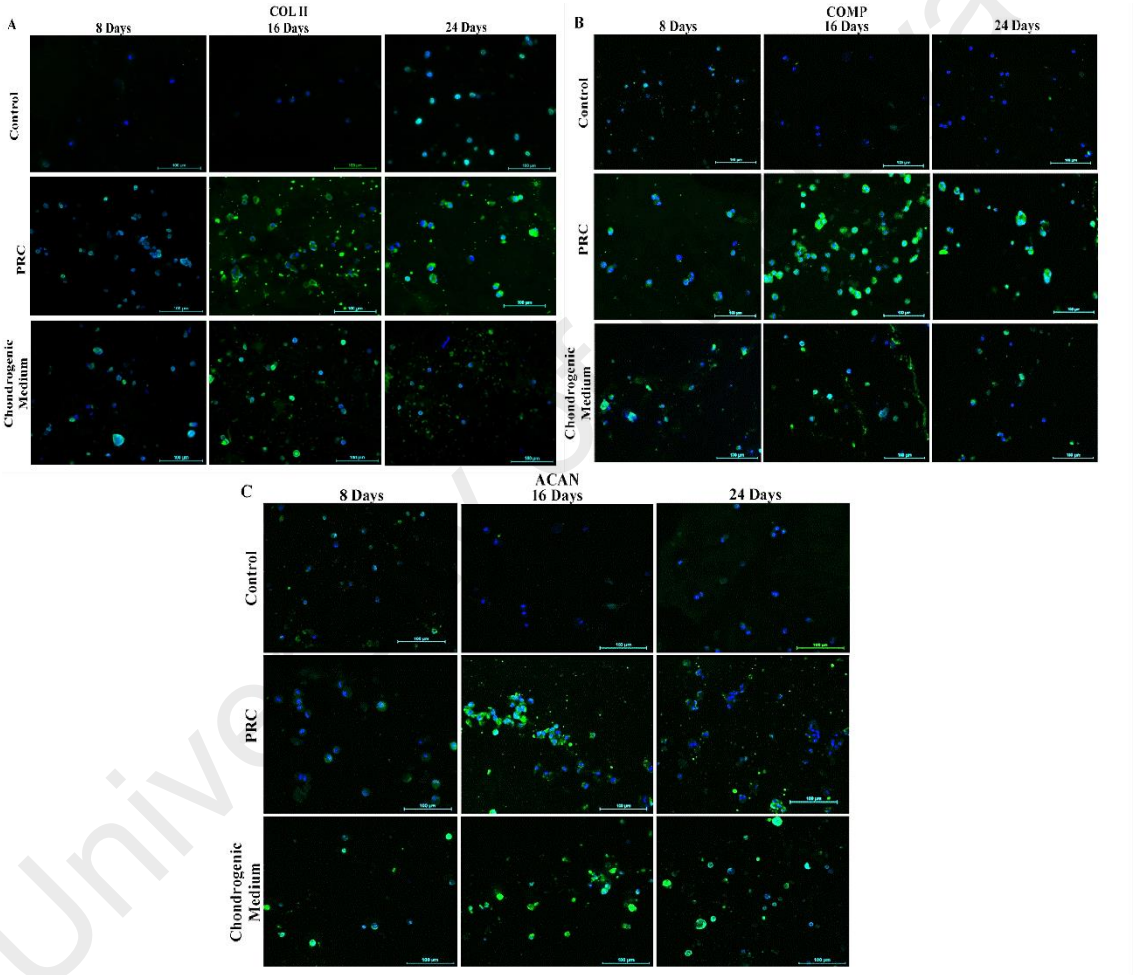

Figure 4.10: Immunofluorescence staining for chondrogenic ECM markers. Qualitative evaluation of chondrogenic differentiation by immunocytochemical staining of the cryostat sections of alginate beads for COL II (A), COMP (B) and ACAN (C) at 8, 16 and 24 days, showing stronger staining of cells in the PRC group. Results are representative of three independent experiments. Scale bar: 100µm.

Concentration of chondrogenic ECM markers

The concentration of COL II, ACAN and COMP were significantly higher ($p< 0.05$) in the PRC group compared to chondrogenic medium (Figure 4.11). At the end of the experiment, the concentration of ACAN in the PRC group increased to 72.19 ± 4.11 ng/mL, whilst that in the chondrogenic medium was 57.90 ± 3.11 ng/mL, and the control was only 24.28 ± 2.64 ng/mL ($p< 0.05$).

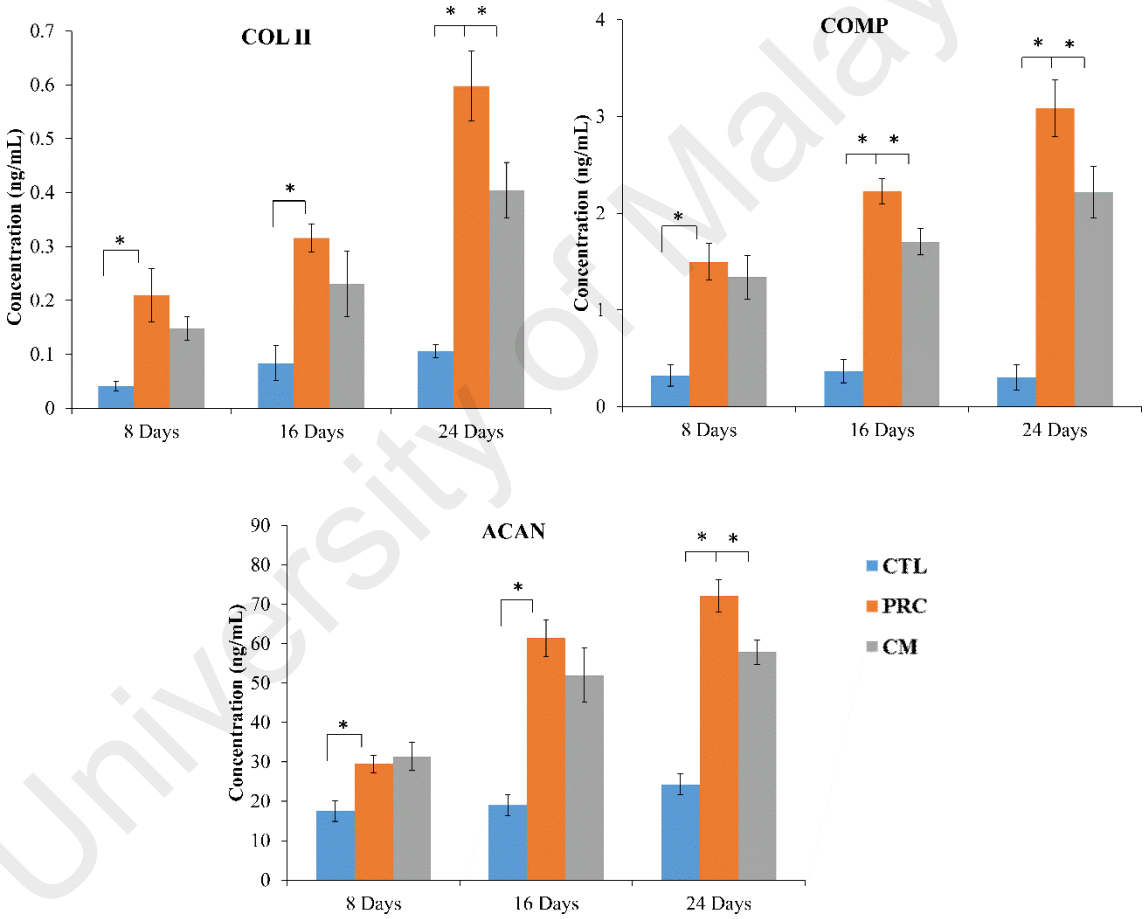

Figure 4.11: Quantification of ECM proteins by ELISA. Concentrations of cartilage ECM proteins were significantly higher in cells cultured in PRC-supplemented medium. Data are represented as mean ± SD (n=3). *p<0.05 denotes statistical significance between the indicated pairs. (CTL: control, CM: chondrogenic medium).

Concentration of glycosaminoglycan

There was a gradual accumulation of GAG observed in cells cultured in PRC and chondrogenic medium, which peaked at day 16 (Figure 4.12). The GAG level in the PRC group was significantly higher compared to that in the chondrogenic medium at day 16 and 24. At the end of the duration of the experiment, the ratio of GAG/DNA was 308.71 ± 41.40 ng/ng in the PRC group compared to 237.70 ± 38.42 ng/ng in chondrogenic medium ($p< 0.05$).

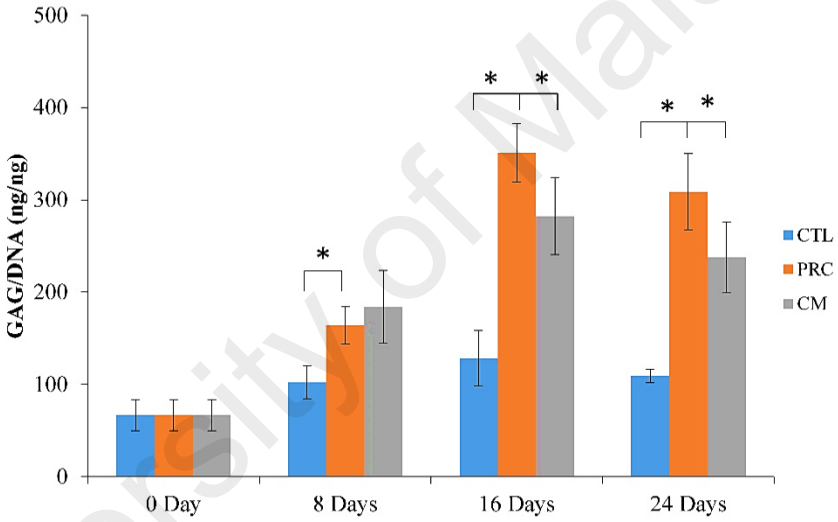

Figure 4.12: Concentration of glycosaminoglycan in cells encapsulated in alginate. Synthesis of GAG was significantly higher in the PRC group compared to control and chondrogenic medium by day 16 onwards. Data are represented as mean ± SD (n=6). *$p< 0.05$ denotes statistical significance between the indicated pairs. (CTL: control; CM: chondrogenic medium).

4.4 Discussion

This study highlights a few major benefits of using PRC as a culture medium for providing abundant pool of pre-differentiated MSCs for application in tissue engineering. PRC alone in serum-free medium was not only able to maintain hMSC proliferation for 24 days, but could also significantly induce osteogenic and chondrogenic differentiation at a greater extent compared to the commercially available differentiation media. This was reflected by higher expressions of the respective lineage-specific markers both at the mRNA and protein levels. The chondrogenic hMSCs treated with PRC also had significantly lower expression of hypertrophic markers compared to standard chondrogenic medium, indicating that PRC might provide additional benefit of delaying the onset of chondrocyte hypertrophy. Therefore, transplantation of cells differentiated in the presence of PRC might have the potential to prevent endochondral ossification, thus producing better cartilage repair. Although PRC was able to maintain adipogenic differentiation of the hMSC during the entire duration of culture, the extent of differentiation was lesser than that induced by standard adipogenic medium.

Cell proliferation was significantly higher in the PRC group compared to control, peaking at day 16, and declined thereafter. The reduction in cell proliferation over time could be due to the fact that cells generally tend to have a low proliferative potential as they differentiate and mature (Birmingham et al., 2012). Once the cells become fully differentiated, they will permanently exit from the cell cycle, and this coincides with proliferation arrest (Ruijtenberg & van den Heuvel, 2016).

This study also confirmed that PRC is potent in inducing osteogenic differentiation and chondrogenic differentiation of hMSCs without altering the temporal pattern of gene expression profile of the cells. It has been demonstrated that the process of MSC differentiation to osteogenic lineage is regulated by master transcription factors RUNX2, osterix, as well as morphogens such as TGFβ/BMPs and FGFs (Javed, Chen, & Ghori, 2010). RUNX2 is a master regulator that acts upstream of osterix, and both are required for the early and late stages of osteoblast differentiation (Nakashima et al., 2002). RUNX2 is also known to regulate the expression of various osteogenic genes such as type I collagen, osteopontin and osteocalcin (Ducy, Zhang, Geoffroy, Ridall, & Karsenty, 1997). The cells initially produce ECM, which mainly consists of collagen type I (Birmingham et al., 2012). With ongoing differentiation, cells start to produce a variety of non-collagenous proteins such as osteopontin and osteocalcin. Finally, the cells transform into mature osteoblasts, as indicated by bone mineralization (Huang et al., 2007; Jaiswal, Haynesworth, Caplan, & Bruder, 1997). In the present study, cells cultured in medium supplemented with PRC underwent osteogenic differentiation as early as 8 days as shown by the high expression of *RUNX2*. This could have further initiated the expression of other osteogenic markers such as collagen type I, osteopontin and osteocalcin.

