EFFECTS OF STROMAL CELL-DERIVED FACTOR-1 ON THE DIFFERENTIATION OF STEM CELLS AND THEIR ROLE IN FRACTURE HEALING

By

CHIH-YUAN HO

SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY UNIVERSITY COLLEGE LONDON

FEBRUARY 2011

JOHN SCALES CENTRE FOR BIOMEDICAL ENGINEERING INSTITUTE OF ORTHOPAEDICS AND MUSCULO-SKELETAL SCIENCE UNIVERSITY COLLEGE LONDON ROYAL NATIONAL ORTHOPAEDIC HOSPITAL TRUST STANMORE MIDDLESEX HA7 4LP UNITED KINGDOM
## Contents

<table>
<thead>
<tr>
<th>Abstract</th>
<th>Page No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgements</td>
<td>5</td>
</tr>
<tr>
<td>List of Figures</td>
<td>7</td>
</tr>
<tr>
<td>List of Tables</td>
<td>8</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>13</td>
</tr>
</tbody>
</table>

### Chapter One: Introduction

1. Bone tissue.................................................................................................................. 16
2. Bone fracture healing................................................................................................. 18
   1.2.1 Intramembranous bone formation................................................................. 20
   1.2.2 Endochondral bone formation........................................................................... 20
3. What is Tissue Engineering?...................................................................................... 20
4. Bone tissue engineering.............................................................................................. 22
   1.4.1 Cells for bone tissue engineering...................................................................... 23
   1.4.1.1 Osteoblasts.................................................................................................. 23
   1.4.1.2 Stem cells.................................................................................................... 23
   1.4.1.2.1 Embryonic stem (ES) cells...................................................................... 24
   1.4.1.2.2 Mesenchymal stem cells (MSCs).............................................................. 26
   1.4.2 Scaffolds for bone tissue engineering............................................................... 33
   1.4.3 Signals in bone tissue engineering...................................................................... 34
   1.4.4 Strategically genetic modification – Gene therapy............................................ 36
5. Chemokines in cell recruitment – stromal cell-derived factor-1.............................. 37
   1.5.1 Controlled cell migration in development....................................................... 38
   1.5.2 Controlled stem cells maintenance and mobilisation in adult life................... 40
   1.5.3 SDF-1 controlled stem cells migration responses to injury............................ 39
6. Study design................................................................................................................ 42

### Chapter Two: Enhanced Osteoblastic Differentiation by Stromal Cell-Derived Factor-1 in Human Mesenchymal Stem Cells

2.1 INTRODUCTION........................................................................................................ 45
2.2 MATERIALS AND METHODS.................................................................................. 46
   2.2.1 Cell isolation and culture.................................................................................. 46
   2.2.2 In vitro osteogenic differentiation...................................................................... 46
   2.2.3 Morphological observation............................................................................... 47
   2.2.4 Alkaline phosphatase activity assay................................................................. 47
   2.2.5 Cell proliferation assay..................................................................................... 48
   2.2.6 Von Kossa staining.......................................................................................... 48
   2.2.7 Statistics.......................................................................................................... 48
2.3 RESULTS.................................................................................................................. 49
   2.3.1 Cell morphology............................................................................................... 49
Chapter Three: SDF-1 Gene Incorporation into MSCs using the Adenoviral Delivery System of SDF-1

3.1 INTRODUCTION.................................................................58
3.2 MATERIALS AND METHODS.................................................61
  3.2.1 Cell isolation and culture..................................................61
  3.2.2 Adenovirus preparation and infection....................................63
  3.2.3 Optimal multiplicity of infection (MOI) for hMSCs and rBMCs........66
  3.2.4 SDF-1 expression measurement...........................................67
  3.2.5 In vitro cell migration assay...............................................67
  3.2.6 Statistics..............................................................................68
3.3 RESULTS....................................................................................69
  3.3.1 Virus production and optimal MOI for hMSCs and rBMCs............69
  3.3.2 SDF-1 expression in hMSC and rBMCs after infection by Ad-SDF-1...73
  3.3.3 In vitro cell migration..........................................................74
3.4 DISCUSSION...............................................................................75

Chapter Four: The in vivo Effect of SDF-1 in Bone Healing

4.1 INTRODUCTION......................................................................78
4.2 MATERIALS AND METHODS.....................................................80
  4.2.1 Cell culture and adenoviral infection......................................80
  4.2.2 General experimental design...............................................80
  4.2.3 Cell distribution and viability on collagen sponge......................81
  4.2.4 Rat femoral defect model.....................................................82
  4.2.5 Measurement of bone mineral content and bone mineral density....84
  4.2.6 Sample collection and histology procedures.............................85
    4.2.6.1 Haematoxylin and Eosin (H&E) staining................................85
    4.2.6.2 Fluorescence in Situ Hybridization staining..........................85
  4.2.7 Statistics.................................................................................86
4.3 RESULTS....................................................................................87
  4.3.1 Cell distribution and viability on collagen sponge......................87
  4.3.2 Three weeks short-term effect of SDF-1 in vivo.........................87
    4.3.2.1 Radiographs of the osteotomy...........................................87
    4.3.2.2 Bone mineralisation after three weeks...............................88
    4.3.2.3 New bone formation.......................................................89
    4.3.2.4 Maintenance of the SDF-1 expressing rBMCs.........................90
  4.3.3 Six weeks long-term effect of SDF-1 in vivo..............................91
    4.3.3.1 Radiographs of the osteotomy...........................................91
    4.3.3.2 Bone mineralisation during the six weeks...........................92
    4.3.3.3 New bone formation.......................................................96
4.4 DISCUSSION..............................................................................99
Chapter Five: Application of Second Cell Transplantation in Rat Femoral Defect Model

5.1 INTRODUCTION

5.2 MATERIALS AND METHODS

5.2.1 Cell culture and adenoviral infection

5.2.2 Experiment design

5.2.3 Statistics

5.3 RESULTS

5.3.1 Radiographs of the osteotomy

5.3.2 Bone mineralisation during the six weeks

5.3.3 New bone formation

5.4 DISCUSSION

Chapter Six: General Discussion

References

Appendix I

Appendix II
Abstract

Critical size bone defects after fracture or malignant tumour resection are still a challenge to repair in orthopaedics. Stem cell therapy combined with cytokines and bone grafts has the potential to improve outcomes. However, this application has its drawbacks preventing it from popular clinical use. The large number of stem cells required for transplantation is often a limiting factor.

The goal of this thesis was to make the stem cells more effective and efficient in bone repair thus potentially reducing the required number of cells. In this study I engineered stem cells by the introduction of a gene to over express stromal cell-derived factor-1 (SDF-1), a pivotal chemokine that has been proved to regulate cell migration, with the hypothesis that these cells would effectively increase the migration of native cells to the site of the repair, thus, enhancing bone repair. In vitro treatment of recombinant SDF-1 to human mesenchymal stem cells induced significantly greater osteogenic differentiation compared with control cells (p=0.024) and increased the migration of non-infected cells in a trans-well migration test (p=0.04). In a rat femoral bone defect model, using a low number of bone marrow cells, resulted in no difference in bone formation compared control defects without cells. Interestingly, the same number of bone marrow cells overexpressing SDF-1 showed significantly (86% increase, p=0.02) more new bone formation within the gap and less bone mineral loss at the area adjacent to the defect site during the early bone healing stage. A greater number of donor cells transfected with SDF-1 remained in the repair site compared with the control non-transfected site. An additional second cell injection of cells at 3 weeks was applied to the fracture but did not result in increased new bone formation but did reduce bone mineral loss at this time point.

This thesis demonstrates that by applying stem cells transfected with SDF-1, bone fracture healing was improved using a low cell number, which is a non-optimal condition for normal stem cell transplantation. This suggests that SDF-1 transfected cells recruited more host’s stem cells into the fracture gap or resulted in greater osteogenic differentiation, preventing bone loss and increasing bone formation.
These findings need further investigations to reveal the mechanism of SDF-1 in bone healing by studying the effects of downstream signaling pathway of SDF-1, the cell type of the recruited cells and the angiogenesis in the defect site.
Acknowledgements

I would like to thank my supervisors Professor Gordon Blunn and Dr. Jia Hua for their guidance and patience in making my PhD a feasible and enjoyable study. I deeply appreciate Professor Blunn giving me this opportunity to study in BME. It would have been impossible for me to complete this thesis without his supervision. I would also like to thank Dr. Hua for his patience and for devoting so much time in discussion with me. His guidance inspired this PhD work. It is impossible to thank enough my two supervisors, and I consider myself very fortunate to have had Professor Blunn and Dr. Hua as my supervisors. I will always appreciate the support they have given to me.

Thank you to Mark Harrison in BME for making the fixators, Rebecca Porter in the IOMS for the knowledge of tissue culture and Gillian Hughes at the RVC for her great help during the surgeries. Your help and brilliant jobs facilitated my in vitro and in vivo work in this thesis. Thank you to lovely Annie and Josie for taking care of me and encouraging me.

Thanks to all the guys in the Zimmer lab. Yvette, thanks for your great care in the first year of my PhD. Siva and Elena, thanks for being my British brother and Spanish sister. Thanks to Michelle, Priya, Carolina, Sorousheh, Jonathan, Lindsey and Michael, you make the Zimmer lab such a heart warming place.

Also, thanks to my friends in the UK and in Taiwan and everyone who helped me to learn and grow during this PhD.

Finally, I would like to thank my family. Thanks to Dad, Mum, Tiffany, Jennifer and Laney for always being supportive and having your heart with me.
## List of Figures

<table>
<thead>
<tr>
<th>Figure no.</th>
<th>Caption</th>
<th>Page no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Structure of long-bone diaphysis (Junqueira and Carneiro 2005).</td>
<td>17</td>
</tr>
<tr>
<td>1-2</td>
<td>Procedures of indirect fracture healing. (A) Hematoma formed to initiate the healing processes. (B) Hard callus forming from periosteum by intramembranous bone formation and soft callus forming by endochondral bone formation. (C) Calcified cartilage is replaced by woven bone which is further replace by lamellar bone through remodelling. (D) Reconstituted shape and strength of normal bone. (<a href="http://www.rci.rutgers.edu/~uzwiak/AnatPhys/APFallLect8.html">http://www.rci.rutgers.edu/~uzwiak/AnatPhys/APFallLect8.html</a>).</td>
<td>19</td>
</tr>
<tr>
<td>1-3</td>
<td>The multipotency of MSCs. MSCs has the ability to self-renew and differentiate towards the mesodermal lineage (solid arrows). MSCs have also been reported that they can transdifferentiate into other lineages (ectoderm and endoderm) (dashed arrows) (Uccelli et al. 2008).</td>
<td>29</td>
</tr>
<tr>
<td>1-4</td>
<td>BMPs signaling pathway. BMPs bind to two types of receptor followed by the transphosphorylation of the type I receptor. The phosphorylated type I receptor can further phosphorylate R-Smad to form complex with co-Smad and translocate into the nucleus and regulate the target genes expression by interaction with transcription factors and transcriptional coactivators (Miyazono et al. 2010).</td>
<td>35</td>
</tr>
<tr>
<td>2-1</td>
<td>Cell morphology after 7 days. Both Group1 and Group 4 showed an osteoblast-like cell shape from the first week of osteoinductive treatment while Group 2 and Group 3 remained in a fibroblastic-like cell shape during the whole time points. Scale bars, 100μm.</td>
<td>49</td>
</tr>
<tr>
<td>2-2</td>
<td>ALP activity of hMSCs was measured in weekly intervals during osteoinductive treatment. The absorbance at 405nm wavelength of each group is shown. Data points has different letter (a, b, c, d) are significantly different to each other (p &lt; 0.05).</td>
<td>50</td>
</tr>
<tr>
<td>2-3</td>
<td>Von Kossa staining after 4 weeks osteoinductive treatment. Only Group 1 and Group 4 showed calcium deposits while Group 2 and Group 3 had no calcium deposit. Scale bars, 100μm.</td>
<td>51</td>
</tr>
<tr>
<td>2-4</td>
<td>Cell proliferation was measured by MTS assay in weekly intervals during the osteoinductive treatment. The absorbance at 490nm wavelength of each group is</td>
<td>52</td>
</tr>
</tbody>
</table>
shown. Data points have different letters (a, b, c, d) are
significantly different to each other (p < 0.05). Only in
data (21 days), there was no significant difference
between each two continuing letters (p > 0.05).

2-5 An overview of BMP and SDF-1 signal transduction
pathway. Arrows stand for activating effect (graph
modified from Franceschi RT 2003).

A concept of adenoviral infection. A new gene is
injected into an adenovirus vector, which is used to
introduce the modified DNA into a human cell. The
adenovirus enters the human cell by receptor-mediated
endocytosis. The endocytosed vector will then enter the
nucleus by binding to nuclear pore complex and release
the genetic materials to make the cell produce the new
protein, for example, the eNOS. CAR, coxsackie virus-
adenovirus receptor; IR, integrin receptor; eNOS,

3-1

Cell morphology of rBMCs of passage 0 (arrow). Cells
harvested from bone marrow show a spindle cell shape
when they attached to the surface of the culture plate.
White spots are hematopoietic cells. Scale bar, 100μm.

3-2

Cell morphology of rBMCs of passage 4 (arrow). Cells
show a flattened cell shape after cell passage. Scale bar,
100μm.

3-4 A concept of adenovirus preparation and infection in
this chapter.

3-5 Production of recombinant adenovirus using the
AdEasy XL adenoviral vector system (Stratagene,
Catalog No. 240010).

3-6 Corning Transwell.

3-7 Normal cell morphology of AD293. Cells can be
cultured at high cell density. Scale bar, 100μm.

3-8 Cytopathic effect of AD293 cells after Ad-SDF-1
infection. Cells become swollen and cylinder-like in
shape (arrows). Cell contact is reduced. Scale bar, 100μm.

3-9 Infection efficiency of adenovirus to hMSCs detected by
X-gal staining of hMSCs β-galactosidase activity
(numbers represent the MOI used). Scale bar on the
bottom right, 100μm.

3-10 Infection efficiency of adenovirus to rBMCs detected by
X-gal staining of hMSCs β-galactosidase activity
(numbers represent the MOI used). Scale bar on the
bottom right, 100μm.

3-11 SDF-1 expression of hMSCs and rBMCs 5 days after
Ad-SDF-1 infection with different MOI (mean ± S.D.).
Data points have different letters are significantly different to each other (p < 0.05).

Cell migration of the non-infected (normal) hMSCs and rBMCs due to the secreted SDF-1 in Ad-SDF-1 infected hMSCs and rBMCs after 6 hours (mean ± S.D.). Both hMSCs and rBMCs show a dose-dependent response to higher SDF-1 concentration secreted in higher MOI groups. Data points have different letters are significantly different to each other (p < 0.05).

The surgery started from shaving of the area around the femur. The area was then disinfected by using Hydrex.

The femur was drilled by a 1.2mm drill, and the 1.5 pins were screwed into the pre-drilled holes.

A three millimeter gap was made.

The wound was closed.

Analysing box for BMC and BMD measurement.

Cell morphology in the inner of the collagen sponge (arrow).

Radiographs of osteotomy gap after three weeks. New bone was fromed within the osteotomy gap (arrows). Scale bar, 5mm.

Total BMC change within the osteotomy gap after three weeks (mean ± S.D.). Groups with different letters are significantly different to each other (p<0.05).

BMD change of the original bone area adjacent to the osteotomy gap after three weeks (mean ± S.D.).

H&E staining of new bone formation after three weeks (arrows). Scale bars, 400μm.