The normal temporal pattern of chondrogenesis was also preserved in the cells treated with PRC, with an early expression of *SOX9* and *TNC*, followed by increasing levels of other cartilage matrix-specific markers such as *COL2A1*, *COMP* and *ACAN*. Studies have shown that chondrogenic differentiation of MSCs *in vitro* simulates the normal progression of chondrogenesis *in vivo*, whereby the process is initiated by cell condensation, followed by formation of aggregates, and subsequent differentiation into cartilage nodules (DeLise, Stringa, Woodward, Mello, & Tuan, 2000). The gene *SOX9*, a

key regulator of chondrogenesis, is reported to be essential for the initial mesenchymal stromal cell condensation. During this phase, there is an upregulation of the transcription factor SOX9 and the ECM protein, TNC (Singh & Schwarzbauer, 2012). SOX9 also regulates the expression of various chondrogenic ECM proteins including COL II and ACAN (Kulyk, Franklin, & Hoffman, 2000; Sekiya et al., 2000). As the differentiation of mesenchymal stromal cells progresses, the markers of cell condensation are replaced by more specific cartilage markers i.e. *COL2*, *ACAN* and *COMP* (Singh & Schwarzbauer, 2012). The expression of *COMP*, a major non-collagenous protein in cartilage, is reported to be an even more sensitive marker of chondrogenesis than *COL2* (Zaucke, Dinser, Maurer, & Paulsson, 2001), but is not widely reported in the literature.

This study also showed significantly lower expressions *COL10* and *RUNX2* in cells cultured in PRC compared to chondrogenic medium. These markers are predominantly associated with hypertrophic cartilage (Leung et al., 2011; Zhou et al., 2006). The gene *SOX9* is known to regulate the transition of chondrocytes to their hypertrophic state. It negatively regulates the expression of *COL10* and *RUNX2* genes, which are commonly expressed by cells undergoing endochondral ossification. In a previous study, Xu *et al*. reported an increase in *COL10* during chondrogenic differentiation of MSC encapsulated in alginate (Xu et al., 2008). Upregulation of *RUNX2* was also observed when MSC-derived chondrocytes were encapsulated in alginate (Fernandes et al., 2013) and agarose (Caron et al., 2013), indicating that the transition of cells to the hypertrophic state is a common phenomenon seen in the 3D culture system. Since the ideal repaired cartilage tissue should be hyaline in nature, the presence of hypertrophic phenotype seen in MSC-derived chondrocytes would be of a great concern for applications of MSC in cartilage repair therapies. Hypertrophy in the neocartilage could ultimately lead to apoptosis, vascular invasion and ossification as sometimes seen in osteoarthritis (Mueller & Tuan,

2008). The extent of reduction of chondrocyte hypertrophic marker induced by PRC in this study was greater than previously reported (i.e. *COL10* was only upregulated by 1.8 fold at day 24). Mardani *et al*. showed a significant increase in the expression of *COL10* (about 45 fold) by day 14 in adipose-derived stem cells cultured in medium containing PRP and TGFβ1. This difference could be explained by the fact that in the previous study, the cells were cultured in a transwell culture and the medium was also supplemented with differentiation inducing supplements such as dexamethasone (Mardani et al., 2013). The use of transwell cultures have been shown to be associated with an increased level of *COL10* expression (Murdoch et al., 2007), and the inclusion of dexamethasone in the culture medium has also been speculated to be a contributing factor towards chondrocyte hypertrophy (Kafienah et al., 2007).

In this study, alginate hydrogel was used as a cell carrier. The structure of alginate is porous as shown by the SEM images, and hence, the cells encapsulated in alginate would be able to respond to the growth factors released from PRC. This argument is supported by the study by Ma et al., which showed that cells encapsulated in alginate were able to respond to TGF-β1 growth factor supplemented in the medium (Ma et al., 2003). Alginate hydrogel are also lacking in ligands recognizable by the cell receptors, thus limiting their interaction with the cell matrix, helping them to maintain a spherical chondrocytic phenotype, mimicking the microenvironment of chondrocytes in the cartilage tissue (Sun & Tan, 2013; Xu et al., 2014). Many studies have shown a strong positive correlation between round cell morphology and maintenance of chondrogenic phenotype. An increase in the expression of chondrogenic markers was observed in cells cultured in aggregates (McBride, Falls, & Knothe Tate, 2008) and high glycosaminoglycan synthesis was also observed in cells in pellet culture (Abbott & Holtzer, 1966). However, it is worth noting that chondrogenic differentiation of cells induced in a pellet culture system are

inclined to have a higher expression of chondrocyte hypertrophy marker, collagen type X (Dashtdar et al., 2016; Yang et al., 2004) and proteolytic enzyme, matrix metalloproteinase 13 (*MMP13*) (Caron et al., 2012). These markers have been implicated in the pathogenesis of osteoarthritis (Wojdasiewicz, Poniatowski, & Szukiewicz, 2014), rendering the application of this culture mass impractical for *in vivo* cartilage repair.

The observation of a relatively lower extent of adipogenesis induced by PRC compared to adipogenic medium could be explained by the fact that differentiation of MSCs to adipocytes and osteoblasts are inversely regulated and the differentiation towards the osteoblast phenotype occurs at the expense of adipocyte phenotype (Chen et al., 2016; James, 2013; Kim & Ko, 2014). A study by Kang et al. showed that the commitment of MSCs to osteogenic and adipogenic lineages were mutually restricted. Growth factor BMP2 induced ALP activity in some cells and lipid accumulation in others, but there were no cells that exhibited both ALP activity and lipid accumulation simultaneously. They also reported that expression of *PPARG* was required for both osteogenic and adipogenic differentiation (Kang et al., 2009). Insufficient expression of *PPARG* seen in heterozygous *PPARG*-deficient mice was found to stimulate osteoblastogenesis (Akune et al., 2004). Hence, based on these observations, it can be speculated that the lower extent of *PPARG* mRNA expression seen in cells treated with PRC compared to adipogenic medium might have led to a preferential differentiation of hMSC to osteogenic over the adipogenic lineage within the same monolayer culture system. In the previous chapter the gene expression levels during 8 days of culture were not markedly high since the cells predominantly underwent proliferation and were just entering the differentiation phase. On the other hand in this chapter, by day 24, the cells had undergone complete differentiation as they were exposed to the differentiation inducing factors for a longer

period of time. Thus, gene expressions of the tri-lineage differentiation markers were observed to be much higher compared to the earlier time point of assessment.

The finding of this study show that PRC has the potential to induce differentiation of hMSCs to multiple lineages, albeit to varying degrees. This multi-lineage potential of PRC can be attributed to the presence of various naturally occurring lineage-specific growth factors released by the platelets. Growth factors such as bone morphogenetic protein (BMP2) and platelet derived growth factor (PDGF) are known to affect bone formation. BMP2 plays a key role in osteoblast commitment and induces MSCs to form osteoblast by increasing ALP activity, and thus plays a role in the early stages of osteogenic differentiation (Hughes, Collyer, Stanfield, & Goodman, 1995). PDGF also regulates osteogenic differentiation by modulating BMP signalling (Li et al., 2014; Lysdahl, Baatrup, Foldager, & Bunger, 2014; Zdunek et al., 2008). BMP2 is also known to regulate adipogenic differentiation. Low concentrations of BMP2 were found to stimulate adipocyte formation while high concentrations favoured osteogenic differentiation (Wang, Israel, Kelly, & Luxenberg, 1993). Other growth factors like transforming growth factor β (TGF-β), and basic fibroblast growth factor (bFGF) are known to support chondrogenesis. Previous study has shown that MSC in pellet culture treated with TGF-β1 expressed less *COL1* and more *COL2* genes (Havlas et al., 2011). bFGF was also found to enhance chondrogenic differentiation of MSCs (Park & Na, 2008). It can therefore be speculated that these growth factors in PRC work synergistically to enhance the multi-lineage potential of hMSC.

This study had few limitations worth noting. The potential of PRC to induce differentiation of MSCs to other lineages like myocytes or tenocytes was not investigated. This was due to unavailability of a standard commercially available positive

differentiation media for the two respective lineages. However, adipogenic, osteogenic and chondrogenic lineages are the most commonly investigated MSC lineages. Hence, it was sufficient to consider only these three lineages. In addition, in this study, the tendency of platelet to favour and direct MSC differentiation to any dominant lineage could not be precisely determined. This would require investigation of the MSC tri-lineage differentiation potential to be performed on monolayer culture system in a single experimental setting. However, this was not ideal in this study as chondrogenesis requires the presence of a 3D scaffold. Since the chondrogenic differentiation potential was assessed separately from adipogenic and osteogenic lineages, it was not possible to determine the dominance of a particular lineage over the other in this study.

In conclusion, the work presented in this chapter fulfilled the study objectives indicating that PRC can maintain the viability of hMSCs until day 24 and produce more superior effect in promoting osteogenesis and chondrogenesis of hMSCs compared to the respective commercially available lineage specific differentiation media. In addition, PRC also renders benefit in delaying hypertrophy in hMSC-derived chondrocyte. Altogether, this study has an implication of potentially advocating the use of PRC as a substitute for standard osteoinductive and chondroinductive medium to obtain sufficient pool of osteogenic and chondrogenic pre-differentiated cells to be applied in bone and cartilage regeneration *in vivo*.

CHAPTER 5: EFFECT OF PRC ON THE REGENERATIVE CAPACITY OF MESENCHYMAL STROMAL CELLS FOR REPAIR OF FULL THICKNESS CHONDRAL DEFECT IN RABBITS

5.1 INTRODUCTION

Having established the positive effect of PRC in enhancing tri-lineage differentiation of hMSC *in vitro* in the previous chapter, there is a need to further prove the potential benefit of PRC in the *in vivo* setting. In this chapter, a rabbit model of focal cartilage defect was chosen as a working model to study the therapeutic advantage of PRC in enhancing the regenerative potential of MSC *in vivo* to improve cartilage repair.

Focal cartilage injuries are particularly prevalent among individuals who participate in high impact sports. It has been reported that injuries involving only the articular surfaces of the cartilage occurs in about 36% of athletes, which is twice more prevalent compared to that occurring in the general population (Flanigan, Harris, Trinh, Siston, & Brophy, 2010). In normal circumstances, articular cartilage possesses the ability to adjust its volume and thickness according to the level of physical activity of the individual. Its ability to evenly distribute the applied load minimizes considerable stress on the underlying subchondral bone (Mithoefer, Peterson, Zenobi-Wong, & Mandelbaum, 2015). This is accomplished through an intricate regulation of extracellular matrix protein synthesis by the chondrocytes (Janostiak, Pataki, Brabek, & Rosel, 2014). However, repeated stress from high impact joint loading activities may impair this physiological process, hence, leading to degenerative changes in the cartilage mass, which would eventually predispose the knee to development of osteoarthritis (OA). Indeed, athletes have 12-fold increased risk of developing OA compared to the general population

(Drawer & Fuller, 2001; Roos, 1998). Treating such articular cartilage defects successfully has continued to present clinicians with a formidable challenge, since the widely used cell-based surgical approach i.e. autologous chondrocyte implantation (ACI), has its inherent limitations such as poor yield of chondrocytes, chondrocyte senescence and chondrocyte de-differentiation, leading to the loss of their capacity to recreate normal hyaline cartilage (Bornes, Adesida, & Jomha, 2014). As an alternative to the use of native chondrocytes, the use of mesenchymal stromal cells (MSCs) in cartilage repair has been increasingly explored, mainly due to their potential to proliferate and differentiate to chondrocytes (Caplan, 2005). The use of MSCs can also overcome the limitations of ACI such as donor-site morbidity and limited matrix production following *in vitro* cell expansion (Bernhard & Vunjak-Novakovic, 2016). Accordingly, various approaches to augment the proliferative and chondrogenic differentiation ability of MSCs have been explored. One promising approach is through the use of platelet-rich plasma (PRP), which constitutes a natural source of various chondroinductive growth factors like TGF-β and FGF (Eppley et al., 2004; Ng et al., 2008). Previous studies have shown that PRP stimulated the proliferation and matrix synthesis of intervertebral disc cells (Akeda et al., 2006). and enhanced articular cartilage repair *in vivo* when used in conjunction with microfracture procedure for the repair of chondral defects (Huh et al., 2014; Milano et al., 2012; Milano, Passino, et al., 2010; Vaisman et al., 2012), or, when used for the treatment of osteochondral defects (Carneiro Mde, Barbieri, & Barbieri Neto, 2013; Liu et al., 2014; Qi et al., 2009; Sun et al., 2010; Xie et al., 2012). Direct PRP injections administered after sub-chondral bone drilling have also led to good functional outcome for the repair of chondral defects in clinical studies (Haleem et al., 2010; Sanchez et al., 2003; Siclari et al., 2012). However, in these studies, it is possible that marrow stimulation would have itself initiated *in situ* cartilage repair by stimulating the stromal cells from the bone marrow to migrate and initiate the repair process, thus, represents a confounding factor

that may have obscured the true effect of the PRC. The isolated effect of PRP itself in enhancing the repair of focal cartilage defects *in vivo* has not yet been established. Therefore, further experiments involving tissue analysis are required to validate the presumed favourable effects of platelets rich preparation in the repair of focal cartilage damage *in vivo*. More importantly, the notion as to whether the regenerative potential of MSC can be further enhanced through the concomitant supplementation of platelet rich concentrate implanted into focal cartilage defects has also not been previously investigated.