New bone area in the osteotomy gap after three weeks (mean ± S.D.). Groups have * are significantly different to each other (p<0.05).

FISH staining of donor cells (400x). Male rat Y chromosome staining are shown in red with nuclei shown in blue.

Radiographs of osteotomy gap after six weeks. Scale bars, 5mm.

Total BMC change within the osteotomy gap from the first week to the third week (mean ± S.D.). Groups have * are significantly different to each other (p<0.05).

Total BMC change within the osteotomy gap from the fourth week to the sixth week (mean ± S.D.).

Total BMC change within the osteotomy gap after six weeks (mean ± S.D.). Groups have * are significantly different to each other (p<0.05).

BMD change of the original bone area adjacent to the osteotomy gap from the first week to the third week.
4-18 BMD change of the original bone area adjacent to the osteotomy gap from the fourth week to the sixth week (mean ± S.D.).

4-19 BMD change of the original bone area adjacent to the osteotomy gap after six weeks (mean ± S.D.).

4-20 H&E staining of new bone formation after six weeks (arrows). Scale bars, 200μm.

4-21 New bone area in the osteotomy gap after six weeks (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

5-1 Radiographs of osteotomy gap after six weeks. Scale bars, 5mm.

5-2 To Total BMC change within the osteotomy gap from the fourth week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

5-3 Total BMC change within the osteotomy gap from the fourth week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

5-4 Total BMC change within the osteotomy gap from the first week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

5-5 BMD change of the original bone area adjacent to the osteotomy gap from the first week to the third week (mean ± S.D.).

5-6 BMD change of the original bone area adjacent to the osteotomy gap from the fourth week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

5-7 BMD change of the original bone area adjacent to the osteotomy gap from the first week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

5-8 H&E staining of new bone formation after six weeks (arrows). Scale bars, 200μm.

5-9 New bone area in the osteotomy gap after six weeks (mean ± S.D.).

5-10 Comparison of BMC change within the osteotomy gap from the third week to the sixth week between the single and double injection of cells (mean ± S.D.). Data were analysed by Student t-test within the same group.

5-11 Comparison of BMD change of the original bone area adjacent to the osteotomy gap from the third week to the sixth week between the single and double injection
of cells (mean ± S.D.). Data were analysed by Student t-test within the same group. Data have * are significantly different to each other (p≤0.05).

| 6-1 | An overview of BMP and SDF-1 signal transduction pathway. Arrows indicate activating pathways (graph modified from Franceschi et al. 2003). | 121 |
List of Tables

<table>
<thead>
<tr>
<th>Table no.</th>
<th>Caption</th>
<th>Page no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Antigen expression of MSCs (Baksh et al. 2004; Gregory et al. 2005).</td>
<td>28</td>
</tr>
<tr>
<td>2-1</td>
<td>Experimental group layout.</td>
<td>46</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
<td></td>
</tr>
<tr>
<td>rBMCs</td>
<td>Rat bone marrow cells</td>
<td></td>
</tr>
<tr>
<td>BMC</td>
<td>Bone mineral content</td>
<td></td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
<td></td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
<td></td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridisation</td>
<td></td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Haematoxylin and Eosin</td>
<td></td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic stem cells</td>
<td></td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
<td></td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
<td></td>
</tr>
<tr>
<td>hMSCs</td>
<td>Human mesenchymal stem cells</td>
<td></td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal cell-derived factor-1</td>
<td></td>
</tr>
</tbody>
</table>
Chapter One
Introduction
1.1 Bone tissue

Bone, composed of intercellular calcified material, the bone matrix, and three cell types: osteocytes, osteoblasts and osteoclasts, is a highly vascularised and metabolically active tissue which remodels throughout its life. As well as providing structural support and protection for vital organs, bone provides a reservoir for calcium, phosphate and other ions, which can be released or stored in a controlled fashion to maintain a constant concentration of these important ions in body fluids. It also harbours the bone marrow, where blood cells are formed (Junqueira and Carneiro 2005).

In mammals, there are four main types of bone. Above the level of the collagen fibril and its associated mineral, there are two distinct forms: woven bone and lamellar bone. Woven bone is primary and usually laid down very quickly, more than 4\(\mu\)m a day, and formed in embryonic development and in the callus that is produced during fracture repair. It is characterised by the variable size and random disposition of fine collagen fibrils. Although highly mineralized, woven bone is often quite porous at the micron level. Lamellar bone is more precisely arranged, and is laid down much slower than woven bone, less than 1\(\mu\)m a day. The collagen fibrils and their associated mineral are arranged in sheets (lamellae), and tend to be oriented in one direction within the lamella. The final degree of mineralization of lamellar bone is less than that of woven bone. Lamellar bone exists in both primary and secondary bone structure (Currey 2002).

The third type of bone, which is primary as well, is known as plexiform, or laminar, or fibrolamellar bone. It is found particularly in large mammals, whose bones have to grow in diameter rather quickly. The fibrolamellar bone is constructed by a less substantial scaffolding of woven bone is laid down quickly to be filled in with lamellar bone. The blood channels are more irregularly disposed in the laminar bone and surrounded by more or less concentric layers of lamellar bone. The structures around the blood vessels are called primary osteons (Currey 2002).

The fourth type of bone is called Harversian systems (secondary osteons). In long bones, the external and internal surfaces are covered by layers of bone-forming cells
and connective tissue called the periosteum and the endosteum (Figure 1-1). The periosteum consists of an outer layer of collagen fibers and fibroblasts, and inner layers of osteoprogenitor cells. The endosteum lines all internal cavities within the bone and is composed of a single layer of flattened osteoprogenitor cells. Between the periosteum and endosteum, there are outer circumferential lamellae, Haversian systems, and inner circumferential lamellae. Haversian systems are composed of collagen fibers arranged in lamellae, which are parallel to each other or concentrically organized around a vascular canal containing blood vessels, nerves and loose connective tissue. Lacunae containing osteocytes are normally found between lamellae. In each lamella, collagen fibers are parallel to each other (Junqueira and Carneiro 2005).

Figure 1-1. Structure of long-bone diaphysis (Junqueira and Carneiro 2005).
1.2 Bone fracture healing

Bone fracture normally occurs due to trauma and other diseases, such as osteoporosis, and congenital deficits, and the risk of fracture generally increases with age. In the US, there are about 8 million bone fractures associated with 5-10% delayed healing or non-union; while in the UK there are about 150,000 fractures due to osteoporosis, with an estimated healthcare cost of £17 billion per annum (Dawson and Oreffo 2008; Jordan and Cooper 2002).

During the repair process, the pathway of normal embryonic development is recapitulated with the coordinated participation of several cell types. The genetic mechanisms regulating fetal skeletogenesis, which include Indian hedgehog (Ihh) and core binding factor 1 (Cbfa1) pathways, also regulate adult skeletal regeneration (Ferguson et al. 1999). It can be divided into direct (primary) and indirect (secondary) fracture healing (Dimitriou et al. 2005).

Direct fracture healing occurs only under the stable condition of fracture with rigid fixation and decreased intrafragmentary strain between the two fragments (McKibbin 1978). Under this stable fixation, bone healing can occur by direct osteonal bridging of the fracture line with minimal or no callus formation (Mow and Huiskes 2005). This process involves a direct attempt to re-establish new Haversian systems by the formation of discrete remodelling units, known as ‘cutting cones’, in order to restore mechanical continuity (McKibbin 1978). In direct osteonal healing, the bone does not increase its diameter through lack of periosteal callus. This limits the load-bearing capacity of the healing bone, which consequently requires a longer period of protection by fracture stabilisation (Mow and Huiskes 2005).

Indirect fracture healing, which occurs more frequently than direct healing, involves the combination of intramembranous and endochondral ossification (Dimitriou et al. 2005). After trauma and fracture, a hematoma occurs, followed by inflammation at the injury site which initiates the healing process (Figure 1-2A). Hard callus formation starts with intramembranous bone formation at the surface of periosteum and endosteum, in an area remote from the fracture. This progresses towards the fracture until the distal and proximal callus wedges unite to achieve mechanical
stability. Meanwhile, endochondral bone formation occurs adjacent to the periosteum at the fracture site, and involves the recruitment, proliferation and differentiation of undifferentiated mesenchymal cells into cartilage (endochondral ossification) (Figure 1-2B). The cartilage then becomes calcified and is eventually replaced by bone. Newly formed bone from intramembranous and endochondral bone formation is initially woven bone, which has an irregular array of collagen fibers, a high mineral content and is replaced by lamellar bone through bone remodelling (Figure 1-2C). Finally, after remodelling, the shape and strength of the normal bone is reconstituted (Figure 1-2D) (McKibbin 1978; Dimitriou et al. 2005; Junqueira and Carneiro 2005; Mow and Huiskes 2005; Kraus and Kirker-Head 2006).

Figure 1-2. Procedures of indirect fracture healing. (A) Hematoma formed to initiate the healing processes. (B) Hard callus forming from periosteum by intramembranous bone formation and soft callus forming by endochondral bone formation. (C) Calcified cartilage is replaced by woven bone which is further replace by lamellar bone through remodelling. (D) Reconstituted shape and strength of normal bone.
(http://www.rci.rutgers.edu/~uzwiak/AnatPhys/APFallLect8.html).
1.2.1 Intramembranous bone formation
Intramembranous ossification involves the formation of bone directly, without first forming cartilage, from committed osteoprogenitors and undifferentiated mesenchymal cells that reside in the periosteum (Einhorn 1998; Dimitriou et al. 2005). The process begins when groups of cells differentiate into osteoblasts. Osteoblasts produce bone matrix and calcification follows, resulting in the encapsulation of some osteoblasts, which then become osteocytes (Junqueira and Carneiro 2005).

1.2.2 Endochondral bone formation
In regions that are mechanically less stable, which allow a range of intrafragmentary motion, for example, from 5% to 15%, and immediately adjacent to the fracture site, endochondral bone formation occurs. During this process, mesenchymal stem cells (MSCs) are recruited and subsequently differentiated into chondroblasts. These cells synthesize and secrete cartilage-specific matrix, including type II collagen and proteoglycans. The cartilage then undergoes hypertrophy and mineralisation in a spatially organised manner. Next, the calcified cartilaginous matrix and chondrocytes are removed by chondroclasts and replaced by the woven bone, which is formed by osteoblasts differentiated from newly recruited MSCs. The woven bone then undergoes remodelling and is replaced by lamellar bone (Barnes et al. 1999; Dimitriou et al. 2005; Junqueira and Carneiro 2005).

Despite the innate capacity of bone tissue to regenerate upon damage, some conditions still make the augmentation of fracture repair desperately required. Such restrictions can include non- or delayed unions, substantial loss of bone tissue from trauma or tumour resection, a requirement of arthrodesis or arthroplasty, or an inability to heal due to disease or old age (Kraus and Kirker-Head 2006).

1.3 What is Tissue Engineering?
Tissue engineering, as defined by Langer and Vacanti, is “an interdisciplinary field of research that applies the principles of engineering and life sciences towards the
development of biological substitutes that restore, maintain, or improve tissue function” (Langer and Vacanti 1993). It is based on the understanding of tissue formation and regeneration and aims to induce new functional tissue by combining knowledge from physics, chemistry, engineering, materials science, biology, and medicine in an integrated manner (Laurencin et al. 1999; Salgado et al. 2004).

To achieve the goal of tissue engineering, researchers face challenges associated with imitating nature. Biologic tissues consist of cells, extracellular matrix and signalling systems. There are three main elements in tissue engineering that are based on the three basic components of biologic tissues; they are cells, scaffolds and signals (Lanza et al. 2000).

Cells are the starting point for any attempt to engineer a tissue or organ substitute. Cells can be autologous, allogeneic, or xenogeneic and each has its advantages and disadvantages. Autologous cells, which are cells from the patient, are considered to be the safest cell source for tissue engineering without the issue of immune rejection. However, the small number of cells which are available has limited their use. Allogeneic cells, which are cells from other patients, can be prepared in advance. This makes these cells more practical in many clinical situations, but their immune acceptance is the main consideration. Xenogeneic cells, which are cells from other species, can be prepared in advance and in large numbers, but immune acceptance and also animal virus transmission are concerns. In addition to the source of cells, the type of the cells and/or engineered cells should be considered in tissue engineering (Lanza et al. 2007).

After selecting the cell source, a three dimensional architecture is often required. Scaffolds for tissue engineering have to present a surface that promotes cell attachment, cell growth and differentiation, and matrix synthesis and maintenance, while providing a porous structure for tissue ingrowth, nutrients and signal exchange (Smith et al. 2009). Also it has to be biocompatible and biodegradable. Thus, scaffolds are often designed from natural biomaterials, such as collagen (Friess 1998), fibrin (Scotti et al. 2009), or hyaluronan (HA) (Nehrer et al. 2009), to mimic the natural condition of extracellular matrix (ECM) (Nöth et al. 2010).
During morphogenesis, the behavior of individual cells within a developing tissue is determined by various signals from the surrounding microenvironment. In tissue engineering, signals are also important for regeneration and repair. The ECM provides biochemical and mechanical signals that can be translocated from cell membrane into cytoskeleton and further into nucleus to direct the cell phenotype. On the other hand, cells can remodel the ECM and affect the microenvironment (Gjorevski and Nelson 2009).

1.4 Bone tissue engineering
Bone loss in large defect from trauma, neoplasia, reconstructive surgery and congenital defects remains a major health problem, which promotes the need for bone regeneration therapies. The long-term clinical goal is to reconstruct bony tissue in an anatomically functional, three-dimensional morphology (Soucacos et al. 2008). Traditionally, bone loss related to disease or trauma has been managed with bone grafts. Autologous bone grafts harvested from the iliac crest are osteoinductive, osteoconductive and non-immunogenic and are considered as the gold standard for bone repair. However, due to its limited supply and the morbidity associated with harvesting this tissue, such as infections, hematomas, vascular and neurologic injuries and iliac wing fractures, then they are of limited clinical use (Arrington et al. 1996). Donor site pain is reported in ranges of 25%-49%, with 19%-27% of patients experiencing chronic site pain two years post-operatively (Fernyhough et al. 1992; Heary et al. 2002). For these reasons, allograft bone is mainly used. However, allograft bone lacks the osteoinductive capacities of autograft bone and, together with the concerns of bone allograft shortage, contamination and immunogenicity, this limits its implementation for orthopaedic applications (Eastlund 2006; Dawson and Oreffo 2008). Thus, current approaches in bone tissue engineering focus on creating the right biological environment *in vivo* to promote bone healing. Bone tissue engineering has been intensely studied using combinations of the three elements: cells, scaffolds and signals.
1.4.1 Cells for bone tissue engineering
Bone formation requires the cellular machinery to create the structural components. Therefore, no strategy of bone regeneration should ignore the introduction of cells during the bone healing process (Kraus and Kirker-Head 2006).

1.4.1.1 Osteoblasts
As the direct bone-forming cell, osteoblasts are the most obvious choice because of their non-immunogenicity from biopsies taken from the patients who will receive the osteoblasts back after *ex vivo* cell expansion (autologous cells) (Kwan et al. 2008). These cells have a relatively limited source and a low expansion rate, thus taking time to achieve the number of cells required to seed on scaffolds (Salgado et al. 2004; Heath 2000). Furthermore, studies showed that osteoblasts have a decreased ability to respond to osteoinductive stimuli with the aging of the patient (Erdmann et al. 1999; Cowan et al. 2003).