Therefore the objective of the work presented in this chapter is to determine the effect of PRC in enhancing the regenerative capacity of MSCs to augment articular cartilage repair *in vivo* in a rabbit model of focal cartilage defect. In this study alginate beads were used as a carrier to facilitate the delivery of MSC, PRC and the combination of the two regenerative promoters to the defect site.

5.2 METHODS

5.2.1 Animals

New Zealand male white rabbits, 6–7 months old, weighing 2–3 kg, were used in this study in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) Review Board (Ethics approval reference number: FIS/12/09/2014/SS (R)). Animals were housed separately under controlled temperature and light conditions and provided free access to drinking water and pellet food. They were kept in standard cages with resting boards that allowed for unrestricted weight-bearing activity. During housing, animals were monitored twice daily for health status. No adverse events were observed. Three rabbits were used for bone marrow isolation and two rabbits were used to determine the fate of the transplanted MSCs *in vivo*. Power analysis indicated that in order to detect an increase of at least 35% in glycosaminoglycan content in the treatment group compared to controls at 6 months, as previously reported in the literature (Tay et al., 2012), with 80% power and an α value of 0.05, a minimum of 4 animals per group were deemed sufficient. Assuming a 25% animal dropout rate, which is consistent with other rabbit surgical studies, 5 animals would be needed in each group. Therefore, a total of 30 rabbits were used in the study. The animals were randomly assigned into three treatment groups: (i) PRC encapsulated in alginate (PRC; n=10), (ii) MSCs isolated from rabbit bone marrow (rbMSCs) encapsulated in alginate (rbMSC; n=10), and, (iii) a combination of PRC and rbMSCs encapsulated in alginate (PRC+rbMSC; n=10). The animals in each treatment group were further divided into 2 groups such that 5 rabbits were sacrificed at each time point (3 and 6 month post-transplantation).

5.2.2 Isolation of allogeneic rabbit MSCs (rbMSCs) from rabbit bone marrow

Three rabbits were sacrificed using an intravenous overdose of pentobarbital. Femur and tibia of both lower limbs of the rabbits were removed, and any adherent tissues were scraped off. All harvested bones were kept on ice in 1X phosphate buffer saline (PBS, pH 7.2, Invitrogen-Gibco, USA) supplemented with 4% penicillin-streptomycin until they were processed (Tay et al., 2012). Within 3 hours, bone marrow was harvested from the femur and tibia. MSCs were isolated according to the protocol described by Pittenger *et al*. in 1999 (Pittenger et al., 1999). Briefly, 2 mL of bone marrow was diluted with the same amount of 1X PBS and slowly layered over 3 mL Ficoll-paque (GE Healthcare – Amersham Biosciences, Piscataway, New Jersey) in a 15-mL Falcon tube (Corning, USA). After 25 minutes of centrifugation at 960x g, mononuclear cells were collected from the interphase. The cells were then washed with prewarmed Dulbecco's modified eagle medium (DMEM, Gibco) and centrifuged at 645x g for 5 minutes. The cell pellet was resuspended in DMEM containing 10% FBS (Invitrogen-Gibco) and 1% penicillin-streptomycin (100 U/mL, Invitrogen-Gibco) and seeded in a T-75 flask and maintained in monolayer culture at 37°C, 95% humidity, and 5% CO_2 for 3 weeks. The culture medium was replaced every 2-3 days. The cells were then serially passaged and cells from passage 3 were used for further experiments.

5.2.3 Characterization of cells isolated from rabbit bone marrow

Cells isolated from rabbit bone marrow were verified to be mesenchymal stromal cells based on immunocytochemical staining for surface markers and cytochemical staining to determine the tri-lineage potential of the cells.

Immmunocytochemical staining for cell surface markers

Immunocytochemical staining was performed as described in chapter 3, section 3.2.1.4 page 74. Rabbit MSCs were incubated with primary mouse anti-rabbit monoclonal antibody against the cell surface markers $CD44^{+}/CD29^{+}$ and $CD45^{-}$ (1:100 dilution, Abcam, Cambridge, UK) at room temperature for 30 minutes. After washing with PBS, cells were incubated with secondary antibody (goat anti-mouse IgG, Dako) at 1:200 dilution for 30 minutes. Cells were then washed with PBS, stained with 3,3′-diaminobenzidine tetrahydrochloride (DAB) chromogen substrate in the dark and examined under light microscopy (Nikon Eclipse TE2000-S; Nikon Corporation, Tokyo, Japan).

Tri-lineage differentiation

The potential of cells to undergo differentiation to adipogenic, osteogenic and chondrogenic lineages was performed as described previously in chapter 3, section 3.2.1.4 page 76.

5.2.4 Creation of full thickness chondral defects

The rabbits were anesthetized prior to surgical exposure of both femorotibial joints. Both knee joints were opened via a medial para-patellar approach along the patellar tendon and all defects were created on the weight bearing region of the medial femoral condyle. Defects measuring 5 mm in diameter and 1 mm in depth were created in both knees of each animal using a custom-made chondrotome as previously described by Kamarul et al (Dashtdar et al., 2011; Kamarul et al., 2008; Tay et al., 2012). The dimensions of the

chondrotome were fixed in order to create a defect of uniform size in all rabbits. The 5 mm diameter defect size was chosen since critically sized defects in rabbits (i.e. those that do not exhibit spontaneous healing) has been reported to be at least 3 mm in diameter, and defect size of 4 to 5 mm have been previously recommended (Ahern, Parvizi, Boston, & Schaer, 2009). Non-calcified cartilage was removed from the defect using a hand curette, with additional precaution taken to retain the calcified cartilage at the base of the defect (Figure. 5.1). Breach of the subchondral region was avoided during this procedure. No bleeding from the surface of the exposed subchondral bone was observed intraoperatively.

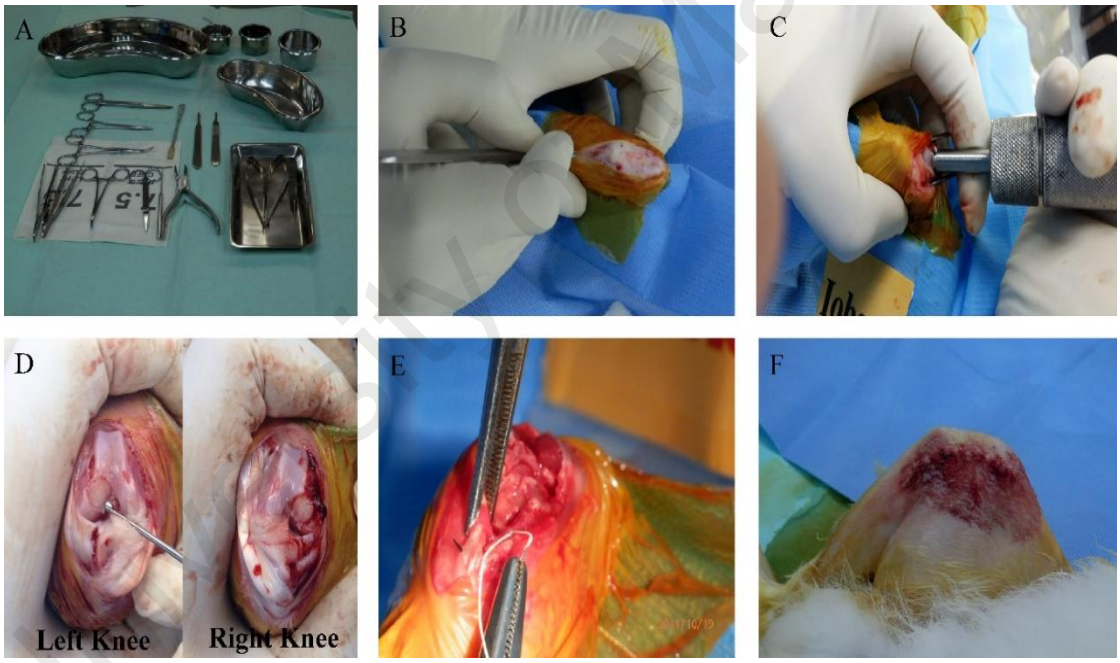

Figure 5.1: Creation of full-thickness chondral defects. (A) Autoclaved surgical set. (B) Incision was made to expose the medial femoral condyle. (C) Full thickness defect creation using custom made chondrotome. (D) The macroscopic appearance of defect created on both knees. (E) Suturing of the defect site. (F) The appearance of a completely sutured defect site.

5.2.5 Isolation and preparation of autologous PRC, rbMSC, and PRC+rbMSC in alginate beads

To prepare autologous PRC, 17 mL blood was drawn from the central vein of the ear of rabbits from the PRC and rbMSC+PRC groups (Figure 5.2). PRC was prepared using double centrifugation method as mentioned in Chapter 3, section 3.2.2.1 page 77. The platelet pellets resuspended in PBS was divided into two portions. One portion was used to determine the platelet number. The amount of platelets in the PRC and rabbit whole blood was determined using Sysmex XE 5000 hematology analyser. The other portion (containing approximately 1500 µL) of PRC was centrifuged at 1740x g and the pellet was resuspended in alginate. PRC was incorporated into alginate beads either alone (PRC group), or in combination with cell pellets containing 1 x 10^6 rbMSCs (PRC+rbMSC group). For the rbMSC group, 1 x 10^6 rbMSCs were resuspended in alginate. The cell suspensions were dropped into a 102 mM calcium chloride solution using a micropipette tip for polymerization and formation of the alginate beads.

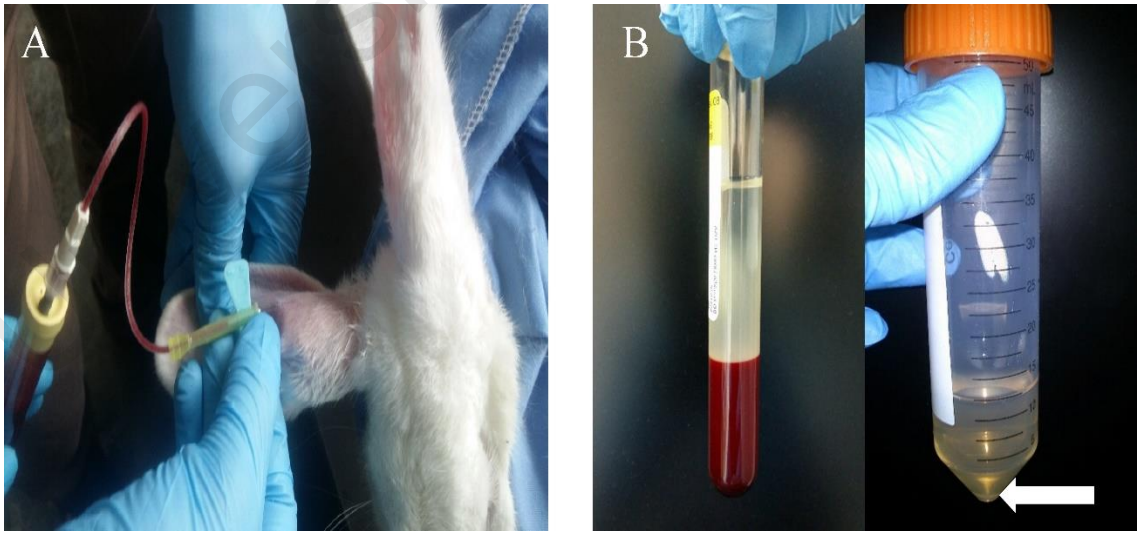

Figure 5.2: Preparation of autologous PRC from rabbit blood. (A) Drawing of blood from the central vein of the rabbit ear. (B) Double centrifugation to isolate the platelet pellet. Arrow indicates the platelet pellet.

5.2.6 Transplantation of alginate constructs into the defect site

All transplantations were performed only in the right knee of each rabbit approximately one month after the defect creation, as done in previous studies (Brittberg et al., 1994; Kamarul et al., 2008). The rabbits were anesthetized and the chondral defects were exposed and identified. A periosteum flap of 7-mm diameter was harvested from the medial aspect of the proximal tibia of the same limb (Figure 5.3). The periosteal flap was then placed on top of the defect and anchored with a suture in each corner using 8-0 Vicryl absorbable sutures. Two beads of either PRC, rbMSC, or PRC+rbMSC were inserted underneath the flap according to the respective treatment group. The remaining opening of the periosteal flap was then sealed using Tisseel fibrin glue (Baxter AG Industries, Vienna, Austria) and sutured (11-0 Vicryl absorbable suture). The wound was closed in layers using absorbable suture and bandages, followed by application of antiseptic solution to prevent infection. The left knees were left untreated (control group). All transplanted rabbits were treated with analgesic and antipyretic, Metacam® (Boehringer Ingelheim, Berks, UK) and Kombitrim (KELA Laboratoria NV, Hoogstraten, Belgium) for 3 consecutive days after the operation, both of which additionally act as broad-spectrum antibiotics for gram-positive and gram-negative bacteria.

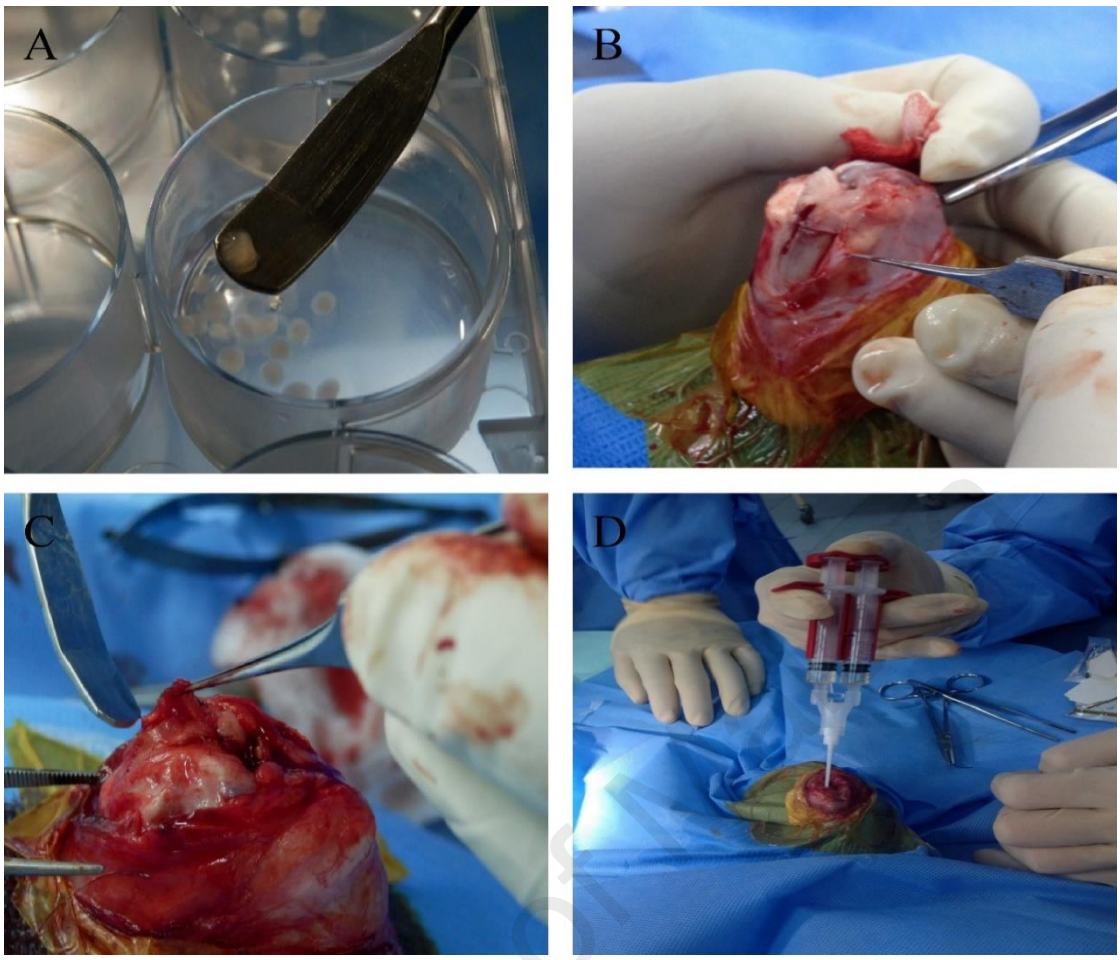

Figure 5.3: Transplantation of alginate beads into the defect site. (A) Prepared alginate beads. (B) Harvesting the periosteal flap. (C) Transplantation of the beads into the defect site. (D) Sealing the defect site with fibrin glue.

5.2.7 *In vivo* tracing of MSCs

To evaluate the fate of the transplanted MSCs, labelling was performed with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Vybrant CFDA SE Cell Tracer Kit [V-12883]; Molecular Probes, Invitrogen) as previously described (K. B. Lee, Hui, Song, Ardany, & Lee, 2007). Briefly, CFDA-SE was dissolved in dimethylsulfoxide, and this mixture was diluted with prewarmed PBS to obtain a working concentration of 5.0 M. Cells cultured *in vitro* were trypsinized and centrifuged to obtain a cell pellet. The supernatant was discarded and the cells were gently resuspended in the pre-warmed

(37°C) PBS containing the chemical dye. The mixture passively diffused into the cultured cells, and an intracellular chemical reaction occurred, causing the colourless and non-fluorescent dye components to form fluorescent dye-protein conjugates. After a 15-minute incubation period at 37°C, the cells were re-pelleted by centrifugation and re-suspended in alginate. The beads were then transplanted to the defect site.

After one month, the tissues were harvested and frozen on dry ice. It was then embedded in optimum cutting temperature compound (ThermoScientific, Denmark). The samples were then cut into 5 µm-thick sections using a cryotome FSE Cryostats (Thermo Scientific, Denmark) and mounted on polysine coated microscope slide (Thermo Scientific, Denmark). The dye-labelled cells were then observed by fluorescence microscopy (Nikon Eclipse Ti-U, Japan) for *in vivo* tracing.

5.2.8 Harvesting and gross examination of the regenerated tissues

After 3 and 6 months of implantation respectively, the knee joints of the rabbits were surgically removed and examined. For gross evaluation, all specimens were examined under direct light microscopy by 2 independent observers; both experienced orthopaedic surgeons, who were blinded to the sample groups. The observers were asked to examine and grade samples according to the modified component of the International Cartilage Repair Society (ICRS) Cartilage Repair Assessment scoring scale (macroscopic appearance subcategory) (Appendix H) (Brittberg, Nilsson, Lindahl, Ohlsson, & Peterson, 1996). Interobserver correlation analysis was performed to determine the reliability of the measurement. Upon completion of the scoring, the specimens were cut into half using a mechanical bone saw (Fein MultiMaster Accu, C & E Fein Gmbh, Stuttgart, Germany) according to the technique previously established by Kamarul et al.

(Kamarul, Ab-Rahim, Tumin, Selvaratnam, & Ahmad, 2011; Kamarul et al., 2008). Half of each specimen was fixed in 10% phosphate-buffered formalin (4% formaldehyde) for histology and immunostaining, while the other half was utilized for the analysis of glycosaminoglycan (GAG) content.

5.2.9 Histologic Examination and immunohistochemical staining

Specimens fixed in buffered formalin were decalcified in 10% formic acid, embedded in paraffin and sectioned into 5-µm thick sections, which were subsequently placed on glass slides, dried overnight, and stored at 4ºC. The sections were further deparaffinised in xylene and dehydrated in ethanol in a stepwise manner from 70% up to 100%. The samples were stained with hematoxylin and eosin for general morphologic evaluation and safranin O – fast green to detect proteoglycan. The O'Driscoll cartilage scoring system (Appendix I) was used for histologic and histochemical assessment of the repaired cartilage specimens (O'Driscoll et al., 2001). Two independent histologists blinded to the group status performed the scoring. An interobserver correlation analysis was performed to determine the reliability of the method.

Immunohistochemical staining for collagen type II was performed using DAKO ARK™ peroxidase for mouse primary antibodies (DAKO, Denmark). The histological slides were incubated in primary antibody solution (anti–collagen type II, Merck Millipore, USA), followed by secondary antibody and substrate-chromogen solution in accordance with the manufacturer's instructions. The images were examined under the microscope (Nikon Eclipse Ti-U; Japan).

5.2.10 Glycosaminoglycan Assay

Protein and glycosaminoglycan (GAGs) were determined using Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Alfred Nobel Drive, Hercules, CA) and Blyscan sulfated glycosaminoglycan assay kit (Biocolor Ltd, County Antrim), respectively. Specimens were dissected into small pieces using a scalpel and digested using papain (Worthington, USA) at 65°C overnight; the assay was performed according to the manufacturer's protocol. Aliquots of each sample were mixed with dimethylmethylene blue (DMMB) dye and reagents (Biocolor). The amount of GAG was quantified by measuring the absorbance of the aliquots at 656 nm using a spectrophotometer and extrapolating their levels from a standard plot of chondroitin sulphate provided by the manufacturer.

5.2.11 Statistical Analysis

Statistical analysis was performed using SPSS version 17 (SPSS Inc, Chicago, IL, USA). A paired t-test was used to compare the platelet number in whole blood and PRC considering that the sample was obtained from the same animal. The values of morphological Brittberg and histological O'Driscoll scores, as well as GAG concentrations for all tissue samples were presented as mean ± standard deviation. Shapir-Wilk test was used to test normality of the samples. All data were normally distributed, hence, parametric analysis were performed. One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to determine the significant differences between the 3 studied groups. Independent sample t-test was used to determine the significance in the quantitative results between 3 and 6 months within each study group.

Intraclass correlation coefficient analysis was performed to determine the interobserver correlation coefficient for the ICRS and O'Driscoll scores. P-values of less than 0.05 were considered significant.

5.3 RESULTS

5.3.1 Yield of platelets in PRC prepared from rabbits' blood

The mean platelet concentration in rabbit whole blood was 273 ± 8 × 10^3/µl. The mean platelet concentration in PRC was 1399 ± 68 × 10^3/µl; this represented a 5-fold higher platelet count in the PRC compared to whole blood (p = 0.001) (Table 5.1).

Table 5.1: Concentration of various cell types in rabbit whole blood and PRC.

Variable	Whole blood	PRC
Platelets × 10^3/µ L	273±8	1399±68*
Leukocytes × 10^3/µ L	4.8±0.3	0.48±0.1*
RBCs × 10^6/µ L	4.40±0.47	0.04±0.02*

Denotes statistically significant differences ($p < 0.05$) between the variables in the same row. Data are represented as mean ± SD of five independent experiments.

5.3.2 Verification of rbMSC characteristics

Cells were stained positive for MSC surface markers CD44 and CD29 (Figure 5.4). No staining for the hematopoietic lineage marker, CD45, was detected in the cells.

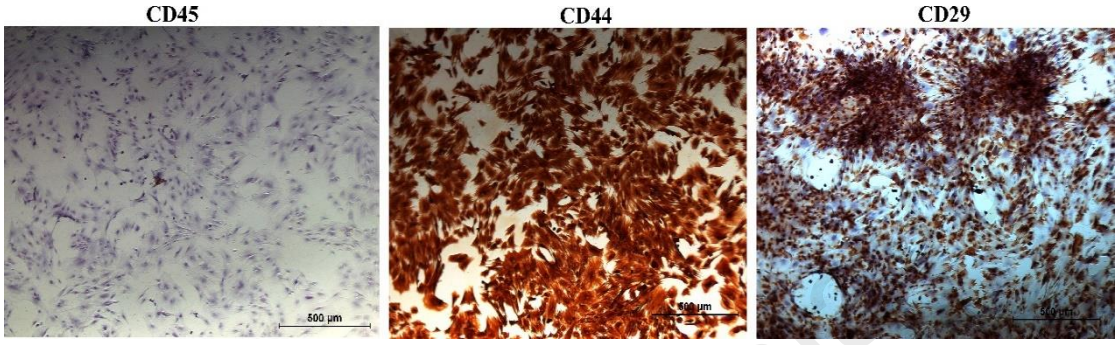

Figure 5.4: Immunocytochemical staining for cell surface markers. Brown colour indicates positive staining. The cells were also counterstained with haematoxylin. Scale bar: 500 µm.