The difficulties of using autologous osteoblasts lead to the thought of the use of allogeneic or xenogeneic cells. However, the immunogenicity of these cells, the possibility of the transmission of infectious agents, such as animal viruses, and ethical problems have limited this approach (Sprangers et al. 2008).

1.4.1.2 Stem cells
Stem cells are undifferentiated cells with an unlimited or prolonged self-renewal ability and they are able to differentiate to at least one type of highly differentiated descendant (Watt and Hogan 2000). Researchers have used the expression of a single or a combination of molecular markers to describe stem cells, however, it is very difficult to determine a unique pattern of expression for specific types of stem cell (Shostak 2006). To date, there is still no universally acceptable phenotypical definition for the term stem cell. Although it is hard to classify stem cells phenotypically, they can be classified according to their potency. The hierarchic order of stem cells range from totipotency to pluri- and multi-potency, to unipotency (Becker and Jakse 2007). Totipotent cells, for example the cells of the morula, can
give rise to both embryonic and extra-embryonic tissues. Pluripotent cells have the ability to differentiate into all three germ layers. Multipotent cells have more restricted potency and are only able to differentiate into the cells of one germ line. Unipotent cells have the least potency.

**1.4.1.2.1 Embryonic stem (ES) cells**

Embryonic stem cells are derived from pre-implantation embryos formed during infertility treatment of couples for *in vitro* fertilization (IVF). They are donated by the couples, with informed consent, for research on embryonic stem cells. Human embryos are usually eight cells surrounded by an acellular glycoprotein shell at three days in culture *in vitro* after insemination with sperm. At the fourth day, it is known as a morula, which consists of 16-32 cells in a grapelike cell cluster. By the fifth to sixth day, the embryo becomes a hollow ball of more than 64 cells, known as a blastocyst, that has an outer layer of trophectoderm cells and a cluster of around 10-30 internalised cells, which are termed the inner cell mass (ICM) (Lanza *et al.* 2007). The first human ES cell line was formed by isolation of ICM from blastocysts using immunosurgery followed by culture on irradiated mouse embryonic fibroblasts as a feeder layer (Thomson *et al.* 1998). More human ES cell derivations were described later and involved different methods for isolation of ICM and different cell lines for the feeder layer (Cowan *et al.* 2004; Hovatta *et al.* 2003).

Undifferentiated ES cells are characterised as being nearly unlimited self-renewing, immortal, and able to differentiate into cells in all three embryonic germ layers, which are endoderm, mesoderm and ectoderm (Brignier and Gewirtz 2010). With its differentiation ability, ES cells have been intensely studied for use in regenerative medicine for the treatment of certain diseases, such as Parkinson’s disease, diabetes, heart disease (Fricker-Gates and Gates 2010; Calne *et al.* 2010; Iacobas *et al.* 2010).

Embryonic stem cells have the ability to differentiate into all three embryonic germ layers and are described as pluripotent. During the *in vitro* culture, when human ES cells are permitted to overgrow in two-dimensional culture, cells begin to pile up and undergo spontaneous differentiation (Sathanathan *et al.* 2002). A wide range of
differentiating cell types form and all three embryonic germ layers can be found in these flat cultures (Conley et al. 2004; Reubinoff et al. 2000; Sathananthan and Trounson 2005). In addition to the spontaneous differentiation, ES cells can undergo specific differentiation by exposure to selected growth factors, or by co-culture of ES cells with cell types capable of lineage induction (Lanza et al. 2007).

In bone tissue engineering, because ES cells can be expanded and manipulated in vitro with relative ease, they are an ideal cell source for bone repair (Waese et al. 2008). ES cells can differentiate into osteogenic cells when cultured in the presence of serum and dexamethasone, ascorbic acid and β-glycerophosphate, or co-cultured with primary bone-derived cells (Ahn et al. 2006; Maniotopoulos et al. 1988; Buttery et al. 2001). However, there are many issues that need to be resolved before they can be applied clinically. One issue is the culture conditions used for maintaining the undifferentiated ES cells, which is usually on mouse embryonic fibroblast (MEF) feeder layer culture with medium that contains non-human serum. These non-human components may be a source of contamination for foreign proteins and increase the risk of transmitting infectious disease (Waese et al. 2008). To overcome this issue, human fibroblasts can be used to replace the MEF, and a serum free culture condition has been developed from recombinant serum components (Richards et al. 2002; Ludwig et al. 2006).

Ironically another issue limiting the use of ES cells in clinical treatment is their dominant differentiation ability. With their pluripotency, ES cells have been shown to form teratomas, tumour containing tissues from the three primary germ layers, in vivo after injection in an immune-compromised animal (http://stemcells.nih.gov/info/basics/basics3.asp). This teratoma can be formed mainly by the differentiated ES cells and has interactions with the host tissue through the developing blood vessels (Gertow et al. 2004). Thus, the understanding of how to control the in vivo cell behavior of ES cells, and that the ES cell-derived cell type is free of undifferentiated cells are of paramount importance for potential clinical applications (Waese et al. 2008).
Apart from the scientific hurdles, human ES cells have also drawn much attention from many sectors of the public. Wide ranging debates and points of view among religious, historical, cultural and medical groups have arisen over the development of ES cell research (Leist et al. 2008). One of the issues is the definition of pregnancy. Some believe that life begins with fertilisation of the ovum, and the destruction of an embryo is thought to be tantamount to infanticide. On the other hand, proponents insist the potential benefit to humankind from this research can mitigate the concerns and argue that ES cells are made from unwanted fertilised ovum that will be destroyed in any event (Leist et al. 2008).

The scientific and ethical issues associated with the ES cells means that there is considerable work still required if these cells are going to be used clinically to bridge the bench to the bed.

1.4.1.2.2 Mesenchymal stem cells (MSCs)
An ideal cell source for tissue engineering should fulfill the criteria, including availability, easy access to the source cells, capacity for extensive self-renewal or expansion to generate sufficient quantity, capacity to differentiate into cell lineages of interest, and lack of or minimal immunogenic or tumorigenic ability (Lanza et al. 2007). Because of the issues associated with the use of ES cells that researchers need to address before for their clinical application, MSCs have become an alternative cell source in regenerative medicine with multipotent differentiation capacity and less ethical issues.

*Historical development of MSCs*
Since the 1950s, the finding that the injection of bone marrow cells into irradiated animals could prevent hematological insufficiency (Lorenz et al. 1951) led to many studies, which developed therapeutic bone marrow transplantation. However, these experiments demonstrated that bone marrow contained regenerative cells for the hematopoietic system, but did not address the source of cells for connective tissue regeneration. Other experiments involving the transplantation of whole bone marrow to ectopic sites demonstrated the osteogenic potential of cells from bone marrow
(Urist and McLean 1952; Tavassoli and Crosby 1968). Alexander Friedenstein and colleagues then isolated cells characterised as fibroblast colony-forming cells (FCFC) that were responsible for the osteogenic response from the bone marrow of guinea pig (Friedenstein et al. 1970). In the 1980s, Castro-Malaspina et al. isolated FCFC from human bone marrow and characterised their in vitro characteristics as having strong adherence properties and distinguished these cells from macrophages and endothelial cells (Castro-Malaspina et al. 1980). These cell characteristics substantiated the human bone marrow fibroblasts, also called colony forming units-fibroblastic (CFU-F), from many of the similar findings of cells from guinea pig bone marrow. Following numerous studies, the term mesenchymal stem cell was first used by Arnold Caplan (Caplan 1991). About 1 in 100,000 nucleated cells in bone marrow are MSCs (Cui et al. 2007; Mirabet et al. 2008). This low number of MSCs in bone marrow means that cells have to be isolated and expanded in number to be used for any treatment and this requires considerable time in cell culture.

**Identification of MSCs**

Although there is no one single marker or a combination of markers that is unique and exclusive to MSCs, experimentally, MSCs express a number of surface markers, including STRO-1, SB-10, SH2, SH3, Thy-1 (CD90), transforming growth factor-β (TGF-β) receptor type 3 endoglin (CD105), hyaluronic acid receptor (CD44), integrin α1 subunit (CD29), CD133, and activated leukocyte-cell adhesion molecules (ALCAM, CD166). MSCs are negative for hematopoietic markers, CD34, CD45, and CD14 (Table 1-1) (Baksh et al. 2004; Gregory et al. 2005).
### Antigens positive for MSCs

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>STRO-1</td>
<td>Surface marker for immature mesenchymal cells.</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2 domain, a structurally conserved protein domain contained within many intracellular signal-transducing proteins.</td>
</tr>
<tr>
<td>SH3</td>
<td>Src Homology 3 domain, a conserved sequence in several protein families such as PI3 Kinase and Ras GTPase activating protein.</td>
</tr>
<tr>
<td>CD29</td>
<td>Receptor for vascular cell adhesion molecule-1 (VCAM-1).</td>
</tr>
<tr>
<td>CD44</td>
<td>Cell surface marker involved in binding of hyaluronic acid and cell adhesion.</td>
</tr>
<tr>
<td>CD90</td>
<td>Cell surface protein expressed in hematopoietic stem cells, MSCs and neurons.</td>
</tr>
<tr>
<td>CD105</td>
<td>Receptor responses to TGF-β1.</td>
</tr>
<tr>
<td>CD133</td>
<td>Targeted to membrane protrusions on stem cells. Function currently unknown.</td>
</tr>
<tr>
<td>CD166</td>
<td>Cell surface marker involved in cell adhesion.</td>
</tr>
</tbody>
</table>

### Antigens negative for MSCs

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>Receptor for lipopolysaccharide.</td>
</tr>
<tr>
<td>CD34</td>
<td>Hematopoietic stem cell marker involved in cell adhesion.</td>
</tr>
<tr>
<td>CD45</td>
<td>Tyrosine phosphatase.</td>
</tr>
</tbody>
</table>

**Table 1-1. Antigen expression of MSCs (Baksh *et al.* 2004; Gregory *et al.* 2005).**

To fulfill the two major functional definitions of stem cells, MSCs can be expanded many fold and retain their ability to differentiate. Karyotyping of passage 12 MSCs that have undergone about 30 population doublings, found no chromosomal aberrations (Pittenger *et al.* 1999). Furthermore, MSCs, described as multipotent, have shown the capacity to differentiate into several mesenchymal lineages such as cartilage, bone, fat, muscle, tendon, and hematopoietic supporting marrow stroma (Caplan 1991; Prockop 1997; Pittenger *et al.* 1999). Moreover, studies showed that MSCs also have endodermic and neuroectodermic differentiation potential (Figure 1-3) (Kopen *et al.* 1999; Petersen *et al.* 1999; Uccelli *et al.* 2008).
MSCs has the ability to self-renew and differentiate towards the mesodermal lineage (solid arrows). MSCs have also been reported that they can transdifferentiate into other lineages (ectoderm and endoderm) (dashed arrows) (Uccelli *et al.* 2008).

**Cell source and heterogeneity of MSCs**
Bone marrow is the most studied cell source of MSCs. Although bone marrow is relatively easily accessible, the potential donor site morbidity, pain and low incidence have prompted researchers to find alternative sources. MSCs have been isolated from different tissues including adipose, muscle, periosteum, synovium, immobilised peripheral blood, cord blood, and placenta (Baksh *et al.* 2004; Gregory *et al.* 2005; Tuan *et al.* 2003). However, MSCs from different tissues show a similar phenotypic characteristic and it is not clear if these are the same MSCs. Studies have shown different chondrogenic and osteogenic differentiation potentials in MSCs.
isolated from bone marrow, synovium, periosteum, muscle, and adipose tissue (Sakaguchi et al. 2005). Therefore, it is important to understand the ability of each tissue derived MSCs for specific cell differentiation in order to obtain the best cell source strategy in further research.

Not only do MSCs isolated from different tissues express different properties, individual colonies derived from single MSC exhibit a heterogeneous nature in cell proliferation and differentiation potentials. Gronthos et al. reported that only a minor proportion of colonies derived from adult human bone marrow continued to grow beyond 20 population doublings, while the larger number of the colonies exhibited early senescence (Gronthos et al. 2003). Even derived from a single colony, Kuznetsov et al. demonstrated that only 58.8% of the MSCs could form bone when seeded on hydroxyapatite-tricalcium phosphate ceramic scaffolds after being implanted into immunodeficient mice (Kuznetsov et al. 1997). With this heterogenous nature of MSCs, it is challenging to select the most potent cells for clinical application in tissue regeneration.

**Immunosuppression and immunomodulation of MSCs**

From a tissue engineering’s point of view, it is always an advantage if a suitable cell source is available during the early treatment of wound or trauma. Autologous MSCs have the advantage in regenerative medicine because of their multipotent differentiation capacity. However, the rarity of MSCs and the reduction of the quantity as well as the quality of MSCs with age and disease make it more feasible to consider using allogeneic MSCs for repairing damaged tissue (Lanza et al. 2007). The first concern in using allogeneic MSCs is the immune-compatibility. Allogeneic cells are normally detected and deleted by the host immune system. Surprisingly, MSCs have shown suppressing and modulatory effects on host immune response.

Innately, MSCs express low to intermediate major histocompatibility complex (MHC) class I molecules, and do not express MHC class II molecules on the cell surface (Götherström et al. 2004; Le Blanc et al. 2003). The expression of MHC class I molecules helps to protect MSCs from deletion by natural killer cells. The lack of surface MHC class II expression enables the MSCs to escape recognition by
alloreactive CD4⁺ T-cells. Apart from the pattern of surface molecule expression, MSCs have been shown to alter the immune system in two mechanisms by 1) cell-cell interaction with immune cells and 2) secreting soluble factors to create a local immunosuppressive environment (Lanza et al. 2007).

Under co-culture conditions, MSCs suppress T-cell proliferation and activation. This inhibition exists even when proliferation-inducing stimulants are present (Bartholomew et al. 2002; Di Nicola et al. 2002; Tse et al. 2003). In addition, MSCs can affect the differentiation and maturation of dendritic cells (Beyth et al. 2005; Zhang et al. 2004) and also alter the phenotype and suppress the proliferation, cytokine secretion and cytotoxicity of natural killer cells (Sotiropoulou et al. 2006).

MSCs have also been shown to secrete factors such as hepatocyte growth factor (HGF), TGF-β1, IL-10, and prostaglandin E2, which affect immune cells (Aggarwal and Pittenger 2005; Di Nicola et al. 2002). HGF and TGF-β1 are thought to be related to the inhibition of T-cell proliferation (Di Nicola et al. 2002), while the IL-10 is a cytokine for regulatory T-cells and can suppress inflammatory immune response (Aggarwal and Pittenger 2005).

Due to their immunosuppressive and immunomodulatory properties, MSCs have been considered a potential cell source applied in vivo to prevent rejection and to promote transplant and patient survival. However, recent studies have reported the possibility that MSCs induce an immune response in specific conditions (Natuta et al. 2006). Thus, before allogeneic MSCs can be applied in clinical treatments, it is necessary to better understand the biology and mechanism of their immunomodulation effect.

**MSCs in bone tissue engineering**

Under some circumstances bone fracture can be healed by applying autogenous bone or osteoconductive bone graft substitutes such as collagen composites and various ceramics, but these applications are not effective in critical-sized defects, which is a more challenging healing environment (Boden 2005). Numerous studies in animal models of critical-sized fracture healing have reported that applying MSCs with various kinds of delivery vehicles will improve or complete bone healing (Niemeyer...
et al. 2010; Zhang et al. 2010). Clinical trials using autogenous bone marrow-derived MSCs seeded on a macroporous hydroxyapatite scaffold have been reported (Quarto et al. 2001) in patients with a defect size from 4 to 7cm in long bones. These defects were treated with autologous MSCs, which were isolated from bone marrow and expanded \textit{ex vivo}. The results showed abundant callus and good integration at the interface with host bone in the second month after the surgery. It also reduced the healing period compared with the traditional treatment of bone defects with bone graft. A good integration of the implants was maintained after 6-7 years reported by the follow-up study (Marcacci et al. 2007).