The tri-lineage differentiation results further confirmed that the cells were MSCs (Figure 5.5). Adipogenesis was confirmed by the presence of fat droplets stained red with the oil red O stain (Figure 5.5A). The presence of calcium mineralization stained red with alizarin red S stain, confirmed differentiation of the cells to osteogenic lineage (Figure 5.5B). Cells grown in pellet culture produced cartilage extracellular matrix rich in sulphated glycosaminoglycan, which was stained red with the safranin O stain (Figure 5.5C).

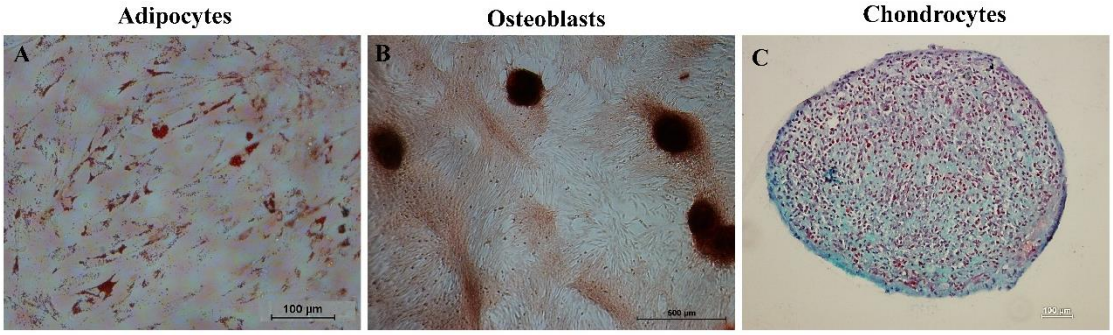

Figure 5.5: Tri-lineage differentiation potential of cells isolated from the bone marrow. (A) The oil droplets produced by adipogenic cells were stained red with oil red O stain, while the nucleus was counter stained with haematoxylin (scale bar: 100 µm). (B) Calcium deposits in osteogenic cells were stained red with alizarin red S stain (scale bar: 500 µm). (C) Proteoglycan synthesized by chondrogenic cells were stained red with safranin O/fast green (scale bar: 100 µm). Figure shows the representative images of the experiments.

5.3.3 Verification of the presence of rbMSCs at the defective site

Fluorescent images of the cartilage tissue sections showed the presence of CFDA-SE-labelled rbMSCs at the defective site, one month after transplantation (Figure 5.6).

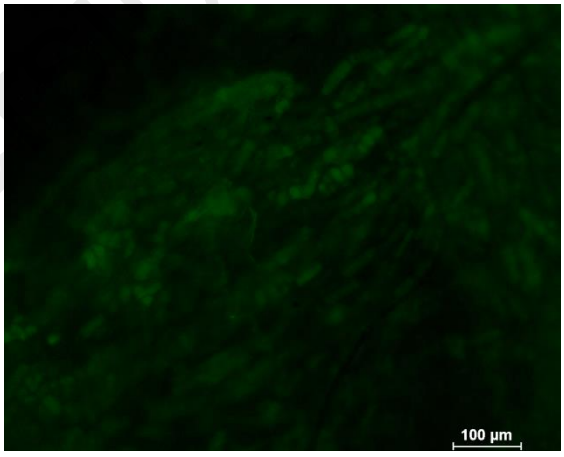

Figure 5.6: *In vivo* tracing of the transplanted cells. Fluorescent microscopy images showed the presence of transplanted rbMSCs at the defective site. Scale bar = 100µm.

5.3.4 Morphology of the regenerated tissues

The gross morphological appearances of the cartilage tissues assessed at 3 and 6 months post-transplantation are shown in Figure 5.7A. Macroscopic evaluation performed by two independent observers was found to be highly correlated. The correlation coefficient values at 6 months were as follows: PRC group = 0.833 (p = 0.02), rbMSC group = 0.800 (p = 0.028) and PRC+rbMSC group = 0.909 (p = 0.006). By 6 months, the tissues in PRC+rbMSC-treated group exhibited smooth glistening surface with less distinct demarcation of the defect border peripherally. In contrast, the regenerated tissues in the untreated knee had rough surfaces and very distinct wound margins. The mean ICRS scores of all treatment groups were higher at 6 months compared to 3 months post-transplantation (PRC group: p=0.008, rbMSC group: p=0.007, PRC+rbMSC group: p= 0.002). All treatment groups had significantly higher ICRS scores compared to the non-treated knee. At 6 months post-transplantation, the PRC+rbMSC group had significantly higher mean ICRS score (9.8 ± 0.8) compared to the PRC (8.0 ± 0.7) (p = 0.005) and rbMSC groups (7.4 ± 0.5) (p = 0.001) (Figure. 5.7B). There was no significant difference in the ICRS scores between the treatment groups receiving only PRC and rbMSCs.

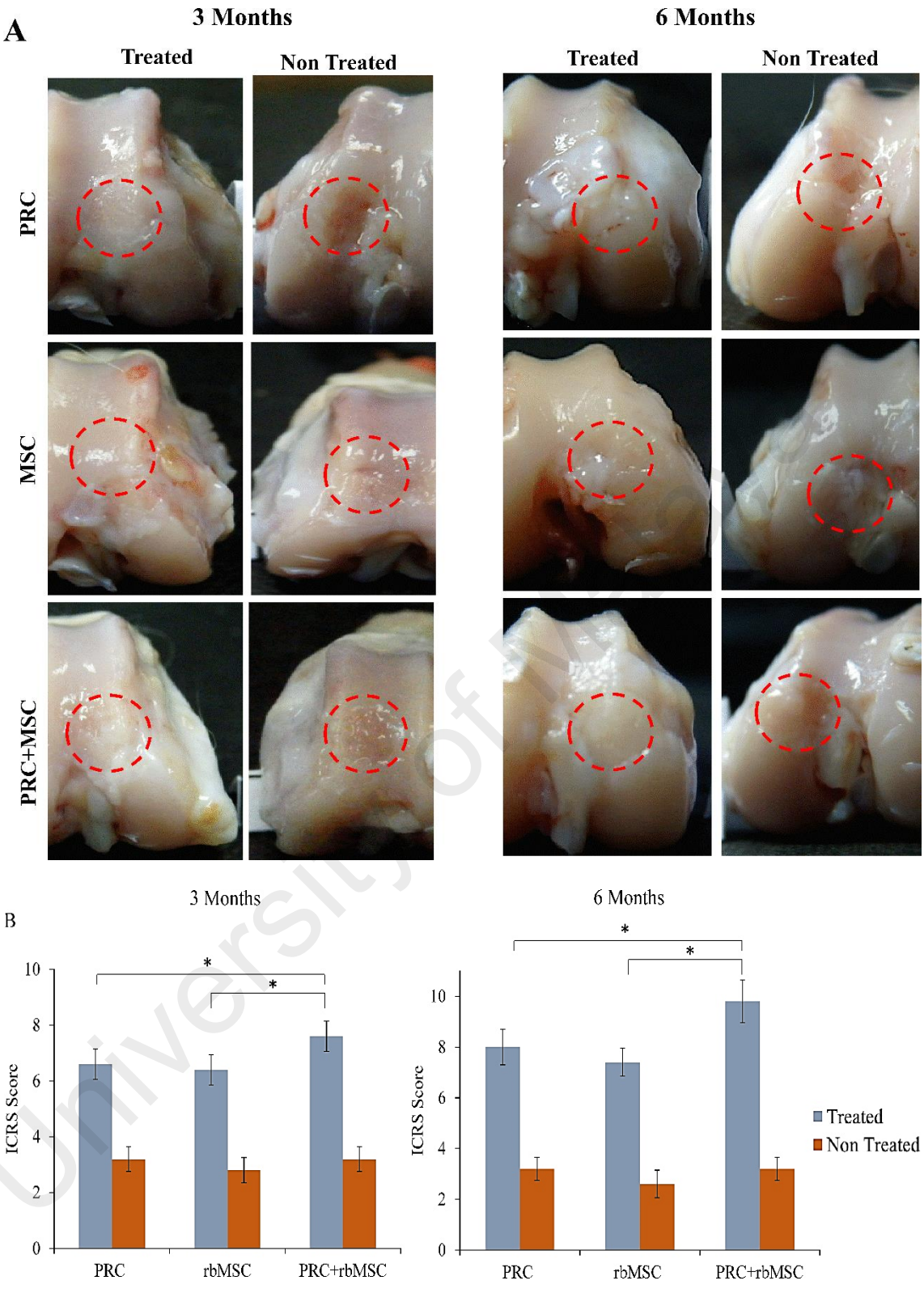

Figure 5.7: Morphological appearance and scoring of the harvested knees. (A) Macroscopic appearance of samples show completely filled defect in the PRC+rbMSC group with good integration to the surrounding healthy cartilage, while samples from non-treated knees showed an irregular surface. (B) ICRS scores were higher in the PRC+rbMSC group at both 3 and 6 month time point. Data are represented as mean ± SD. *p< 0.05 denotes statistical significance.

5.3.5 Histological staining of the regenerated tissues

Microscopic evaluation using the O'Driscoll scoring performed by the two independent observers was also found to be highly correlated. The correlation coefficient values at 6 months for the PRC group = 0.889 (p = 0.022), rbMSC group = 0.949 (p = 0.007) and PRC+rbMSC group = 0.903 (p = 0.018). Safranin O and haematoxylin–eosin (H & E) staining of the regenerated tissues in the PRC, rbMSC and PRC+rbMSC groups at 3 and 6 months post-transplantation are shown in Figure 5.8. The regenerated tissues in the PRC+rbMSC group were heavily stained with safranin O stain, reflecting higher proteoglycan production at both time points compared to the PRC and rbMSC groups. The tissue appeared to be hyaline-like with good thickness, surface regularity and integration with native tissues. In the PRC+rbMSC group, intense safranin O stain staining the chondrocyte extracellular matrix was seen by 6 months post-transplantation (Figure 5.8). On the contrary, the tissue in the defective site of the non-treated knee had less cell density and was characterized by minimal matrix staining by the safranin O stain. O'Driscoll scores of each group are shown in Figure 5.9. In the PRC group, no significant difference in the scores was observed between the two time points of assessment (p = 0.168). At both time points, the PRC+rbMSC group had significantly higher scores than the PRC and rbMSC groups alone. By 6 months, the scores were 17.2 ± 0.9 for the PRC+rbMSC groups compared to 13.3 ± 1.5 for the rbMSC group (p = 0.015) and 10.5 ± 1.7 for the PRC group (p = 0.002).