Apart from bone fracture healing, MSCs are also considered as a potential source for cell-based therapy for metabolic bone diseases (Undale et al. 2009). Osteogenesis imperfecta (OI) is a genetic disorder characterised by defective type I collagen, which leads to osteopenia, multiple fracture, severe bone deformity and shortened stature (Undale et al. 2009). Horwitz et al. used allogeneic bone marrow-derived MSCs to treat six children with severe OI (Horwitz et al. 2002). Patients received two infusions of the allogeneic MSCs, and five of the six patients showed engraftment in one or more site, including bone, skin and marrow stroma, between 4 and 6 weeks after the second infusion. An acceleration of growth velocity during the first 6 months post infusion was also observed.

With their innate regeneration potential and immunosuppressive features, MSCs show great potential in bone tissue engineering. Due to the low cell number when isolated from bone marrow, MSCs have to be expanded in long-term culture in order to acquire the appropriate cell numbers, which normally $5 \times 10^6$ to $10 \times 10^6$/cm$^3$ of cells are applied (Kruyt et al. 2008). These culture conditions often use animal products such as serum and the potential risk of these conditions needs to be investigated, as well as the incidence of tumourogenesis associated with long term culture of MSCs (Undale et al. 2009).
1.4.2 Scaffolds for bone tissue engineering

Two-dimensional tissue culture is the most popular technique to investigate cellular differentiation and extracellular matrix (ECM) deposition. It is now thought that this approach cannot perfectly reflect the situations and the interactions between cells and the environment in vivo, which strongly depends on the 3-dimensional environment in which the cells are organised (Tortelli and Cancedda 2009). Thus, scaffolds with 3-dimensional structure are important for tissue engineering to mimic the real environment in the body. The essential properties for scaffolds in bone tissue engineering include biocompatibility, porosity, pore size, surface properties, osteoinductivity, mechanical properties, and biodegradability (Salgado et al. 2004).

In bone, intimate interactions between osteogenic cells and ECM have been revealed in bone mineral maintenance and responses to various stimuli (Gjorevski and Nelson 2009). The importance of ECM in bone led to the development of a large number of different types of scaffolds such as natural polymeric materials and synthetic polymers (Tortelli and Cancedda 2009).

The advantage of natural biomaterials in bone tissue engineering is the ability to mimic the composition of native ECM, which provides proper biocompatibility and biodegradability and also facilitates cell adherence, migration, and differentiation (Nöth et al. 2010). Natural biomaterials such as collagen are normally more osteoinductive and more osteoconductive than man-made materials. Osteoinductivity is defined as the ability of a material or compound to induce osteogenesis from undifferentiated stem or progenitor cells, whilst osteoconductivity means that a material enhances new bone formation over the surface (Albrektsson and Johansson 2001). Collagen composes 30% of all vertebrate body protein and 13 different types occur naturally. In tendon and bone, more than 90% of the extracellular proteins consist of collagen with a predominant proportion of type I collagen (Friess 1998). As used in tissue engineering, type I collagen shows good biocompatibility, minimal potential for antigenicity and high porosity, which gives space for neohistogenesis (Glowacki and Mizuno 2008). It is also osteoinductive (Mizuno et al. 1997). Type I collagen has been intensively investigated as a scaffold material and can be applied in many different forms including membranes, sponges,
fleeces and hydrogels (Nöth et al. 2010). With its osteoinductive property, type I collagen has been studied in augmentation of new bone formation (Shimoji et al. 2009; Reichert et al. 2009).

Ceramics, such as synthetic hydroxyapatite (HA), β-tricalcium phosphate (β-TCP), and calcium phosphate, are made from an inorganic, non-metallic material that can form a crystalline structure (Nöth et al. 2010; Salgado et al. 2004). They are normally osteoinductive and osteoconductive, and have been considered for bone tissue engineering applications (Quarto et al. 2001; Dong et al. 2002). However, they have some drawbacks due to their brittle property, which provides a low mechanical stability. Their degradation is difficult to predict in vivo, which could compromise the mechanical stability. Also, if these scaffolds are degraded too quickly, an increased concentration of Ca and P could damage cells (Salgado et al. 2004).

1.4.3 Signals in bone tissue engineering

Fracture repair is performed by cellular actions, which are regulated by several growth factors including transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1) and bone morphogenetic protein (BMP). Among these growth factors, BMPs have been considered as the most potent regulators of osteogenic differentiation (Kwong and Harris 2008).

BMP was discovered by Urist in 1965. New bone formation was found in the intramuscular implantations of hydrochloric acid decalcified bone matrix, which led to the identification of BMP in bone matrix (Urist 1965). To date, more than 16 different human BMPs have been identified (Wozney and Rosen 1998). BMPs are members of the TGF-β superfamily and are identified by their ability to induce ectopic bone formation in adult animals (Canalis 2009). BMP-2, -4, -7 and -9 are considered as the most potent osteoblastic differentiation inducers for stem or precursor cells (Haidar et al. 2009).

BMPs bind to two types of serine-threonine kinase receptors, type I and type II. Both type I and type II receptors are required for signal transduction. During the signal
transduction, BMPs bind to heterotetrameric complex of the type I and type II receptors, followed by the transphosphorylation of the type I receptor. The phosphorylated type I receptor can further phosphorylate receptor-regulated Smads (R-Smads), which exist in the cytoplasm through interaction with membrane anchoring protein. For example, Endofin and CD 44. R-Smads form complex with common-partner Smad (co-Smad), Smad-4, and translocate into the nucleus and regulate the target genes expression by interaction with transcription factors and transcriptional coactivators (Miyazono et al. 2010) (Figure 1-4). These target genes includes the runt-related transcription factor-2/core-binding factor-1 (Runx-2/Cbfa-1), which plays a critical role in the differentiation of cells toward an osteoblastic pathway (Lee et al. 2000; Banerjee et al. 2001; Canalis et al. 2003).

![Figure 1-4. BMP's signaling pathway. BMP's bind to two types of receptor followed by the transphosphorylation of the type I receptor. The phosphorylated type I receptor can further phosphorylate R-Smad to form complex with co-Smad and translocate into the nucleus and regulate the target genes expression by interaction with transcription factors and transcriptional coactivators (Miyazono et al. 2010).](image)
BMPs induce the differentiation of stem cells to osteoblastic lineages, and by enhancing the osteoblastogenesis, it also induces the osteoclastogenesis, which indicates a role of BMPs in bone remodelling (Canalis et al. 2003; Canalis 2009; Kaneko et al. 2000). BMP signalling has important functions throughout life. Mice embryos deficient in BMP-2 and 4 are non-viable (Zhang and Bradley 1996; Winnier et al. 1995). Naturally occurring mutations of BMPs also reflect the important functions of BMPs by resulting in a wide range of musculoskeletal disorders (Chen et al. 2004). In postnatal bone formation, blocking of BMP signals by expression of truncated type II receptor transgene led to a decrease in bone mineral density, bone volume, and dynamic bone formation rates (Zhao et al. 2002).

Because of their osteoinductive property, BMPs have been intensely studied for therapeutic utilisation. Animal experiments in rats, rabbits, dogs, and sheep showed that applications of recombinant BMPs, especially BMP-2 and BMP-7, can promote or attain union in large segmental defect models, which would fail to heal in the absence of exogenous BMPs (Barnes et al. 1999; Yasko et al. 1992; Murakami et al. 2002; Cook et al. 1994; Gerhart et al. 1993). Implantation of MSCs, infected by adenovirus carrying BMP-2 gene, showed bone healing in the defect site (Chang et al. 2003). Multiple clinical trials in trauma surgery have demonstrated that BMP-2 and BMP-7 are safe and effective in the treatment of fracture (Ghodadra and Singh 2008; White et al. 2007). BMP-2 and BMP-7 are approved by the Food and Drug Administration (FDA), US, for clinical use under certain circumstances (Axelrad and Einhorn 2009).

1.4.4 Strategic genetic modification – Gene therapy

To study the biological functions of a particular signal in vivo, the most common strategies are either to amplify the signal or to block the signal. These phenomena can be achieved by transferring genetic information, normally in the form of DNA, into the nucleus to modify the cell phenotype (Lanza et al. 2007). This same mechanism can be used to enhance the production of proteins or other factors from cells to treat diseases and is referred to as gene therapy (Verma and Weitzman 2005). The key to successful gene therapy is the gene delivery system, which has to be safe,
without causing any associated pathogenic effects, efficient, capable of transferring genes in a variety of tissues and easy to prepare (Verma and Weitzman 2005).

The vehicles that encapsulate therapeutic genes for delivery are called vectors. Vectors can be categorised in non-viral and viral vectors, which will be discussed in chapter 3 of this thesis. The vector, with its gene cargo, can be administrated *ex vivo* or *in vivo*. The *ex vivo* strategy is performed by transferring the vectors to the targeted cells *in vitro* and returning the genetically modified cells to the patient. In gene therapy, the transferred cell population can be well defined with controllable and predictable transfer efficiency. This is the most suitable strategy for stem cell gene therapy (Lanza *et al.* 2007).

The *in vivo* strategy administers the vector directly to the target organ or via the vascular system into vessels feeding that organ (Lanza *et al.* 2007). This strategy can avoid the tedious *in vitro* processes, such as harvesting cells from the patient, *in vitro* cell culture and returning the genetic modified cells to the patient. Challenges of this strategy includes the induction of immune responses caused by the vector and the uncertain transfer efficiency of the target cell/organ (Lanza *et al.* 2007).

Gene therapy for bone healing has been reported. Use of BMP-2, BMP-4 and BMP-7, with non-viral and viral vectors via *ex vivo* or *in vivo* delivery strategies, has given convincing results in small-animal models (Evans 2010). Although gene therapy has the potential to improve recovery in many diseases, a few failed clinical trails have been reported, which caused death of the patient due to the severe immune responses (Wilson 2009). Before it can be applied in clinical trials, reliable methods and detailed pharmacological and toxicological studies need to be carried out (Evans 2010).

1.5 Chemokines in cell recruitment — stromal cell-derived factor-1

Both organogenesis during development and tissue regeneration in tissue repairing mainly rely on the cellular activity and responses of cells to the extracellular stimuli. Cellular movement and localisation are among the crucial events, which are strongly
regulated by chemokines. Chemokines are a group of small proteins (8-14 kDa) characterised as being able to direct the movement of receptor-presenting cells towards higher concentrations of the chemokine in the environment (Luster 1998), which is termed chemotaxis. This property is shared by practically all members of the chemokine super-family. Depending on the presence and the position of conserved cysteine residues, chemokines are categorised into four subgroups: C, CC, CXC, and CX3C (X can be replaced by any amino acid). The chemokines interact with G-protein-coupled seven-transmembrane receptors (DeVries et al. 2006). Among the chemokines, stromal cell-derived factor-1 (SDF-1, also named CXCL12), and its receptor CXCR4 have been found to play crucial roles in a wide range of development processes and tissue repair.

In bone marrow, SDF-1 is constitutively expressed by endothelial and endosteal bone lining stromal cells and osteoblasts (Imai et al. 1999; Peled et al. 1999a; Jung et al. 2006). The amino acid sequence of SDF-1 is highly conserved during evolution and SDF-1 is cross-reactive between humans and mice (Peled et al. 1999b). This feature indicates its biological importance.

1.5.1 Controlled cell migration in development

The in vivo chemotactic effects of chemokines were first found in the immune system by investigating the accumulation of lymphocytes at sites of immune and inflammatory reactions (Baggiolini 1998). A simple example of chemokine-guided single-cell migration is the migration of the primordial germ cell (PGC). In Zebrafish, the CXCR4-expressing PGCs will arrive at the target location where expression of SDF-1 occurs and the gonad develops in an SDF-1/CXCR4 regulated manner (Doitsidou et al. 2002). This SDF-1/CXCR4 regulated PGCs migration is also found in other organisms, including mouse and chick (Molyneaux et al. 2003; Stebler et al. 2004).

In zebrafish embryonic development, SDF-1/CXCR4 interaction plays a role in the co-ordinated movement between mesodermal and endodermal layers, which controls the proper location and morphology of the tissues and organs that develop from these
germ layers (Raz and Mahabaleshwar 2009). In the absence of SDF-1/CXCR4 signalling, severe failure in endodermal organ development occurs, including defects in the pancreas, liver and intestine duplications (Nair and Schilling 2008).

This SDF-1/CXCR4 regulated cell migration is also revealed in the development of the nervous system (Tiveron and Cremer 2008). In the developing cortex, SDF-1 expression has been described in meninges, which is constant during the entire corticogenic process, and in the sub-ventricular zone, which is highly dynamic in a spatiotemporal pattern (Tiveron and Cremer 2008). Two cell populations during corticogenesis, Cajal-Retzius (CR) cells and GABAergic interneurons, are also a demonstration of CXCR4 expression throughout the cell migration (Borrell and Marín 2006; Stumm et al. 2003). It has been shown that SDF-1/CXCR4 interaction plays a crucial part in the tangential migration of these cells during corticogenesis (Borrell and Marín 2006; Tiveron and Cremer 2006), and interruption of the SDF-1/CXCR4 signalling leads to striking changes in the distribution of inter-neurons (Li et al. 2008).

Apart from guided cell migration, SDF-1/CXCR4 interaction is also involved in the maintenance of cells in specific regions of the brain. During cerebellar development, SDF-1/CXCR4 knock out mice showed a premature migration of granule cells from the external granular layer (EGL, an external cell layer of the cerebellum) into more internal layers of the cerebellum (Ma et al. 1998). This phenomenon has been further analysed and it has been shown that the SDF-1 produced by the meningeal cells, located at external parts of the cerebellum, acts as an attractant to anchor the granule cells close to the brain surface, preventing early premature migration. In later development (postnatal), these cells migrate away from the EGL and this is correlated with the loss of CXCR4 expression from the surface (Reiss et al. 2002; Zhu et al. 2002). This anchoring role of SDF-1/CXCR4 interaction is also demonstrated in the CR cell migration during corticogenesis. A chemical inhibition of CXCR4 after the tangential migration of CR cells led to a substantial displacement of the cells towards the deeper cortical layers (Paredes et al. 2006).
1.5.2 Controlled stem cells maintenance and mobilisation in adult life

In addition to development, SDF-1/CXCR4 interaction plays important roles in adulthood. Apart from bone marrow, SDF-1 expression is also found in the heart, skeletal muscle, liver, brain, and kidney parenchymal cells playing roles related to cell recruitment and maintenance (Askari et al. 2003; Ratajczak et al. 2003; Kollet et al. 2003, Tachibana et al. 1998; Schrader et al. 2002). In bone marrow, reciprocal interactions between hematopoietic cells and stromal cells have been widely studied. SDF-1/CXCR4 interaction has been involved in the homing, retention, and mobilisation of hematopoietic stem cells (HSCs) (Dar et al. 2006). Over-expression of CXCR4 in human hematopoietic progenitors resulted in increased homing and engraftment in murine bone marrow and spleen by the chemoattractive interaction of SDF-1/CXCR4 (Kahn et al. 2004). Inhibition of SDF-1 or CXCR4 by the use of antibodies led to a significant reduction of human CD34+ cells engraftment in murine bone marrow (Peled et al. 1999b; Kollet et al. 2001). These studies demonstrate the important role of SDF-1/CXCR4 interaction in stem cell homing.