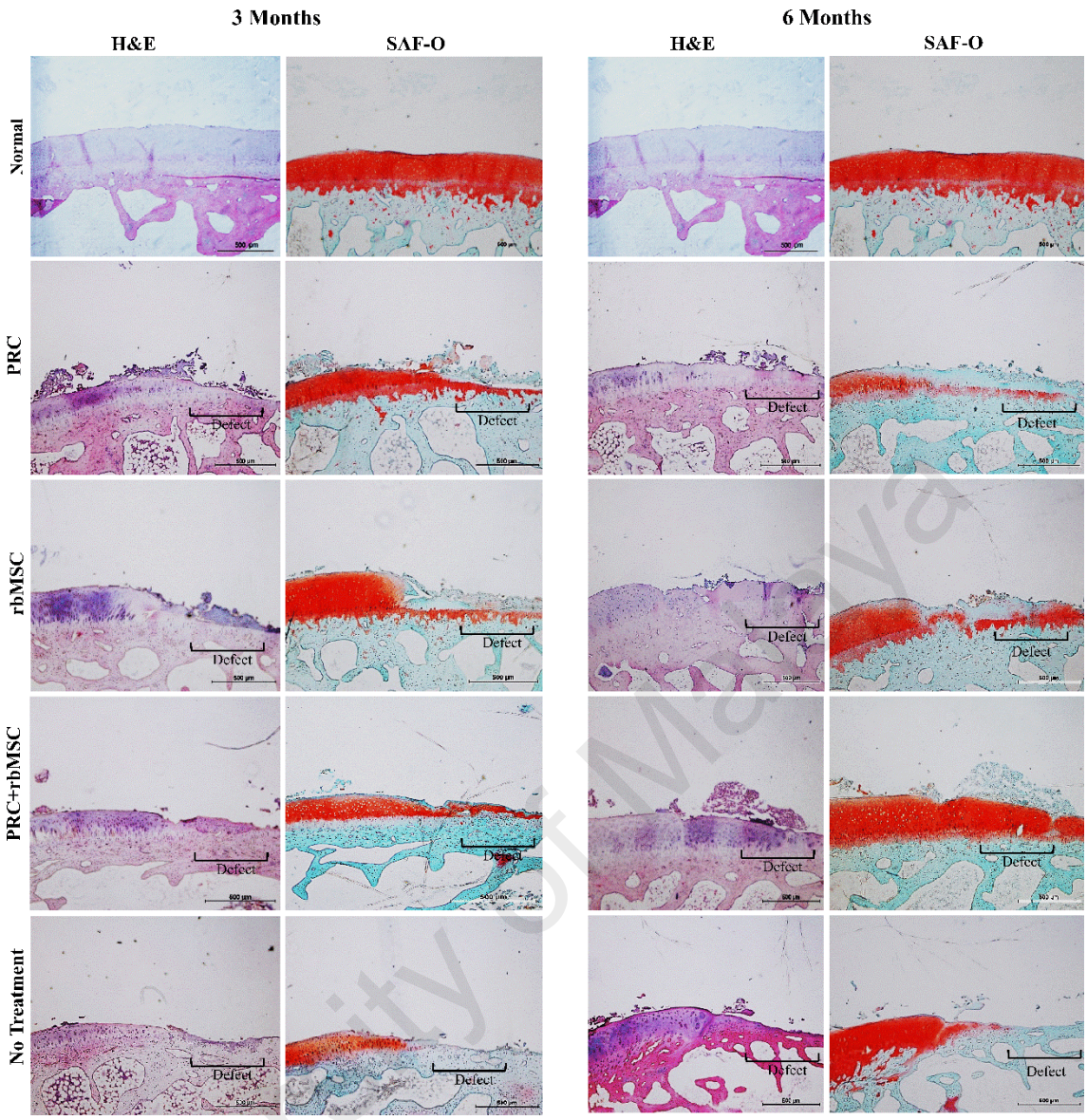

Figure 5.8: Histological staining of stained tissues. Representative sections of the regenerated tissues stained with safranin O (saf-O) and hematoxylin and eosin (H&E) showing intense staining, high cellularity and hyaline-like structure in the PRC+rbMSC group. Safranin O staining was absent in the sections from the non-treated knee, indicating the lack of regeneration. Scale bar = 500 µm.

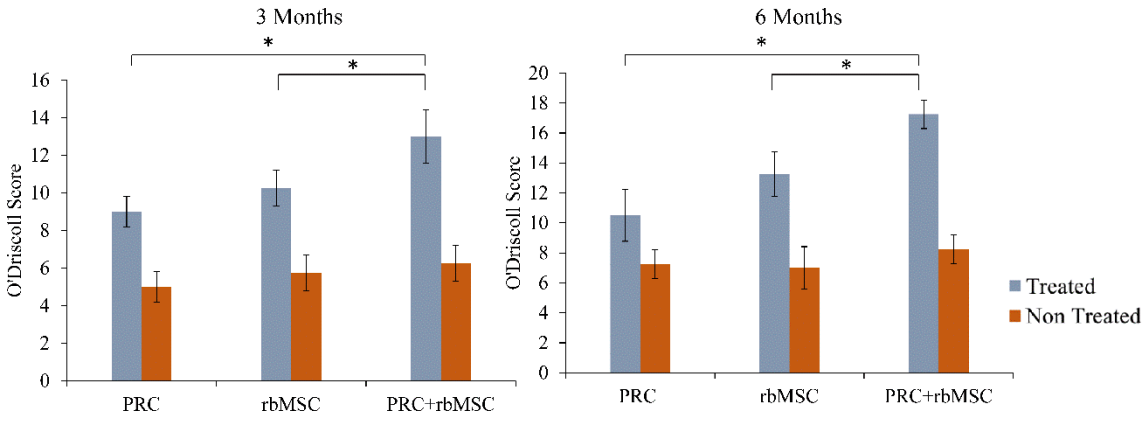

Figure 5.9: Histological scoring of stained tissues. O'Driscoll scores in the PRC+rbMSC group showed 27% and 44% higher scores compared to rbMSC treated groups and PRC treated groups respectively at 3 month time point. After 6 months, it showed a 30% and 64% higher scores compared to rbMSC treated groups and PRC treated groups respectively. Data are represented as mean ± SD. *$p < 0.05$ denotes statistical significance.

5.3.6 Immunohistochemical staining for collagen type II

There was a stronger collagen type II staining in the repaired tissues in the PRC+rbMSC group compared to other groups at 3 and 6 months post-transplantation. In the non-treated knees, no cartilage filling was noted and only some collagen type II stain was seen at the base and border of the defects (Figure 5.10).

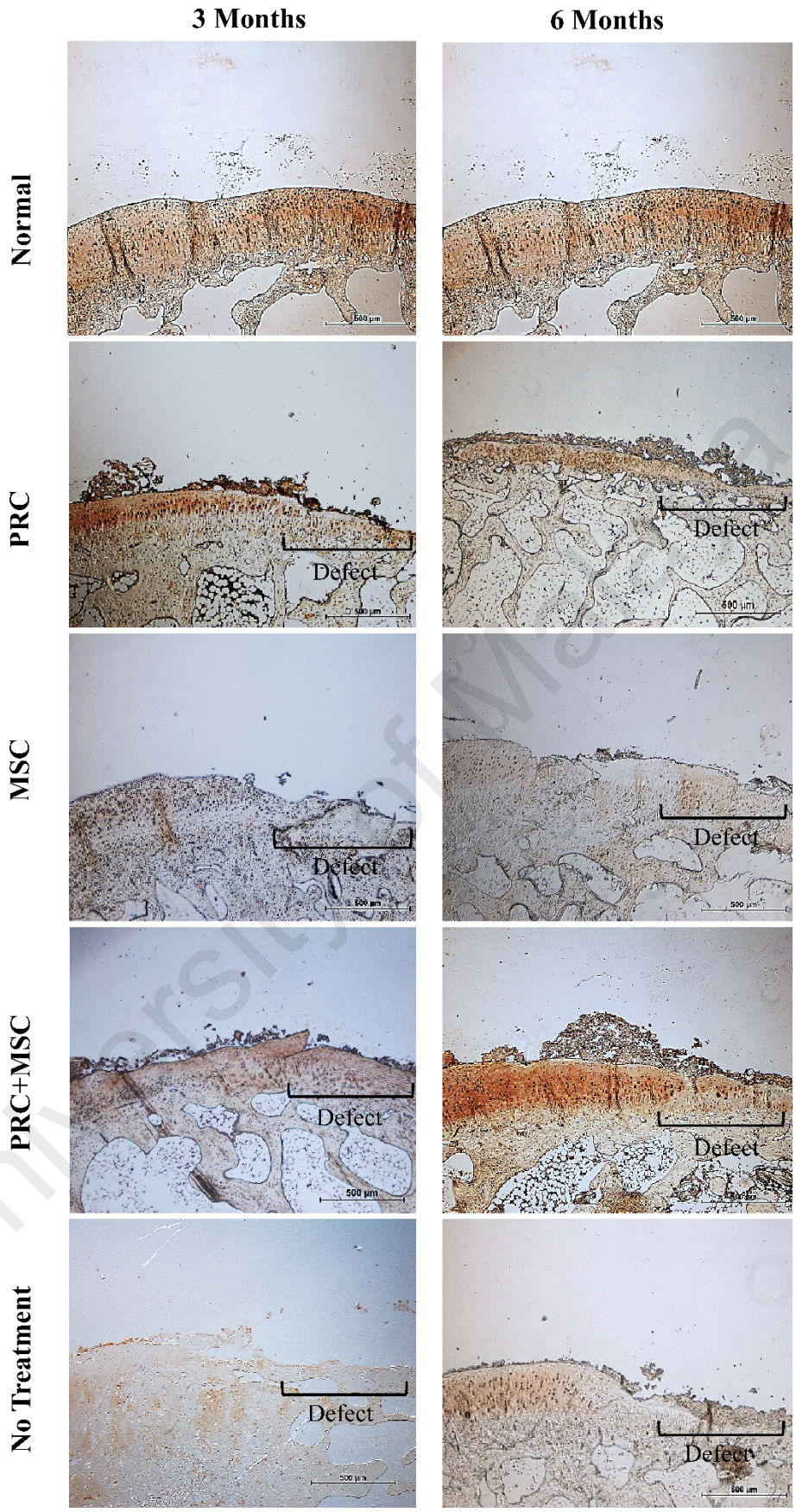

Figure 5.10: Immunohistochemical staining of tissues. Staining of representative sections of the regenerated tissue for collagen type II showing intense staining (stained brown) in the PRC+rbMSC group indicating higher collagen type II content. Scale bar = 500 µm.

5.3.7 Concentration of glycosaminoglycan in the regenerated tissues

GAG content was significantly higher at 6 months compared to 3 months in all treatment groups (PRC group: p=0.002, rbMSC group: p=0.002, PRC+rbMSC group: p=0.003). The mean content of GAG/total protein (µg/mg) were also higher in the PRC+rbMSC-treated knees compared to PRC or rbMSC groups at both time points of evaluation (Figure 5.11), consistent with the morphological and histological scores. Six months after treatment, GAG concentration in the PRC+rbMSC group was 3.4 ± 0.3 µg/mg compared to the rbMSC group (2.6 ± 0.2 µg/mg) (p = 0.030) and PRC group (2.1 ± 0.2 µg/mg) (p = 0.002).

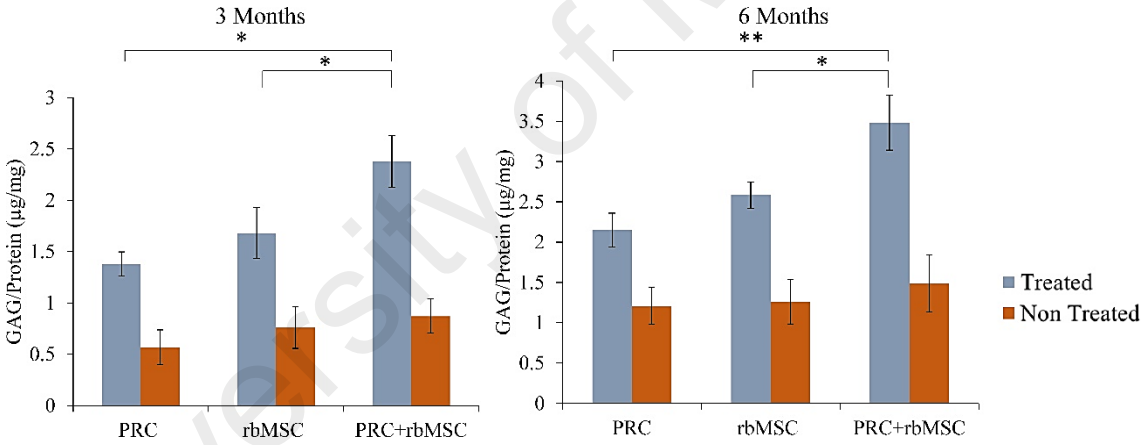

Figure 5.11: Concentration of glycosaminoglycan in the harvested tissues. Glycosaminoglycan/protein content was higher in the PRC+rbMSC compared to the PRC and rbMSC groups at 3 and 6 months post-transplantation. Data are represented as the means ± SD. *p < 0.05 and **p<0.005 denotes statistical significance.

5.4 Discussion

The results of the present study indicate that PRC, rbMSCs and PRC+rbMSCs encapsulated in alginate were efficacious in improving *in vivo* cartilage repair in a focal cartilage defect animal model. Treatment with PRC alone appeared to be equally effective as rbMSCs in filling in the defect; more importantly, the concurrent use of PRC with rbMSCs produced superior cartilage healing compared to PRC or rbMSCs alone in this animal model. This is reflected by significant improvements of the repair outcomes ranging from macroscopic and histological appearance of the regenerated tissue to the amount of extracellular matrix protein (glycosaminoglycan) being produced. In addition, the extracellular matrix of the regenerated tissue in the PRC+rbMSC group was intensely stained with safranin O as well as for collagen type II, indicating regeneration of hyaline-like cartilage at the defective site.