SDF-1/CXCR4 interaction is also important for the retention of stem cells in bone marrow. Normally most HSCs were found in contact with SDF-1 highly expressing cells located near the endosteum. However, an inducible deletion of CXCR4 in a mouse model resulted in severe reduction of HSC numbers in bone marrow, and increased HSC number in the blood stream (Sugiyama et al. 2006). This stem cell maintaining property of SDF-1/CXCR4 interaction is further utilised for therapeutic purposes. Drug AMD3100 is a CXCR4 antagonist which selectively blocks the binding of SDF-1 to CXCR4 and is used to mobilise the HSCs from bone marrow into the peripheral blood in order to collect HSCs for transplantation. This method will also lead to an increased number of MSCs in peripheral blood too. (Miller et al. 2008; Pusic and Dipersio 2010).

Accumulating evidence shows that SDF-1/CXCR4 interaction plays an important role in granulocyte colony-stimulating factor (G-CSF)-induced HSC mobilisation, which is normally used together with AMD3100 in the collection of HSCs for clinical use (Pusic and Dipersio 2010). Five day consecutive daily treatment of G-CSF resulted in a severely reduced SDF-1 protein level in bone marrow (Petit et al. 2006; Peled et al. 1999b; Kahn et al. 2004).
which could be attributed to the increased secretion of proteolytic enzymes that cleaved SDF-1 protein or to the sharply reduced SDF-1 mRNA expression in osteoblasts, the major source of SDF-1 in bone marrow (Petit et al. 2002; Semerad et al. 2005; Nervi et al. 2006). This reduced protein level of SDF-1 in bone marrow, together with the normal SDF-1 level found in the peripheral blood (Petit et al. 2002), resulted in a gradient of SDF-1 concentration between the bone marrow and peripheral blood and mobilised the HSCs towards the peripheral circulation. This HSC mobilisation caused by the imbalance of SDF-1 concentration between the bone marrow and circulation, can be also achieved by enhancing the SDF-1 level in the circulation by intravenous injection of an adenoviral vector expressing SDF-1 (Hattori et al. 2001).

In addition to HSCs in bone marrow, other types of stem cell were isolated from bone marrow including MSCs, tissue committed stem cells (TCSC) and side population (SP) cells (Kucia et al. 2005; Challen and Little 2006). Bone marrow derived MSCs have demonstrated the expression of CXCR4 and the ability to respond to the chemotaxis of SDF-1 (Sordi et al. 2005). Also, it is well known that stem cells are retained in many adult tissues, where the microenvironment is called stem cell niche, and can be expanded in order to cope with stressful situations such as damage or infection (Miller et al. 2008). The maintenance and migration of these stem cells in non-hematopoietic niche may also be regulated in a similar SDF-1/CXCR4 chemotaxis manner. So, for example, the niche of stellate cells within rat liver is composed of sinusoidal endothelial cells, which release SDF-1 to attract stellate cells via CXCR4 (Sawitza et al. 2009).

1.5.3 SDF-1 controlled stem cells migration responses to injury
During injury and tissue repair, migration of the repairing cells from the surrounding and remote locations plays an important role in the process. Studies have reported that this cell migration can be initiated by chemokines and receptor interactions (Fox et al. 2007). An increased secretion of SDF-1 protein at the wound site is found following many different kinds of tissue damage, which forms an immediate gradient of SDF-1 concentration between the injured site and the peripheral area (Wang et al.
2006). For example, an increased protein level of SDF-1 in rat liver was found 24 hours after sub-lethal irradiation, which led to increased HSCs migration to the liver. This phenomenon was diminished by neutralising anti-CXCR4 antibodies (Kollet et al. 2003; Dalakas et al. 2005). Similar findings were also reported in injuries in the heart (myocardial infarction), retina, brain, and in vascular repair. By an enhanced SDF-1 level, improved cell migration, including myoblasts, MSCs or endothelial progenitor cells, towards the injured sites was demonstrated (Askari et al. 2003; Abbott et al. 2004; Lai et al. 2008; Ji et al. 2004; Stellos and Gawaz 2007).

One major difference between initiation of bone formation in developmental skeletogenesis and in regenerative skeletogenesis is the significant role of inflammation in the healing process (Sundelacruz and Kaplan 2009). Kitaori et al. recently reported the importance of SDF-1/CXCR4 interaction in fracture healing. In a murine femoral defect model with autologous bone graft, increased expression of SDF-1 mRNA and protein were found in periosteum two days postoperatively. Intravenous injection of pre-labelled bone marrow stromal cells demonstrated the cell migration towards the fracture site was regulated by SDF-1/CXCR4 interaction, while the cell migration was significantly reduced when CXCR4 antagonist was applied (TF14016). New bone formation was also reduced after treatment with SDF-1 neutralising antibody or TF14016 (Kitaori et al. 2009). These results indicate the crucial role of SDF-1/CXCR4 interaction in fracture healing by the recruitment of MSCs to the fracture site.

1.6 Study design
Presently, autologous stem cells are the recommended source for stem cell therapy in tissue engineering. An adequate number of cells is an important factor. However, it is often difficult to derive the required number of cells in a short period of time, especially when large defects are to be treated. For example, critical-sized bone defect healing is still a challenge in orthopaedics. Long-term *ex vivo* expansion of stem cells is time consuming, and timing for applying cells in the therapeutic process may be crucial. Increased culture time also raises the risk of contamination and mutagenesis. Therefore, in this thesis, I focus on increasing the efficiency of MSCs
used in bone tissue engineering by utilising SDF-1, which due to its stem cell recruiting property may compliment the cells which have been introduced from the recipient’s stem cell pool.

Before applying SDF-1 in bone tissue engineering, an understanding of the effect of SDF-1 in stem cell osteogenic differentiation is necessary. In chapter 2, I demonstrate the role of SDF-1 in stem cell osteogenic differentiation \textit{in vitro}. Before the \textit{in vivo} study, the establishment of functionally SDF-1 over-expressing rat bone marrow cells is described in chapter 3. Chapter 4 then assesses the bone healing effect of these SDF-1 over-expressing cells in a rat femoral defect model with a relatively low cell number applied. Chapter 5 investigates the effect of double injection of the SDF-1 over-expressing cells in bone fracture healing.

\textbf{Aim}

The aim of this study is to improve fracture healing by using a small number of cells, which enhances the local SDF-1 protein level in the fracture site.

\textbf{General hypothesis}

The hypothesis of this thesis is that bone healing will be enhanced by transplanting low amount of SDF-1 overexpressing bone marrow cells, which would not be achieved by the same amount of normal bone marrow cells.
Chapter Two
Enhanced Osteoblastic Differentiation by Stromal Cell-Derived Factor-1 in Human Mesenchymal Stem Cells
2.1 INTRODUCTION

By studying the mechanism of cell migration during embryonic development, the interaction between SDF-1 and CXCR4 has been implicated in many developmental procedures. Abnormal phenotypes have been observed in the SDF-1/CXCR4 gene deletion models, of which all can be explained by abnormal cell migration during development (Miller et al. 2008). It has been also reported that SDF-1 and CXCR4 play roles in stem cell homing and are related to short-term and long-term engraftments of stem cells (Lapiodot et al. 2005).

Although a number of studies focus on the character of SDF-1/CXCR4 in cell migration and growth, the understanding of SDF-1/CXCR4 in osteogenic differentiation of stem cells is unclear. In bone tissue engineering, the number of studies that indicate a role for SDF-1/CXCR4 interaction on osteogenic differentiation or new bone formation is limited. Retroviral transduced bone marrow stromal stem cell lines secreting high SDF-1 levels displayed an enhanced ability to form ectopic bone in vivo when mixed with hydroxyapatite/tricalcium phosphate ceramic particles and implanted into subcutaneous pockets on the dorsal surface of nonobese diabetic/severe combined immunodeficient (NOD/SCID) mice (Kortesidis et al. 2005). Furthermore, blocking SDF-1/CXCR4 signalling strongly inhibited bone morphogenetic protein 2 (BMP2)-induced osteogenic differentiation of ST2 bone marrow stromal cells (Zhu et al. 2007). On the other hand, SDF-1 promotes the development and survival of human osteoclast and the osteoclastogenesis of human oral cancer cells (Wright et al. 2005; Tang et al. 2008a). According to the above studies, it is likely that SDF-1/CXCR4 effects osteogenic differentiation. The purpose of this study is to investigate the possible role of SDF-1 in osteogenic differentiation of human mesenchymal stem cells (hMSCs). Will SDF-1 induce or enhance the osteogenic differentiation in hMSCs? The hypothesis of this study is that SDF-1 will improve the osteogenic differentiation of hMSCs.
2.2 MATERIALS AND METHODS

2.2.1 Cell isolation and culture

Human mesenchymal stem cells (hMSCs) were a gift from Professor Oscar Lee (National Yang-Ming University, Taiwan) and obtained from the iliac crest of one of the healthy donors (n=14) with informed consent. The phenotypes of these cells were previously described by flow cytometry and the multipotency of the cells was also tested (Lee et al. 2004). Briefly, the cells are negative of: CD13, CD34, CD45, CD133 and positive of: CD29, CD44, CD71, CD73, CD90, CD105, SH-2 and SH-3. hMSCs were cultured in growth medium, which consists of Iscove’s modified Dulbecco’s medium (IMDM) (Sigma-Aldrich, MO) and 10% fetal bovine serum (HyClone, UT) supplemented with 10 ng/mL fibroblast growth factor-basic (bFGF), 100 units of penicillin, 1000 units of streptomycin, and 2 mmol/L L-glutamine (Sigma-Aldrich, MO). Medium was changed twice per week during cell culture.

2.2.2 In vitro osteogenic differentiation

To investigate whether SDF-1 has an effect on osteogenic differentiation, four groups were included in my study with 3 repeats in each group (Table 2-1).

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Positive control</td>
<td>Cells were treated with osteogenic medium consisting of Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 0.1 M dexamethasone, 10mM β-glycerol phosphate, and 0.2 mM ascorbic acid (Sigma-Aldrich, MO)</td>
</tr>
<tr>
<td>2</td>
<td>Negative control</td>
<td>Cells were treated with IMDM only</td>
</tr>
<tr>
<td>3</td>
<td>SDF-1 group</td>
<td>Cells were treated with IMDM plus 30ng/ml human recombinant SDF-1 (R&amp;D system, MN)</td>
</tr>
<tr>
<td>4</td>
<td>Positive control plus SDF-1 group</td>
<td>Cells were treated with osteogenic medium plus 30ng/ml human recombinant SDF-1</td>
</tr>
</tbody>
</table>

Table 2-1. Experimental group layout.
Seventh- to ninth-passage hMSCs were used and seeded at 5000 cells/cm² density which is a compromise between better calcium deposit formation and cell detachment during osteogenic differentiation (Jaiswal et al. 1997). Medium was changed twice per week and cells were harvested and analysed after 1, 2, 3 and 4 weeks treatment.

2.2.3 Morphological observation
To observe the morphological changes of the cells during the osteogenic differentiation, cells seeded at 5000 cells/cm² density in 6-well plates in different groups were photographed under the light microscope at weekly intervals.

2.2.4 Alkaline phosphatase activity assay
Alkaline phosphatase (ALP) is a membrane-bound ectoenzyme that hydrolyses monophosphate esters. It is expressed on the cell membrane of hypertrophic chondrocytes, osteoblasts and odontoblasts. By hydrolysing its substrates, which are inhibitors of hydroxyapatite formation during extracellular matrix mineralization, ALP is considered to be an early marker for osteogenic differentiation of osteoblast precursors (Orimo 2010; Hoemann et al. 2009).

Total cell proteins were extracted from cell lysates, which were lysed by using radioimmuno precipitation assay (RIPA) buffer (150mM sodium chloride, 50mM Tris, 0.5% (w/v) DOC, 0.1% (w/v) sodium dodecyl sulfate, 1% (w/v) NP-40) (Sigma-Aldrich, MO). hMSCs seeded in 25T flask were scraped off the surface using a cell scraper with 500μl phosphate buffered saline (PBS). The cells were collected by 2000rpm centrifugation for 5 minutes at room temperature. One hundred μl RIPA buffer was used to lyse the cells for 30 minutes on ice. Proteins were collected by harvesting the supernatant after 14000rpm centrifugation for 15 minutes at 4°C. Protein quantification was performed using a commercial protein assay kit (PIERCE, IL). Ten μg total proteins were combined with distilled water to approach 50 μl protein samples and then mixed with 50μl ALP assay reagent (5mg p-nitrophenyl phosphate dissolved in the solution which consists of 95% (v/v) 2M Diethanolamin and 5% (v/v) 20mM Magnesium Chloride) (Sigma-Aldrich, MO). Absorbance at
405nm wavelength was read at 5 minutes using a microplate reader (Bio-Rad Model 3550, CA). There were three replicates at each time point in each group.

### 2.2.5 Cell proliferation assay

Cell proliferation was estimated by using a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (CellTiter 96® AQueous One Cell Proliferation Assay, Promega, WI), which is a colorimetric method based on the production of coloured formazan. To prevent cell confluence and overgrowth, hMSCs were seeded at a lower cell density of 4000 cells/cm² in each well of 24-well plates. Three hundred μl basal medium (IMDM) and 60μl MTS reagent were mixed and added into each well, after removing the culture medium. Cells were incubated in the dark at 37°C for 90 minutes. One hundred μl of the supernatant of each sample were removed and the absorbance was measured at 490nm wavelength by a microplate reader. There were three replicates at each time point in each group.

### 2.2.6 Von Kossa staining

Calcium phosphate deposition is considered a functional in vitro endpoint of osteogenic differentiation. By utilising silver nitrate, Von Kossa staining can visualise the phosphate within the deposited mineral (Hoemann et al. 2009).

hMSCs were seeded at 5000 cells/cm² density in 6-well plates for each time point. Culture medium was removed before washing the cells twice by PBS and fixing with 4% formaldehyde for 20 minutes, followed by washing twice with water before covering the cells with 1% silver nitrate and incubating under UV light for 45 minutes. After washing out the silver nitrate solution, calcium deposits were observed under the light microscope.

### 2.2.7 Statistics

Results were first analysed by Kruskal-Wallis test with the level of significance of p<0.05. Significantly different results were further analysed by Mann-Whitney U test with the significant level of p<0.05.
2.3 RESULTS

2.3.1 Cell morphology

Cell morphology of hMSCs became more flattened and had less space between cells in Group 1 and Group 4 after 7 days of culture (Figure 2-1). In Group 3, cell morphology was still spindle and fibroblastic-like, and these were similar to the cell shape in Group 2, suggesting that SDF-1 alone had no effect.

Figure 2-1. Cell morphology after 7 days. Both Group 1 and Group 4 showed an osteoblast-like cell shape from the first week of osteoinductive treatment while Group 2 and Group 3 remained in a fibroblastic-like cell shape during the whole time points. Scale bars, 100μm.
2.3.2 ALP activity after SDF-1 treatment

Figure 2-2 showed ALP activities of all study groups at all time points. There were no differences between Groups, 2 and 3. However, the ALP activity of hMSCs in Group 4 was significantly higher than Group 1 up to the third week (p=0.024). After 4 weeks treatment, there was no significant difference between Group 1 and Group 4.

Figure 2-2. ALP activity of hMSCs was measured in weekly intervals during osteoinductive treatment. The absorbance at 405nm wavelength of each group is shown. Data points has different letter (a, b, c, d) are significantly different to each other (p < 0.05).
2.3.3 hMSCs Von Kossa staining

Both osteo-induction groups, Group 1 and Group 4, were calcium positive for Von Kossa staining after 4 weeks (Figure 2-3). hMSCs in Group 4 had more calcium deposits than Group 1. There was no calcium deposited in Group 2 or Group 3.

![Figure 2-3. Von Kossa staining after 4 weeks osteoinductive treatment. Only Group 1 and Group 4 showed calcium deposits while Group 2 and Group 3 had no calcium deposit. Scale bars, 100μm.](image-url)
2.3.4 Cell proliferation

Figure 2-4 showed that the cell proliferation in Group 1 and Group 4 had almost doubled when compared with Group 2 and Group 3. The differences were significant at all time points except the third week. There was no significant difference between Group 1 and Group 4 nor between Group 2 and Group 3.

Figure 2-4. Cell proliferation was measured by MTS assay in weekly intervals during the osteoinductive treatment. The absorbance at 490nm wavelength of each group is shown. Data points has different letter (a, b, c, d) are significantly different to each other (p < 0.05). Only in data (21 days), there was no significant difference between each two continuing letters (p > 0.05).
2.4 DISCUSSION

The chemo attractive effect of SDF-1 to the cells, which express its receptor CXCR4 on the surface, is well known. However, the effects of SDF-1 on osteogenic differentiation in stem cells remain unknown. In my study, hMSCs were cultured with SDF-1 or with a combination of osteoinductive medium and SDF-1, to investigate the effect of SDF-1 on stem cell osteogenic differentiation. Morphologically, cells cultured in osteoinductive medium (Group 1 and Group 4) showed a flattened more iso-diametrical cell shape with greater cell contact, which are common signs of osteogenic differentiation (Declercq et al. 2005). In contrast, cells cultured with SDF-1 alone (Group 3) remained in a spindle and fibroblast-like shape during the whole treatment, as in the negative control (Group 2). In the ALP assay, cells grown in SDF-1 alone (Group 3) remained at the same level of the negative control (Group 2) during the whole experiment. The groups containing osteoinductive medium showed an expected up-regulated ALP activity. According to the morphology and ALP activity assay, SDF-1 on its own showed no effect in hMSCs osteogenic differentiation. This was also consistent with the result of Von Kossa assay for calcium deposits.

Although it did not show any effect of SDF-1 alone on stem cell osteogenic differentiation, the most interesting finding in this study is that SDF-1 can enhance differentiation under specific conditions. When cells were cultured with SDF-1 and osteoinductive medium (Group 4), they showed a significantly higher ALP activity than the positive group (Group 1) during the first three weeks, indicating an enhanced early osteogenic differentiation. Calcium deposits were only found in Group 1 and Group 4 at the fourth week. Larger amounts of calcium deposits in Group 4 may also be due to the enhanced effect of SDF-1. Because of the cell proliferation pattern was similar between Group 1 and Group 4 (Figure 2-4, no significant difference), it suggests that there are more calcium deposits per cell in Group 4, which may due to a more mature osteogenic differentiation. These results may indicate an enhanced role of SDF-1 in osteogenic differentiation.

One possible explanation of the observation in this study is that SDF-1 can affect osteogenic differentiation through signal transduction. SDF-1 has been shown to
stimulate cell proliferation and recruit CXCR4 expressing cells in a dose dependant manner. Studies in human meningioma cells and pre-B cells showed that SDF-1 enhanced cell proliferation via the activation of the extracellular signal regulated kinase1/2 (ERK-1/2) signalling pathway (Acharya et al. 2009; Barbieri et al. 2006). Activation of the ERK-1/2 pathway also leads to nuclear translocation of nuclear factor-κB (NF-κB) in prostate cancer cells and osteosarcoma cells, which increases the cell migration (Ganju et al. 1998; Kukreja et al. 2005; Huang et al. 2009). The ERK-1/2 pathway is one of the mitogen-activated protein kinase (MAPK) cascades, which are important signalling pathways that convert extracellular signals into cellular responses and regulate proliferation, differentiation, cell activation and immune responses (Pearson et al. 2001; Pleschka 2008). The activation of ERK-1/2 signalling also contributes to osteoblast differentiation by the phosphorylation of Runx2, a crucial transcription factor for osteoblast differentiation (Raucci et al. 2008; Franceschi et al. 2007).

Runx2 can up-regulate osteocalcin gene expression by binding to the osteoblast-specific element 2 (OSE2) in the osteocalcin promoter. Moreover, OSE2-like elements are also present in the promoter of many osteoblast genes, such as collagen type I, osteopontin (Ducy et al. 1997). Over expression of Runx2 up-regulated the gene expression of collagen type I and osteocalcin, and enhanced ALP activity (Byers and Garcia 2004). The regulation of Runx2 can be affected by many different signalling pathways (Franceschi and Xiao 2003). Bone morphogenetic proteins (BMPs), which are the best described inducers of osteoblast and chondrocyte differentiation, by binding to its type I and type II BMP receptors, will up-regulate Runx2 gene expression through the Smads signalling pathway (Ducy et al. 1997; Javed et al. 2008). On the other hand, post-translational modification and protein-protein interactions can also regulate this factor. As mentioned above, the ERK-1/2 signalling pathway can activate Runx2 by phosphorylation. Transgenic expression of constitutively active MEK1, the up stream MAPK kinase of ERK-1/2, in osteoblasts also accelerated skeletal development (Franceschi et al. 2007).

In this study, the osteoinductive medium, which consists of dexamethasone, β-glycerol phosphate and ascorbic acid, has been applied to induce osteogenic
differentiation in many types of cells by increasing Runx2 gene expression (Bielby et al. 2004; Justesen et al. 2004; Mimori et al. 2007). Therefore, the up-regulated ALP activity in Group 4 may be due to the synergy between the increased gene expression of Runx2, induced by the osteoinductive medium, and the activation of the Runx2 by SDF-1 through the activated ERK-1/2 pathway. Interestingly, Franceschi et al. showed a similar increased ALP activity when co-transducing osteoblasts with BMP and Runx2 viruses compared with either virus alone (Franceschi and Xiao 2003). Mimori et al. showed that an ERK-1/2 inhibitor suppressed ascorbic acid-induced ALP gene expression, whereas Runx2 was not affected (Mimori et al. 2007). All these findings indicate that SDF-1 can enhance the osteogenic differentiation through the ERK-1/2 pathway. However, the result of the Group 3 suggests that SDF-1 will have this enhancing effect only in a Runx2 induced environment (Figure 2-5).

Figure 2-5. An overview of BMP and SDF-1 signal transduction pathway. Arrows stand for activating effect (graph modified from Franceschi RT 2003).
According to this Runx2 dependant enhancing effect of SDF-1 in osteogenic differentiation, it is interesting to investigate whether combining BMPs and SDF-1 will have synergistic effects in stem cell osteogenic differentiation. BMP signalling through the Smads cascade can increase the transcription and translation of Runx2. Therefore, by the ability of SDF-1 to activate Runx2 through the ERK1/2 pathway, the combination of BMPs and SDF-1 may further enhance stem cell osteogenic differentiation.

Both osteoinductive groups showed increased cell proliferation, which may be due to the transient amplified cells during the osteogenic differentiation. In Group 3, SDF-1 alone didn’t show effects in cell proliferation, and this is in contrast to other studies, which showed enhanced cell proliferation in SDF-1 overexpressing cells (Kortesidis et al. 2005; Hwang et al. 2006). This may indicate that a constantly high SDF-1 level is necessary to enhance cell proliferation.

From the results in this chapter, it has shown that SDF-1 has the ability not only to recruit the CXCR4 expressing cells, as been well reported by literatures, but also to enhance stem cell osteogenic differentiation. These two properties of SDF-1’s multicharacters have inspired the applications of SDF-1 in bone tissue engineering. One concept of this is whether the locally enhanced level of SDF-1 in bone injured sites can improve the bone healing by recruiting more stem cells into the sites and enhancing osteogenic differentiation. To answer this question, a reliable SDF-1 overexpressing system needs to be established.
Chapter Three
SDF-1 Gene Incorporation into MSCs using the Adenoviral Delivery System of SDF-1
3.1 INTRODUCTION

For bone regeneration, direct delivery of growth factors, for instance BMP-2, 4 and 7, to the site of repair has disadvantages such as a short half-life and instability of the proteins, which result in low efficacy with the need of repeated administration (Hao et al. 2009). It is also not financially feasible. Thus, gene therapy has been focused on transfection of autologous cells with therapeutic genes in vitro before in vivo transplantation, which leads to consistent expression of target genes (Hao et al. 2009).

Gene therapy involves the transfer of genes to and into cells and either modulates the expression of the target genes or directly affects production of proteins. In orthopaedic research, gene therapy has resulted in progress of the treatment of chronic and acute genetic and non-genetic disorders. Gene therapy relies on delivery mechanisms to enable the genes of interest to enter the target cells and in some cases, the nucleus. The vehicles that encapsulate therapeutic genes for delivery are called vectors. To achieve most clinical applications, the vectors must be non-toxic, induce the minimal immunogenic response and be able to have high transfer efficiency in a wide range of cell types (Nixon et al. 2007).

According to the delivery methods, there are viral and non-viral vectors for gene delivery. Viruses are potent vectors because of their ability to invade cells and to deliver genetic material to the nucleus for incorporation into the genome. The most common viruses used in gene therapy are adenovirus, retrovirus, lentivirus and adeno-associated virus (AAV), and each has various advantages and disadvantages (Lind and Bünger 2005). Non-viral delivery systems can be physical, mechanical or chemical. Electroporation can increase cell membrane permeability to facilitate DNA intracellular flux by the electrical energy. Lipofection is a method where DNA material is coated with cationic lipids, which are readily taken up by cells due to their electrochemical properties. Non-viral delivery systems are generally less efficient than viral techniques due to the poor incorporation of the desired DNA material into the genome (Lind and Bünger 2005).
Among the viruses in gene therapy, adenoviruses are DNA-containing, non-enveloped viruses. The adenovirus genome consists of a single piece of linear double stranded DNA, approximately 36 kb long and flanked by two short-inverted terminal repeats. Adenoviruses enter cells by receptor-mediated endocytosis. Once the virus genome is released into the nucleus, the viral early genes are transcribed, leading to DNA replication, late transcription, synthesis of viral structural proteins and virus assembly (Seth 2000, 2005; Witlox et al. 2007). By replacing viral sequences in adenovirus DNA by foreign cDNA, recombinant adenoviruses, which carry genes of interest, can be generated (Figure 3-1) (Imperiale and Kochanek 2004).

Figure 3-1. A concept of adenoviral infection. A new gene is injected into an adenovirus vector, which is used to introduce the modified DNA into a human cell. The adenovirus enters the human cell by receptor-mediated endocytosis. The endocytosed vector will then enter the nucleus by binding to nuclear pore complex and release the genetic materials to make the cell produce the new protein, for example, the eNOS. CAR, coxsackie virus-adenovirus receptor; IR, integrin receptor; eNOS, nitric oxide synthase. (Khurana and Meyer 2003).
Adenovirus is the most popular viral gene delivery system. Unlike retrovirus, which will only infect the dividing cells and randomly insert the genetic material into the genome, adenovirus will infect both dividing and non-dividing cells and does not incorporate genes into host DNA. Therefore, adenovirus gene transfer gives higher infection rates and, in term of safety, less risk of mutagenesis (Lind and Bünger 2005). In bone tissue engineering, adenovirus carrying bone morphogenetic protein-2 gene (Ad-BMP-2) has been used in fracture healing by increasing new bone formation. A locally increased level of BMP-2, which resulted from in vivo transduction by Ad-BMP-2, showed significantly improved healing of segmental bone defects (Betz et al. 2006). Either direct delivery of adenovirus carrying BMP-7 (Ad-BMP-7) or implantation of dermal fibroblasts transduced ex vivo with Ad-BMP-7 to subcutaneous sites, led to a robust osteogenic response (Franceschi et al. 2000, 2004).

Therefore, in the studies of this thesis, adenovirus will be a useful and feasible gene delivery vector to investigate whether increased local levels of SDF-1 improve fracture healing in vivo. Before the in vivo study, a reliable viral system of Ad-SDF-1 transfer needs to be established and tested. In this chapter, I focus on the infection ability of the adenovirus and the optimal multiplicity of infection (MOI) for rat bone marrow cells and hMSCs. Whether the Ad-SDF-1 infected cells can over-express and secrete functional SDF-1 was also tested.
3.2 MATERIALS AND METHODS

3.2.1 Cell isolation and culture

Rat bone marrow cells (rBMCs) were harvested from young adult male Wistar rats, a general multipurpose rat model. Rats were killed by cervical dislocation. The skin covering the thigh area was shaved and then disinfected using Hydrex. Under aseptic conditions, an incision was made in the skin to expose the muscles overlying the femur, which were dissected away. The femurs were removed and soaked in culture medium which was composed of Dulbecco's Modified Eagle Medium (DMEM), 10% fetal calf serum, 100 unit/ml Penicillin, 100 mg/ml Streptomycin (Sigma-Aldrich, MO). Under a tissue culture hood, the femora were removed from the medium and both ends of the bone were resected using a sterile scalpel. The marrow was flushed out with 5 ml culture medium by using a 10 ml syringe with a 20 gauge needle. The flushing was repeated several times to obtain the maximum amount of bone marrow cells. The cells were collected in a 25 cm² flask after being pumped through a cell strainer. The flask was then placed into an incubator at 37°C, 95% air and 5% CO₂. The media was changed after 4 days and non-adherent cells were removed, together with the media (Figure 3-2). The media was changed twice a week thereafter. After 10-14 days of primary culture, the cells reached confluence and were passaged using Trypsin-EDTA (Sigma-Aldrich, MO). Cells were then passaged about every 7-8 days. Passage 4 and passage 5 cells were used for studies (Figure 3-3).
Figure 3-2. Cell morphology of rBMCs of passage 0 (arrow). Cells harvested from bone marrow show a spindle cell shape when they attached to the surface of the culture plate. White spots are hematopoietic cells. Scale bar, 100μm.

Figure 3-3. Cell morphology of rBMCs of passage 4 (arrow). Cells show a flattened cell shape after cell passage. Scale bar, 100μm.
3.2.2 Adenovirus preparation and infection

The concept for preparing the adenovirus carrying SDF-1 gene and the procedures in this chapter are shown in Figure 3-4. Human SDF-1 gene is first combined with the adenoviral vector and transfected into the packaging cell line to produce the adenovirus. Viral particles were then collected. The infection efficiency of the adenovirus to hMSCs and rBMCs was tested in vitro. The SDF-1 overexpressing rBMCs were transplanted into a rat model, described in Chapters 4 and 5.