In our study, the use of non-activated PRC alone without any confounding effect of other marrow stimulation techniques was able to induce cartilage repair. Although the mechanism has not been explicitly delineated in this study, this outcome may be attributed to the reparative role of numerous growth factors from the platelets in inducing resident chondrocytes near the defect site to undergo proliferation, migration and matrix synthesis. Many of these growth factors, including TGF-β and FGF regulate chondrocyte metabolism and chondrogenesis. Studies have shown that TGF-β stimulate the expression of cartilage matrix genes, resulting in increased synthesis of proteoglycan and collagen type II in the monolayer culture of chondrocytes (Smith et al., 2000; Trippel, Ghivizzani, & Nixon, 2004). Cuevas et al. also showed that growth factor FGF enhanced the healing of intra-chondrial lesions *in vivo* by stimulating chondrocyte proliferation and formation of extracellular matrices (Cuevas, Burgos, & Baird, 1988).

The combination of PRC and rbMSCs encapsulated in alginate produced superior healing of the cartilage as compared to other groups. The presence of proinflammatory cytokines such as TNF-α, IL-1α or IL-1β found in injured tissues can stimulate MSCs to release high levels of immunosuppressive factors such as TGF-β1 and HGF (Ma et al., 2014). Both TGF-β1 and HGF work together to suppress T cell proliferation and modulate the immune response (Seo & Jung, 2016). Study by Copland et al also showed that the immune potential of MSCs was enhanced when cultured in fibrinogen-depleted PRP (Copland, Garcia, Waller, Roback, & Galipeau, 2013). MSCs also have ability to influence the fate of other cell types through paracrine signalling. It has been reported that MSCs can secrete many growth factors and proteases like VEGF, HGF, IGF1, bFGF, TGF-β, and PDGF, which help in the repair of injured cells and tissues (Xiang, He, Wang, & Gui, 2009). These growth factors are also present in platelets, and hence, it can be speculated that PRC can enhance the immune response and the paracrine activity of MSCs.

Study by Xie et al. showed that when introduced concomitantly, the presence of PRP in close proximity to the transplanted MSCs resulted in enhanced chondrogenic differentiation of the transplanted MSCs (Xie et al., 2012). Enhanced healing may have also been attributed to the stimulatory effect of the growth factors in PRC, providing a favourable environment for survival, proliferation, and chondrogenic differentiation of the transplanted rbMSCs. Previous studies have shown that efficacy of stem cell therapies are often limited by two critical factors, which include: i) lower survival capacity of the transplanted cells due to being exposed to harsh acidic, hypoxic and avascular microenvironment with high levels of cytokine, most notably IL-1 and TNFα at the defect site, and, ii) lack of control over their differentiation into the terminal cell types, which is needed to replace the lost tissues (Hua, Liu, Tao, & Yang, 2015; Rodrigues, Griffith, &

Wells, 2010). Since platelet growth factors such as TGF-β are known to stimulate MSC proliferation, protect the cells from apoptosis and induce expression of chondrogenic markers in pellet cultures (Longobardi et al., 2006), the use of PRC in combination with rbMSC may help to overcome both of these challenges to achieve a greater therapeutic outcome. Apart from stimulating the rbMSC, the growth factors released from PRC may also have a direct effect on the resident chondrocytes at the defect site as discussed previously. Hence, stimulation of both the rbMSC and resident chondrocytes may have produced a synergistic effect, leading to more superior repair outcomes seen in the PRC+rbMSC group. In addition, it has previously been shown that when MSCs were co-cultured with chondrocytes, the chondrocyte-derived soluble factors could direct the chondrogenic differentiation of MSCs (Fischer, Dickhut, Rickert, & Richter, 2010). Therefore, in this study, it is plausible that soluble growth factors released by the resident chondrocytes could have also further induced the chondrogenic differentiation of the transplanted rbMSCs probably through their paracrine effect.

Although the specific mechanism of action of MSCs in cartilage repair has not yet been clearly defined, a number of mechanisms have been proposed to be involved, which include establishment of cell-cell contact, paracrine signalling, chondrogenesis, immunomodulatory activity, or a combination of these mechanisms. MSCs are known to actively secrete cytokines, chemokines, and growth factors that work as paracrine signals (Elnakish et al., 2012; Wiafe, Metcalfe, & Adesida, 2015) that aid in tissue repair. This may explain the effectiveness of rbMSCs alone in regenerating cartilage, as seen in our study.

In the present study, the regenerative promoters were encapsulated in alginate beads. This offers a number of additional benefits as previous report has indicated that alginate

protects the transplanted cells from the host immune system (Buzhor et al., 2014) apart from providing an ideal microenvironment to induce chondrogenesis. Study also shows that the use of alginate increases bioavailability and the clinical efficiency of PRP and enables localised sustained release of the mitogenic and angiogenic stimuli at the site of injury (Lin et al., 2006). The growth factors contained in PRP preparation administered via direct intra-articular injection have been speculated to be rapidly degraded *in vivo*, whereas a more sustained release of growth factors from the platelets could be expected when PRP is incorporated in microspheres such as alginate beads, resulting in better cartilage repair (Saito et al., 2009). Another potential drawback of direct intra-articular injection of PRP is a high possibility of the platelets being dispersed after the injection. This might lead to an insufficient amount of mitogenic and angiogenic stimuli being delivered at the defect site (Bornes et al., 2014). Although this study did not determine the effect of alginate alone on cartilage regeneration, it is expected that alginate will not significantly contribute to cartilage repair, as evident in a previous study (Dashtdar et al., 2015).

Despite our attempt to produce a robust experimental design, this study has limitations that are worth noting. First, autologous MSCs might have been a better treatment option instead of allogeneic sources since it has been suggested that chondrogenic MSCs from an allogeneic source increased immunogenicity and anti-donor immune response of the MSCs (Ryan et al., 2014). However, harvesting MSCs from the same animal can only be performed in larger animal models. Extraction of autologous MSCs from rabbit bone marrow would have been too challenging due to the small size of the animal. Considering that the use of allogeneic MSCs has been adopted in previous studies (Dashtdar et al., 2015; Tay et al., 2012), similar approach taken in this present study would be considered valid. Second, although we demonstrated that the technique used to enrich the platelet

concentrate provided approximately five-fold higher concentration of platelet than that of whole blood, which is already considered an optimal level for PRC to be therapeutically effective (Everts et al., 2006), the concentration of growth factor(s) in the PRC were not specifically measured. It is probable that the exact concentrations of the numerous growth factors in PRC may differ between individuals. Nevertheless the mitogenic and differentiation inducing factors was still present in relatively higher proportion in most PRC preparation. In view of the lack of studies that have investigated the extent of healing produced by the synergistic action of PRC and MSCs in an animal model of chondral defect, this study is valuable in providing proof of concept that may advocate transplantation of PRC and rbMSCs encapsulated in alginate as a treatment modality for *in vivo* cartilage repair. A notable advantage of this study is the fact that the PRC was not pre-activated *in vitro*, and hence, it is expected that the growth factors would have been retained within the platelets until they are transplanted into the defect site. Once transplanted, the platelets would have to be activated *in vivo* when it comes in contact with collagen found abundantly in musculoskeletal tissues. This gradual *in vivo* activation of platelets is advantageous as it results in a more sustained release of the growth factors (Mishra et al., 2009).

In conclusion, the findings of this study indicate that transplantation of PRC and rbMSC produced synergistic effects that resulted in superior cartilage repair compared to treatment with PRC or rbMSC alone. PRC appears to enhance the regenerative potential of MSCs *in vivo* to further improve the repair of full-thickness cartilage defect. Hence, transplantation of PRC and MSCs encapsulated in alginate could be further developed as an alternative approach to effectively treat focal cartilage lesions in clinical settings.

CHAPTER 6: SUMMARY AND CONCLUSION

6.1 Summary of the findings and general discussion

The use of MSCs for repair and regeneration of damaged tissues holds a great promise. However, their wide application is currently limited by the need for greater expansion of the pre-differentiated cells that are free from xenogenic components. PRC has recently emerged as a potentially valuable adjunct that could considerably enhance the therapeutic potential of MSC, addressing the aforementioned limitation. However, several questions remain to be answered in order to further develop the potential use of PRC in clinical practice. This thesis aims to investigate the effect of PRC in serum-free medium on the proliferative and differentiation potential of MSCs *in vitro* and the regenerative potential of MSC to repair tissue *in vivo,* in which a rabbit model of focal cartilage defect was used as a working model.

Several optimisation was initially made in the preparation of the PRC in order to study its effect in the most optimal culture condition. The results indicate that a single application of PRC in serum-free medium at an optimal dose of 15% was able to increase proliferation and direct early differentiation of hMSCs without the aid of external lineage-specific growth factors in the culture medium within 8 days of culture. Activation of PRC prior to its *in vitro* application was not a pre-requisite to achieve the proliferative and differentiation effect.

Having established the positive effects of PRC on proliferation and differentiation of hMSCs in a short-term culture, we investigated its effect on a longer term culture (24 days). The results showed that 15% non-activated PRC could induce osteogenic,

chondrogenic and adipogenic differentiation of hMSCs despite its infrequent supplementation. When compared to commercially available lineage-specific media, PRC appeared to induce greater osteogenic and chondrogenic differentiation compared to the respective standard differentiation media. However, the extent of adipogenic differentiation in the PRC group was lesser compared to standard adipogenic medium. Furthermore, the expression of hypertrophic markers in the PRC group was lower compared to cells cultured in commercially available chondrogenic medium, suggesting that supplementation of PRC offers additional benefit of potentially regenerating cartilage without formation of endochondral ossification.

The final part of this study evaluated the potential of PRC in enhancing the regenerative capacity of MSCs in the *in vivo* setting, in which a rabbit model of full thickness focal chondral defect was employed as a working model. In order to determine the extent of enhancement of MSC repair potential by PRC, the contribution from the individual therapeutics was compared to the combination of the two. The results showed that transplantation of PRC+rbMSC in combination produced superior repair of cartilage defects compared to PRC or rbMSC group.

The results support the hypothesis outlined in this thesis and indicate that PRC is sufficiently potent to enhance MSC regenerative potential both in the *in vitro* and *in vivo* settings, without the need for concurrent supplementation of external mitogenic factors such as those in fetal calf serum or other lineage-specific differentiation medium. The absence of the requirement to activate platelets prior to their application is an advantage as it provides greater ease of preparation without the need to add bovine thrombin, which has potential immunological and haematological side effects (Lawson, 2006). The use of non-activated platelets could also result in a longer therapeutic effect as the growth factors

would be gradually released and accumulated within the physiological environment *in vivo* to exert a more sustained actions (Mishra et al., 2009).

In general, the enhanced cell proliferation and differentiation of MSCs induced by PRC as shown in this study are most likely due to the presence of various growth and differentiation promoting factors in PRC such as TGF-β, PDGF AA, PDGF BB, PDGF AB, BMP, FGF, and VEGF. However, the factors involved and their contribution in regulating MSC proliferation and differentiation are yet to be investigated. The observation of PRC-induced tri-lineage differentiation *in vitro* raises a concern regarding the directionality of cell differentiation *in vivo*. It is speculated that when used *in vivo*, growth factors released from platelets influence the fate of surrounding cells through the process of paracrine signalling and they also provide a pro-healing microenvironment (Rubio-Azpeitia & Andia, 2014). Hence, growth factors released from PRC when transplanted at the cartilage defects, stimulates the surrounding chondrocytes and enhances the cartilage repair. As mentioned in the previous chapter, growth factors present in platelets like TGF-β and FGF regulate chondrocyte metabolism by stimulating the expression of cartilage matrix genes, resulting in increased synthesis of proteoglycan and collagen type II in chondrocytes (Cuevas et al., 1988; Smith et al., 2000; Trippel et al., 2004). When PRC is used for the treatment of bone defects, they have the potential to influence osteoblast and initiate early bone healing by stimulating matrix production and bone remodelling (Oryan, Alidadi, & Moshiri, 2016). The growth factor BMP present in platelets is known to be osteoinductive and also required for early bone development. It increases bone formation by driving osteoblast differentiation and stimulating angiogenesis in the developing bone (Zhang et al., 2009a).

Results of the *in vivo* study highlight the value of using PRC and MSCs to enhance cartilage repair. Supplementation of PRC alone may have promoted cartilage repair through paracrine effect of the growth factors in PRC, which regulates the proliferation and ECM production of the resident chondrocytes surrounding the defect. On the other hand, cartilage regeneration after transplantation of MSCs alone could be attributed to both the chondrogenesis undergone by MSCs as well as the release of cytokines and growth factors from MSCs to the surrounding tissues. Ideally, this would have contributed to the tissue replacement and repair. Therefore, it is expected that the combination of PRC and MSCs together would confer the cumulative benefit of the individual therapeutics. When used in combination, PRC has the ability to enhance tissue repair through a number of potential mechanisms. These most likely include enhancement of MSC proliferation and chondrogenesis by the growth factors released from platelets, direct paracrine effect of platelet growth factors on the surrounding tissues, and release of growth factors from MSCs themselves into the surrounding tissues. These synergistic actions could therefore provide favourable environment for more superior cartilage repair. This therapeutic strategy would also be applicable to enhance repair of other injuries.

The novelty of this study lies on the fact that, unlike previous studies that implied the need for an additional supplementation of differentiation media in order for PRC to exert its effect, this study involved the use of only PRC in serum-free medium, enabling determination of its absolute effect, without any confounding factors. The approach of supplementing PRC at a longer interval (every 8 days), to induce osteogenic differentiation renders a benefit of avoiding unnecessary and frequent acquisition of large volume of blood for PRC preparation. This would be a more practical approach for potential application of PRC in clinical settings. An additional strength of the study is related to the creation of defect in the animal model. In the *in vivo* chondral defect model,

the injuries were limited to the chondral surface and were not extended into the sub-chondral bone plate. This allows determination of the therapeutic outcome of PRC+MSC transplantation on cartilage repair, without confounding effects from marrow stimulating procedures.

6.2 Clinical implications

The protocol for PRC-induced cellular expansion and differentiation would provide sufficient amount of pre-differentiated cells, which are devoid of any xenogenic components, as part of a novel treatment strategy for tissue repair. Less volume of blood would be required from patients for PRC isolation, as the study showed that PRC was able to exert favourable effects even when infrequently supplemented. Results of the *in vivo* study imply that transplanting PRC+MSC encapsulated in alginate can be considered as an alternative treatment modality in the clinical setting for full-thickness chondral defects, such as those acquired during sports injury. These kind of injuries are prevalent amongst individuals participating in high impact joint loading sports activities. Since in this study the mixture of PRC and MSC are encapsulated in alginate, the growth factors in PRC would be allowed to accumulate and exert their effect on the cells without any interference, as the entire 3D construct would be protected from the harsh microenvironment at the defect site. The synergistic action of all these factors would be beneficial to provide a better cartilage healing in clinical settings. Finally, transplantation of the two regenerative promoters i.e. PRC and MSC for the repair of full-thickness defects could be done in a one-step surgery since there is no need for harvesting of autologous cartilage to isolate chondrocytes. This would reduce donor-site morbidity. In general, the findings of this study therefore have an implication of widening treatment options particularly for patients in the field of sports medicine.

6.3 Future Work

The present study can be further extended in several directions. First, it would be interesting to understand the underlying mechanism of PRC and identify the major growth factor contributing to the positive effects observed in this study. Cells cultured in PRC can be treated with recombinant noggin, which is a BMP-2 antagonist. After 24 days, the level of gene expression of osteogenic markers in the treated cells would clarify the role of BMP2 growth factor and whether PRC exerts its effect on osteogenesis through the BMP signalling pathway.

In addition, the role of PRC on chondrogenic differentiation of hMSC can also be determined by blocking Smad2/3 or Smad1/5/8 phosphorylation in PRC-treated hMSC culture by the addition of SB-505124, which inhibits the TGF-β type I receptor serine/threonine kinase known as activin receptor-like kinase (ALK). After 24 days, the cell lysates can then be used for gene expression analysis to demonstrate the subsequent changes in the temporal pattern of expression of chondrogenic markers.

Further studies are also needed to elucidate the mechanism of action of the combination of PRP and MSC on chondral defects *in vivo* before translating this model to clinical practice. Analysing the differential expression of the chondrogenic proteins in the regenerated tissues in the PRC+MSC and MSC groups could provide an insight on the cellular processes that might have been altered by PRC, which could have resulted in the enhanced tissue regeneration. This can be done using Marix-Assisted Laser Desorption Ionization (MALDI) Tissue-TOF. The regenerated tissue sections can be stained by H&E or immunohistochemistry and coregistered to the mass spectrometric analysis. The mass signals (*m/z*-species) detected can be visualized as colour intensity maps assigning

molecular patterns to cell types. These colour signals allow the detection of patterns, which represent the distribution of molecules of interest in the tissue. This also helps in analysing the spatial distribution of proteins. Also, stiffness tests to determine the biomechanical properties of the repair tissue could be carried out as part of a multi-faceted functional assessment of the repaired tissue.

6.4 Conclusions

In conclusion, the work presented in this thesis fulfilled all of the study objectives indicating that a single application of PRC in serum free medium could enhance proliferation and induce early differentiation of hMSCs to osteogenic, adipogenic and chondrogenic lineages, without the aid of additional growth factors in the culture medium. PRC was also able to maintain cell proliferation and induce complete tri-lineage differentiation of hMSCs during a longer culture period of 24 days, and during this course of differentiation period, the effect of PRC in promoting osteogenesis and chondrogenesis was superior compared to the standard lineage-specific media. PRC also has a better capacity to delay chondrogenic hypertrophy compared to the standard differentiation inducing media. When its effect was studied *in vivo,* PRC could enhance the regenerative potential of allogeneic MSCs and augment the repair of articular cartilage damage in a rabbit model of focal full thickness cartilage defects. Thus, transplantation of PRC and MSC encapsulated in alginate could be considered as a potential option to effectively treat focal cartilage lesions in clinical settings. However, more work is required to further verify the effect of PRC and delineate its mechanism of action before application of PRC can be fully adopted in clinical practice.

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LIST OF PUBLICATIONS

1. **Samuel S**, Ahmad RE, Ramasamy TS, Karunanithi P, Naveen SV, Murali, MR, Abbas AA, Kamarul T. 2016. Platelet-rich concentrate in serum free medium enhances osteogenic differentiation of bone marrow-derived human mesenchymal stromal cells. PeerJ 4:e2347; DOI 10.7717/peerj.2347 (ISI Indexed Publication).

2. **Shani S**, Ahmad RE, Naveen SV, Murali MR, Puvanan K, Abbas AA and Kamarul T. 2014. Platelet Rich Concentrate Promotes Early Cellular Proliferation And Multiple Lineage Differentiation Of Human Mesenchymal Stromal Cells In Vitro. Scientific World Journal. Article ID 845293, 12 pages, 2014. (*SCOPUS-Cited Publication*).

Platelet-rich concentrate in serum free medium enhances osteogenic differentiation of bone marrow-derived human mesenchymal stromal cells

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ABSTRACT

Previous studies have shown that platelet concentrates used in conjunction with appropriate growth media enhance osteogenic differentiation of human mesenchymal stromal cells (hMSCs). However, their potential in inducing osteogenesis of hMSCs when cultured in serum free medium has not been explored. Furthermore, the resulting osteogenic molecular signatures of the hMSCs have not been compared to standard osteogenic medium. We studied the effect of infrequent supplementation (8-day interval) of 15% non-activated platelet-rich concentrate (PRC) in serum free medium on hMSCs proliferation and differentiation throughout a course of 24 days, and compared the effect with those cultured in a standard osteogenic medium (OM). Cell proliferation was analyzed by alamar blue assay. Gene expression of osteogenic markers (Runx2, Collagen1, Alkaline Phosphatase, Bone morphogenetic protein 2, Osteopontin, Osteocalcin, Osteonectin) were analyzed using Q-PCR. Immunocytochemical staining for osteocalcin, osteopontin and transcription factor Runx2 were done at 8, 16 and 24 days. Biochemical assays for the expression of ALP and osteocalcin were also performed at these time-points. Osteogenic differentiation was further confirmed qualitatively by Alizarin Red S staining that was quantified using cetylpyridinium chloride. Results showed that PRC supplemented in serum free medium enhanced hMSC proliferation, which peaked at day 16. The temporal pattern of gene expression of hMSCs under the influence of PRC was comparable to that of the osteogenic media, but at a greater extent at specific time points. Immunocytochemical staining revealed stronger staining for Runx2 in the PRC-treated group compared to OM, while the staining for Osteocalcin and Osteopontin were comparable in both groups. ALP activity and Osteocalcin/DNA level were higher in the PRC group. Cells in the PRC group had similar level of bone mineralization as those cultured in OM, as reflected by the intensity of Alizarin red stain. Collectively, these results demonstrate a great potential of PRC alone in inducing proliferation of hMSCs without any influence from other lineage-specific growth media. PRC alone has similar capacity to enhance hMSC osteogenic differentiation as a standard OM, without changing the temporal profile of the differentiation process.

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Research Article

Platelet Rich Concentrate Promotes Early Cellular Proliferation and Multiple Lineage Differentiation of Human Mesenchymal Stromal Cells *In Vitro*

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Platelet rich concentrate (PRC) is a natural adjuvant that aids in human mesenchymal stromal cell (hMSC) proliferation *in vitro*; however, its role requires further exploration. This study was conducted to determine the optimal concentration of PRC required for achieving the maximal proliferation, and the need for activating the platelets to achieve this effect, and if PRC could independently induce early differentiation of hMSC. The gene expression of markers for osteocytes (ALP, RUNX2), chondrocytes (SOX9, COL2A1), and adipocytes (PPAR-γ) was determined at each time point in hMSC treated with 15% activated and nonactivated PRC since maximal proliferative effect was achieved at this concentration. The isolated PRC had approximately fourfold higher platelet count than whole blood. There was no significant difference in hMSC proliferation between the activated and nonactivated PRC. Only RUNX2 and SOX9 genes were upregulated throughout the 8 days. However, protein expression study showed formation of oil globules from day 4, significant increase in ALP at days 6 and 8 ($P \leq 0.05$), and increased glycosaminoglycan levels at all time points ($P < 0.05$), suggesting the early differentiation of hMSC into osteogenic and adipogenic lineages. This study demonstrates that the use of PRC increased hMSC proliferation and induced early differentiation of hMSC into multiple mesenchymal lineages, without preactivation or addition of differentiation medium.

1. Introduction

The current resurgence of interest in the field of tissue engineering and regenerative medicine has driven many researchers to explore the potential use of cell-based therapy, especially that involving the use of adult mesenchymal stem or stromal cells (MSCs). MSCs have the ability to self-renew, modulate immune responses, and exhibit multilineage differentiation potential. Due to these distinct characteristics, the potentials of MSCs in clinical applications have been widely speculated, and in many studies, the use of these multipotent cells has demonstrated good outcomes. However, in order for MSC treatment to be effective, the cells need to be of sufficient numbers, usually in the order of millions, in addition to having the ability to undergo directed lineage differentiation. Thus, it has become apparent that the use of adjuncts that can dramatically improve the proliferation and differentiation of the MSCs *in vitro* is of paramount importance. Of the many known biological products that has been previously described, platelet rich concentrate (PRC), that is, enriched levels of platelets relative to whole blood [1], has recently emerged as a potential tool that may result in these desirable outcomes [2]. The regenerative property of the platelets is reckoned to be the result of the release of various growth factors, cytokines, and chemokines as the platelets are activated after being exposed to certain factors such as

LIST OF POSTER PRESENTATIONS

1. Platelet Rich Concentrate Stimulates Chondrogenic Differentiation of Human Mesenchymal Stromal Cells in a three-Dimensional Hydrogel *In Vitro*, Poster Number PP18, 3rd Asian Cartilage Repair Society Meeting, 29th-31st October, 2015, Nexus, Bangsar, Malaysia. (Asian)

2. Platelet Rich Concentrate Increases Cellular Proliferation and Drives the Multiple Lineage Differentiation of Human Mesenchymal Stromal Cells *In Vitro*. Poster Number D4, 4th UTAR Seminar on Stem Cell and iPSC Research (SCiP) 2015 & 1st Joint UTAR-UST (Taiwan) Symposium on Stem Cell Research, 3rd-4th August 2015, UTAR, Malaysia. (International)

3. Effect of Platelet Rich Concentrate on the Proliferation and Differentiation Potential of Human Mesenchymal Stromal Cells: An *In Vitro* Study, Poster Number PB02B, 45th Malaysian Orthopaedic Association, 22nd- 24th May 2015, Shangri-La Hotel, Kuala Lumpur, Malaysia. (International)

4. Effect of Platelet Rich Concentrate on Human Mesenchymal Stromal Cells Proliferation and Differentiation Potential: A Preliminary *In Vitro* Study, Best Poster at the Pharmacology & Physiology International Scientific Congress (PPISC) organized by **Malaysian Society of Pharmacology and Physiology (MSPP)**, 22nd- 24th August 2014, Putra World Trade Centre, Kuala Lumpur, Malaysia. (International)

5. Platelet Rich Concentrate Promotes Early Cellular Proliferation And Multiple Lineage Differentiation Of Human Mesenchymal Stromal Cells *In Vitro*, 44th Malaysian Orthopaedic Association, 29th May – 1st June 2014, Hilton/ Le Méridien, Kuala Lumpur, Malaysia. (International) **(Oral)**

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