Figure 3-4. A concept of adenovirus preparation and infection in this chapter.
Human SDF-1 cDNA were obtained from National Yang-Ming University VYM Genome Research Center, Taiwan. The production of adenovirus encoding human SDF-1 gene (Ad-SDF-1) was carried out using AdEasyTM XL Adenoviral Vector System (Stratagene, CA). Human SDF-1 gene and the pShuttle-CMV vector were both cut by restriction endonucleases Not I and Xho I to form two types of specific sticky end. Human SDF-1 cDNA was then cloned into the pShuttle-CMV vector through the matches of Not I and Xho I cutting sides by using Thermus aquaticus (Taq) DNA ligase. The incorporated shuttle vector was then linearised with Pme I restriction endonuclease and transformed into BJ5183-AD-1 competent cells, which is a prokaryotic host strain. Transformants are selected for kanamycin resistance, and recombinants were subsequently identified by restriction digestion. After a recombinant was identified, its copies were largely expended in bulk using the recombination-deficient XL 10-Gold strain. Purified recombinant plasmid DNA was digested with Pac I restriction endonuclease to expose its inverted terminal repeats (ITRs), and was then used to transfect AD-293 cells, a cell line modified from human embryonic kidney cells, where deleted viral assembly genes, which are the E1 and E3 genes, are complemented in vivo (Figure 3-5).
Figure 3-5. Production of recombinant adenovirus using the AdEasy XL adenoviral vector system (Stratagene, Catalog No. 240010).
After showing serious cytopathic effect, normally 2 to 3 days after transfection, the cells were collected by flushing with PBS. The cell suspension was transferred to centrifuge tubes and subjected to four rounds of freeze/thaw by alternating the tubes between liquid-nitrogen and a 37°C water bath. After centrifugation of the cellular debris, Ad-SDF-1 was collected by harvesting the supernatant. The Ad-SDF-1 was then mixed with an equal volume of 2-times virus stock solution (20mM Tris, 4mM magnesium chloride and 8% sucrose; Sigma-Aldrich MO) and stored in -70°C. The virus titer was measured by plaque assay (see Appendix I). Cell infection was performed by adding a targeted amount of virus within 200μl serum-free medium to the cells in a 24-well plate. The plate was then incubated at 37°C for 2 hours, and the medium was then changed back to growth medium.

In this study, the adenovirus carrying the LacZ gene (Ad-LacZ), which encodes β-galactosidase in *E. coli*, with a similar producing procedure from Professor Yu Sue’s Laboratory, National Yang-Ming University, was also used to determine the optimal multiplicity of infection for different cell types.

### 3.2.3 Optimal multiplicity of infection (MOI) for hMSCs and rBMCs

Thirty thousand hMSCs (passage 9) or rBMCs (passage 5) were seeded in each well of 24-well plates and cultured at 37°C in an incubator overnight. Cells were then infected by Ad-LacZ with different MOI from 0 to 500 (6 replicates for each MOI group). After infection, cells were cultured for two days before testing the activity of β-galactosidase. To detect the expression of β-galactosidase, an assay was used which digests X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) into galactose and an insoluble blue product (5,5’-dibromo-4,4’-dichloro-indigo). Cells with β-galactosidase activity appear blue and can be easily distinguished.

To do this, cells were gently washed with cold phosphate buffered saline (PBS) twice for 5 minutes each time. Two hundred and fifty μl of fix solution (2% formaldehyde and 0.2% glutaraldehyde; Sigma-Aldrich, MO) was than added into each well for 5 minutes at room temperature. After two more cold PBS washes, 250 μl of staining solution (20mM potassium ferricyanide, 20mM potassium ferrocyanide, 2mM
magnesium chloride and 0.5 mg/ml X-gal; Sigma-Aldrich, MO) was added into each well. The cells were kept in the dark at 37 °C overnight. Blue cells were counted under the light microscope.

3.2.4 SDF-1 expression measurement
Thirty thousand hMSCs (passage 11) or rBMCs (passage 5) were seeded in each well of 24-well plates and cultured at 37°C in an incubator overnight. Cells were then infected by Ad-SDF-1 with different MOI from 0 to 500 (6 replicates for each MOI group). After two days in normal culture medium, the medium was changed to serum-free DMEM for 3 days. The concentration of secreted SDF-1 in the medium was detected by enzyme-linked immunosorbent assay (ELISA) of SDF-1, which was determined by the absorbance at 450nm wavelength (R&D systems, MN) (see Appendix I).

3.2.5 In vitro cell migration assay
Thirty thousand hMSCs (passage 9) or rBMCs (passage 4) were seeded in 24-well plates and cultured at 37°C in an incubator overnight. Cells were then infected by various MOI of Ad-SDF-1 according to the optimal MOI assay for each cell type (for hMSC: 0, 125 and 250; for rBMCs: 0, 250 and 500; 6 replicates for each MOI group) and cultured in normal medium. On the fourth day after infection, another 4,500 cells were seeded on the upper surface of a polycarbonate membrane with 0.8μm porosity in a trans-well chamber (Corning, Fisher Scientific, UK) (Figure 3-6) and cultured at 37°C in an incubator overnight. After 5 days, the chamber was combined with infected cells for 6 hours. Before combination, the cells that had spontaneously migrated to the opposite side of the membrane were removed using cotton buds. Cells that migrated to the opposite site of the membrane after 6 hours were fixed and stained with Toluidine blue (see Appendix I), and cell counted.
Figure 3-6. Corning Transwell (http://www.ub.es/biocel/wbc/images/cultivo/transwell.jpg).

3.2.6 Statistics

Results were analysed with one-way analysis of variance (ANOVA) test at the level of significance of $p \leq 0.05$. For ANOVAs with significant F tests, a Tukey’s post-hoc procedure was performed to compare the significance between the two groups.
3.3 RESULTS

3.3.1 Virus production and optimal MOI for hMSCs and rBMCs

A cytopathic effect is one of the easiest and earliest signs of adenovirus production. After the linearised recombinant adenovirus plasmid had been transfected into AD293 cells, a cytopathic effect was observed. The original AD293 cells, which were modified human embryonic kidney 293 cells, showed a flattened and polygonal cell shape. These cells could be cultured at high cell density and were fully congruent. Once the cytopathic effect appeared, cells became swollen and cylinder-like in shape. Cell contact was highly reduced (Figure 3-7 and Figure 3-8). This indicates the production of the Ad-SDF-1 in the AD293 cells.

Figure 3-7. Normal cell morphology of AD293. Cells can be cultured at high cell density. Scale bar, 100 μm.

Figure 3-8. Cytopathic effect of AD293 cells after Ad-SDF-1 infection. Cells become swollen and cylinder-like in shape (arrows). Cell contact is reduced. Scale bar, 100 μm.
To test the tolerance of different cells for adenovirus infection and the most efficient MOI, Ad-LacZ was used. hMSCs and rBMCs were infected by different MOI and the cell’s β-galactosidase activity was tested (Figure 3-9 and Figure 3-10). Both hMSCs and rBMCs showed positively stained cells. From very low MOI, an increased amount of blue cells in MOI groups higher than 175 was shown. Seventy per cent of these cells were infected at MOI 500.
Figure 3-9. Infection efficiency of adenovirus to hMSCs detected by X-gal staining of hMSCs β-galactosidase activity (numbers represent the MOI used). Scale bar on the bottom right, 100μm.
Figure 3-10. Infection efficiency of adenovirus to rBMCS detected by X-gal staining of hMSCs β-galactosidase activity (numbers represent the MOI used).

Scale bar on the bottom right, 100μm.
3.3.2 SDF-1 expression in hMSC and rBMCs after infection by Ad-SDF-1

SDF-1 expression in hMSCs and rBMCs was estimated at the fifth day after the infection by Ad-SDF-1 of various MOI (Figure 3-11). SDF-1 expression of hMSCs was up-regulated with MOI 25 and reached maximum expression at MOI 250. Severe cell damage, which led to cell death, was observed from MOI 250 to 500. In rBMCs, the expression of SDF-1 was up-regulated using MOI 125 and reached the maximum at MOI 500. Severe cell damage was not observed for all different MOI.

Figure 3-11. SDF-1 expression of hMSCs and rBMCs 5 days after Ad-SDF-1 infection with different MOI (mean ± S.D.). Data points have different letters are significantly different to each other (p < 0.05).
3.3.3 In vitro cell migration

Both hMSCs and rBMCs showed significant and dose-dependent chemoattractive activity to higher SDF-1 concentration, which was secreted by the Ad-SDF-1 infected stem cells (Figure 3-12). In hMSCs, there were twice as many cells in the infected group compared with the non-infected group (MOI 0). The MOI 125 group and the MOI 250 group secreted the same level of SDF-1 and showed the same level of response in hMSCs migration. Furthermore, 2-3 times more rBMCs migrated to the opposite side of the membrane in the MOI 250 group and the MOI 500 group compared with the non-infected group.

![hMSCs Migration assay](image)

![rBMCs migration assay](image)

Figure 3-12. Cell migration of the non-infected (normal) hMSCs and rBMCs due to the secreted SDF-1 in Ad-SDF-1 infected hMSCs and rBMCs after 6 hours (mean ± S.D.). Both hMSCs and rBMCs show a dose-dependent response to higher SDF-1 concentration secreted in higher MOI groups. Data points have different letters are significantly different to each other (p < 0.05).

74
3.4 DISCUSSION

The purpose of this chapter is to establish a reliable system for modifying hMSCs and rBMCs in order to make these cells overexpress SDF-1. Adenovirus, because of its high infection efficiency for a wide range of cell types and its non-integrating character, is an optimal system for this purpose.

The cytopathic effect of AD293 cells during the production procedure of Ad-SDF-1, indicated the assembly of virus particles within the cells. In order to amplify the amount of Ad-SDF-1, serial infections were performed by collecting the viruses from small amounts of AD293 cells and then infecting a larger amount of AD293. For example, the viruses collected from one 25T flask of AD293 cells were then added into another 75T flask with confluent AD293 cells. During this amplifying procedure, the cytopathic effect was the sign of a successful infection, which represents functional adenoviruses were produced.

Adenovirus infects cells by the attachment of Coxsackie adenovirus receptor (CAR) on the cell surface. The expression pattern of CAR varies, not only at different developmental stages and with different tissues, but also between species (Tallone et al. 2001). Thus, the sensitivity of the target cell to adenoviral infection will vary according to the cell type and the condition of the cell. It is necessary to test the optimal MOI for hMSCs and rMBCs to obtain the balance between the infection rate and cell damage. The infection rate of each cell was dose dependant and reached about 70% rate at MOI 500. However, cell damage in hMSCs appeared using an MOI 250 but was not seen in rBMCs, which showed a higher tolerance to adenoviral infection of rat cells.

After confirming the adenovirus’ infection ability on both types of cell, we next quantified the expression of SDF-1 of the infected cells and, also, tested the function of the secreted SDF-1 in vitro. The expression of SDF-1 in both types of cell showed a dose dependant effect, respectively. In rBMCs, SDF-1 expression kept arising after MOI 125, however, enhanced SDF-1 expression halted after MOI 125 in hMSCs. Severe cell damage, was again observed after MOI 250 in hMSCs, which reflects the nature of SDF-1 expression. In the functional test, the secreted SDF-1 can
successfully increase cell migration towards the infected cells in a dose dependant manner. The reduced cell migration at MOI 250 in hMSCs may be due to the cell damage, which resulted in a similar concentration of secreted SDF-1 in the medium (Figure 3-11).

Comparing the tolerance to adenovirus, the expression of SDF-1 and the activated cell migration, hMSCs started expressing SDF-1 with less virus particles and had more cell migration at the same MOI 250 compared with rBMCs. This indicates that hMSCs are more sensitive to adenoviral infection. However, hMSCs are less infected at high MOI due to induced cell death and, therefore, are unable to secrete a high level of SDF-1. rMBCs, by contrast, are more tolerant of the high MOI and have the ability to cause more cell migration.

In this chapter, a reliable adenoviral gene modifying system has been established. The effect of SDF-1 in bone tissue engineering in vivo will then be tested by transplantation of cells, which are infected by Ad-SDF-1. Because of the higher tolerance to adenoviral infection and higher expression of SDF-1, and also, to avoid the possibility of varying results due to different species, rBMCs will be used for the following chapters.
Chapter Four

The *in vivo* Effect of SDF-1 in Bone Healing
4.1 INTRODUCTION

Stem cell migration plays a crucial role during development and repair processes. Studies have revealed that the SDF-1/CXCR4 interaction is one of the important factors that mediate cell migrations. SDF-1, which belongs to the CXC chemokine family, and its G protein coupled receptor CXCR4 are widely expressed during the development in the embryo (Miller et al. 2008). Deletion of the SDF-1/CXCR4 genes causes many different phenotypical deficits included in β-lymphopoiesis and myelopoiesis (Nagasawa 2007; Zou et al. 1998), cardiogenesis (Zou et al. 1998), angiogenesis (Tachibana et al. 1998) and neurogenesis (Tran et al. 2007). These conditions are all related to the abnormality of the stem/progenitor cell migration during development. SDF-1/CXCR4 interaction is also critical in repair and regeneration. SDF-1 is expressed by bone marrow endothelium cells and enhances the retention of CXCR4 expressing hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) in the stoma (Kaplan et al. 2007). Many studies have shown that during tissue repair, local SDF-1 expression is up regulated and acts as a signal to recruit the CXCR4 expressing cells from circulation and/or bone marrow. Up-regulated SDF-1 expression level was also observed in human heart transplantation in response to ischemic injury and more recipient’s cells were recruited into the injury site (Yamani et al. 2005) indicating that SDF-1 leads to the recruitment of stem cells to the site of expression.

Many clinical conditions require regeneration and implantation of bone. In the past few years, gene- and stem cell-based therapy have been extensively studied for use in the regeneration of massive bone defects as an alternative to current solutions, such as bone grafting and protein-based therapy (Kimelman et al. 2007). For instance, the use of autologous bone graft is considered as the gold standard for use in repairing bone defects, but the use of autogenic bone may cause donor site morbidity which may lead to infection, bleeding and pain. It is also only successful with relatively small bone defects (Mastrogiacomo et al. 2005). Direct delivery of osteoinductive factors, such as bone morphogenetic proteins, has shown improved results in bone healing, but the need of expensive doses of the factors to achieve the improvement is the drawback for its general application (Kimelman et al. 2007). MSCs have been considered to be a potential cell source for bone tissue engineering due to their
proliferation, differentiation and immunosuppression properties (Salgado et al. 2004). The clinical application of bone tissue engineering is often limited by the cell number and immune response of implanted cells. For example, the combination of MSCs and allograft has shown improved bone formation around revision hip replacements (Korda et al. 2008). However, a $100 \times 10^6$ cell dose was applied. For clinical situations, the relatively small number of MSCs isolated from bone marrow aspirates means that the time taken to achieve the expansion required for re-implantation may be excessive. A high passage number may also affect the ability of the cells to differentiate.

This chapter will focus on using the homing abilities of the native stem cells, which are strongly mediated by SDF-1/CXCR4 interaction, to enhance bone repair. The idea is that by making stem cells more efficient then a smaller number of cells may be used. An SDF-1 over-expressing rat bone marrow cells (rBMCs) model has been established and reported in chapters 3 of this thesis. An amplified local SDF-1 concentration was formed by implantation of SDF-1 overexpressing rBMCs in a rat femoral defect model.

The hypothesis of this study is that the locally increased level of SDF-1 in fracture sites will improve the bone healing in vivo through better bone formation and mineralisation in the fracture site.
4.2 MATERIALS AND METHODS

4.2.1 Cell culture and adenoviral infection

rBMCs were harvested from femora of young male Wistar rats. rBMCs were expanded \textit{ex vivo}, and cells of passage 3 were used in the experiment. SDF-1 over-expressing rBMCs (rBMC-SDF-1) were engineered by infection of adenovirus carrying human $SDF-1$ gene at the multiplicity of infection (MOI) 500. Procedures of primary rat cell culture and adenoviral infection are as described in chapter 3 of this thesis.

4.2.2 General experimental design

In this study, all the operations were performed in the standard animal operation theatre in the Royal Veterinary College. A project license for the use of experimental animals was obtained from the Home Office (PPL 70/6964). It has been reported that the healing of a small osteotomy gap (1.5mm) on rat femora will take about 6 weeks (Strube \textit{et al}. 2009). Thus, to investigate the short and long-term effect of SDF-1 \textit{in vivo}, two time points were used at 3 weeks and 6 weeks. For the 3 weeks investigation, eighteen adult female Wistar rats were divided into three groups with 6 rats in each group: (1) rBMC-SDF-1, (2) rBMCs and (3) control. A three millimeters gap in the middle of the femur was created during surgery and stabilised by an external fixator. In two groups, $2.4 \times 10^5$ male rBMCs or rBMC-SDF-1 were suspended in 200$\mu$l medium and seeded on a collagen sponge (4mm x 4mm x 7mm) (Helistat, COLLA-TEC, NJ) and transplanted into the gap. For the control group, sponges soaked in 200$\mu$l medium without cells were used.

For the 6 weeks experiment and also to study the direct effect of injection of the Ad-SDF-1, a fourth Ad-SDF-1 group was added. In the Ad-SDF-1 group, $1.2 \times 10^8$ Ad-SDF-1 virus particles suspended in 200$\mu$l culture medium were seeded onto the collagen sponge. All of the other three groups were in the same condition as the three groups in the 3 weeks short-term experiment. Rats were sacrificed at 3 and 6 weeks after operation and the femora were harvested.

X-rays of the bone defect site were taken after rats were sacrificed. Bone mineral content (BMC) within the gap and bone mineral density (BMD) of the original bone
area adjacent to the gap were measured by dual-energy X-ray absorptiometry (DXA) scanning. The area of new bone formation was measured using histomorphometry on Hematoxylin and Eosin stained sections longitudinally made from the middle of the femur, which can show nuclei in blue and collagen (represents bone) in pink, and quantified by imaging analysis system. The preparation of harvested samples is described in section 4.2.6 of this chapter. To test the maintenance of the donor cells in the early stage of the bone healing, and to avoid varying results caused between different species or by artificial cell labelling, detection of specific chromosomal DNA sequences was performed. This method has been widely used and can distinguish particular cells from admixtures of karyotypically different cell lines (Devillee et al. 1988). In this study, transplantation of male rat bone marrow cells into female rats was performed. By the genetic difference of the sexual chromosome between the XY male chromosome pair and XX female chromosome pair, and by detecting the male specific Y chromosome from female double X chromosome using rat Y chromosome specific DNA probe around the osteotomy site, the positively detected cells will reflect the donor cell maintenance and distribution. This method has been widely used in in vivo studies for donor cell tracing after allogenic cell transplantation (Kassmer and Krause 2010). Also, this natural difference between the two genders reduces the possibility of biologic diversities of the donor cells due to artificial cell labelling, which is normally approached by using gene inserting viral transduction to obtain long-term performance, for example, retrovirus carrying green fluorescent protein. In this chapter, fluorescence in situ hybridization (FISH) of male rat Y chromosome was performed in the 3 week groups.

4.2.3 Cell distribution and viability on collagen sponge

To test the compatibility of collagen sponge for cell delivery in this study, cell morphology on collagen sponge was observed by scanning electron microscopy (SEM) (JEOL JSM-5500LV). Two hundred and forty thousand rBMCs were seeded on the same size of collagen sponge and SEM was performed at day 3. Collagen sponge with rBMCs was fixed, washed, serially dehydrated and dried. The sponge was cut into two pieces and the inner surface was faced up when the sponge was mounted onto SEM stubs. It was then sputter coated with an 8nm coating and earthed with silver dagganite. Images of the inner sponge were then taken.
4.2.4 Rat femoral defect model

Rats were first anesthetised by inhalation of isoflurane. The anesthetised rats were weighed (weight between 200 and 250 grams) and the left femur was shaved. The skin over the left hind leg was disinfected and draped (Figure 4-1). A three-centimeter skin incision was made positioned 0.5 cm laterally behind and parallel to the femur to expose the muscles. The muscles covering the femur were then gently separated to expose to femur. The femur was held firmly in place by clamps during the surgery. After adjusting the position between the femur and the drill platform, four holes were drilled with a 1.2mm drill. The 1.5mm pins were then screwed into the pre-drilled holes (Figure 4-2). After the pins were inserted into the bone, four tiny holes on the skin were made, allowing the pins passed through the skin. The pins were then capped and fixed by an external fixator, and the clamps were released. A three-millimeter osteotomy was made between the second and the third pin (Figure 4-3) by a small diamond handsaw. Male donor cells were seeded on collagen sponge (method as described above) and incubated at 37°C one day before the operation. Cell distribution and viability was observed by SEM photography as described above. After the collagen sponge with/without cells seeded on was inserted into the gap, the wound was then closed layer by layer (Figure 4-4). Antibiotics (Ceporea 0.1ml) and analgesics (Vetergesic 0.05mi) were given after the operation and the following day.

Figure 4-1. The surgery started from shaving of the area around the femur. The area was then disinfected by using Hydrex.
Figure 4-2. The femur was drilled by a drill with 1.2mm in diameter, and the pins with 1.5mm in diameter were screwed into the pre-drilled holes.

Figure 4-3. A three millimeter gap was made.

Figure 4-4. The wound was closed.
4.2.5 Measurement of bone mineral content and bone mineral density

Bone mineral content (BMC) and bone mineral density (BMD) were measured by dual-energy X-ray absorptiometry (DXA) scan (QDR-1000, Hologic, USA) at the first, third and sixth week after operation. DXA scan, by sending X-rays from two different sources with different energy levels to the sample and calculating the amount of X-ray gets through to detector, can accurately measure the bone density of the sample. Inhalation anaesthesia was given to the rats prior to and during scanning. The left leg of the rats was fixed by a clamp, which held the external fixator, in the same position during DXA scanning at each time point. Calibration of the DXA scanning was performed using a phantom before the scanning of the rats. The phantom was composed of anthropomorphic spine and the scanner was calibrated based on the known density of the phantom. To make sure the accuracy of the DXA machine, the measurement of BMD of the phantom has to be within the limit lines, which is ±1.5% of mean of the database recorded by the same phantom, before measuring the rats. To measure the BMC and BMD of the rat femora, the extra high-resolution programme was used. A constant region area was analysed which included the osteotomy gap with parts of the original bone for BMC (red boxes) and peripheral bone formed adjacent to the gap for BMD (yellow boxes) (Figure 4-5). The results are shown in sum of the measurements from proximal and distal analysing boxes. Measurements were performed every 3 weeks.

Figure 4-5. Analysing box for BMC and BMD measurement.
4.2.6 Sample collection and histology procedures

After the rats were sacrificed, the left femur was retrieved. The specimens were fixed in 10% formal saline, then dehydrated by a series of alcohol and decalcified by ethylenediaminetetraacetic acid (EDTA), which acts as a hexadentate ligand and chelating agent that can sequester metal ions such as Ca$^{2+}$. Decalcification was confirmed by radiography. After decalcification, specimens were dehydrated, treated with chloroform to de-fat the tissues and then embedded in wax. Samples were labeled on the longitudinal middle line of the femur before embedded in wax to indicate the location of the sectioning area. Sections measuring 7μm thick were made using a sledge microtome.

4.2.6.1 Haematoxylin and Eosin (H&E) staining

Samples were de-waxed in two changes of xylene and placed in two changes of 100% alcohol and then hydrated in serial dilutions of alcohol. After the hydration, samples were immersed in the nuclear stain, haematoxylin, for 5-10 minutes. The excess stain was washed off by immersing the slides in tap water for 5 minutes. Samples were then differentiated in 0.5% acid (HCl)-70% alcohol and washed by water. After removing the acid-alcohol, samples were counterstained by immersing in 1% eosin for 3-4 minutes and then washed by water and dehydrated by serial dilutions of alcohol. Finally, samples were cleaned by xylene and mounted under coverslips using Pertex Mounting Medium (CellPath plc, UK). Samples were observed under the light microscope and the area of new bone was measured by image analysis system (KS-300, Zeiss, UK).

4.2.6.2 Fluorescence in Situ Hybridisation staining

Fluorescence in Situ Hybridisation (FISH) staining of rat Y chromosome was performed using a commercial kit (Cambio, Cambridge, UK), which will co-stain by DAPI. Probe of rat Y chromosome was denatured in 65°C water bath for 10 minutes and held at 37°C. Sample slides were de-waxed by xylene, dehydrated by 100% ethanol and dried out at room temperature. Pepsin solution was then added to the sample for 5 minutes and washed away by buffer saline. Samples were rinsed with distilled water and dehydrated by ethanol. Pre-warmed denaturation solution was added to cover the sample in a 65°C oven for 2 minutes. After the denaturation,
samples were soaked in ice-cold 70% ethanol for 4 minutes and dehydrated by ethanol. Five microlitres of pre-warmed probe was added to the samples, which were then covered with coverslips and sealed using rubber cement. The sealed slides were incubated in a 37°C humidified chamber overnight. The rubber cement and the coverslips were then taken off, and the samples were washed using buffer saline and stringency wash solution and detergent wash solution at 45°C. One hundred and fifty microlitres of detection solution was added to the sample, which was then incubated at 37°C for 20 minutes. Samples were washed using detergent wash solution, covered by storage solution and sealed with coverslips using nail varnish. Sample slides were then observed under the fluorescence microscope (KS-300, Zeiss, UK).

4.2.7 Statistics
Results were analysed with one-way analysis of variance (ANOVA) test at the level of significance of $p \leq 0.05$. For ANOVAs with significant F tests, a Tukey’s post-hoc procedure was performed to compare the significance between two groups.
4.3 RESULTS

4.3.1 Cell distribution and viability on collagen sponge
The cell distribution of the rBMCs on the collagen sponge was observed before the operation by SEM scanning. After 3 days culture on the collagen sponge, cells adhered to the collagen sponge and migrated into the inner of the sponge. Cells showed a flattened and elongated shape (Figure 4-6).

![Figure 4-6. Cell morphology in the inner of the collagen sponge (arrow).](image)

4.3.2 Three weeks short-term effect of SDF-1 in vivo

4.3.2.1 Radiographs of the osteotomy
Radiographs of each group were taken three weeks after surgery (Figure 4-7). All groups showed new bone formation within the osteotomy gap. Both rBMCs and rBMC-SDF-1 groups showed more bone formation in the radiographs.

![Figure 4-7. Radiographs of osteotomy gap after three weeks. New bone was formed within the osteotomy gap (arrows). Scale bar, 5mm.](image)
4.3.2.2 Bone mineralisation after three weeks

BMC change of the osteotomy area was measured by DXA scanning. The rBMC-SDF-1 group showed significantly increased BMC compared to both the control and rBMCs groups (p=0.003 with the control group and p=0.0029 with the rBMCs group) (Figure 4-8). BMD around the osteotomy gap was also measured to show the effects of the different treatments on the original bone. All groups showed reduced BMD after three weeks with no significant difference among the groups (Figure 4-9).

![Figure 4-8. Total BMC change within the osteotomy gap after three weeks (mean ± S.D.). Groups with different letters are significantly different to each other (p<0.05).](image)

![Figure 4-9. BMD change of the original bone area adjacent to the osteotomy gap after three weeks (mean ± S.D.).](image)
4.3.2.3 New bone formation

Histology of the osteotomy site after three weeks showed new bone formation in all three groups with the rBMC-SDF-1 group showing greater bone formation (Figure 4-10). The rBMC-SDF-1 group produced significantly more new bone than the rBMC group (p=0.02), but no difference with the control group (p=0.08). There was no significant difference between the control group and the rBMC group (p=0.8) (Figure 4-11). The new bone formation in most samples of the control group was only found on one side of the fracture, whilst in the other two groups bone formation was more uniform (Figure 4-10).

![Figure 4-10. H&E staining of new bone formation after three weeks (arrows). Scale bars, 400 μm.](image)

![Figure 4-11. New bone area in the osteotomy gap after three weeks (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).](image)
4.3.2.4 Maintenance of the SDF-1 expressing rBMCs

The maintenance of the donor cells in the osteotomy gap after 3 weeks was detected by FISH. The rBMC-SDF-1 group showed more donor cells in the fracture site than the rBMCs group (about 2.5 fold) (Figure 4-12). Interestingly, most of the donor cells in the rBMC-SDF-1 group were found in more osteocyte-like shaped cells embedded in new bone. However, donor cells in the rBMCs group were found mainly in regions with less compact bone structure and remained in a round shape within cell clusters.

Figure 4-12. FISH staining of donor cells (400x). Male rat Y chromosome staining are shown in red with nuclei shown in blue.
4.3.3 Six weeks long-term effect of SDF-1 in vivo

4.3.3.1 Radiographs of the osteotomy

Radiographs of each group were taken six weeks after surgery (Figure 4-13). All groups showed new bone formation within the osteotomy gap.

Figure 4-13. Radiographs of osteotomy gap after six weeks. Scale bars, 5mm.
4.3.3.2 Bone mineralisation during the six weeks

BMC and BMD were measured every three weeks during the experiment. The BMC in the first three weeks showed a similar trend to the results reported in 4.3.2.2. The rBMC-SDF-1 group showed almost four times significantly more BMC increase than the control group (p=0.008), and also a higher increase than in the rBMCs group (p=0.088) (Figure 4-14). The BMC of the rBMCs group and the Ad-SDF-1 group had a higher increase than the control group but without significant difference.

During the second three weeks (from the fourth week to the sixth week), all groups showed a decrease of BMC (Figure 4-15). However, the rBMC-SDF-1 group had the least decrease compared with the control group (p=0.08). The overall BMC change after six weeks showed that only the rBMC-SDF-1 group had an increased BMC compared with the control group (p=0.003) (Figure 4-16).

BMD change of the peripheral bone adjacent to the osteotomy gap was also measured at the same time points. Both the rBMC-SDF-1 and Ad-SDF-1 groups showed increased BMD in the first three weeks, while the control and rBMCs groups showed reduced density (Figure 4-17). During the second three weeks, all groups had further BMD loss (Figure 4-18). Although there is no significant difference in bone mineral density change among all groups after six weeks (Figure 4-19), the rBMC-SDF-1 group had the least BMD loss compared with the control group (p=0.167) and the rBMCs group (p=0.140).
Figure 4-14. Total BMC change within the osteotomy gap from the first week to the third week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

Figure 4-15. Total BMC change within the osteotomy gap from the fourth week to the sixth week (mean ± S.D.).
Figure 4-16. Total BMC change within the osteotomy gap after six weeks (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

Figure 4-17. BMD change of the original bone area adjacent to the osteotomy gap from the first week to the third week (mean ± S.D.).
Figure 4-18. BMD change of the original bone area adjacent to the osteotomy gap from the fourth week to the sixth week (mean ± S.D.).

Figure 4-19. BMD change of the original bone area adjacent to the osteotomy gap after six weeks (mean ± S.D.).
4.3.3.3 New bone formation

Staining for new bone formation after six weeks showed a similar trend to the new bone formation in the three week experiment (Figure 4-20). The rBMC-SDF-1 group had the most new bone formed among all the groups (p=0.029 when compared with the control group) (Figure 4-21).
Figure 4-20. H&E staining of new bone formation after six weeks (arrows). Scale bars, 200μm.
Figure 4-21. New bone area in the osteotomy gap after six weeks (mean ± S.D.).

Groups have * are significantly different to each other (p≤0.05).
4.4 DISCUSSION

In this study, according to the new bone formation of the control group 6 weeks after the operation, which is 0.4mm² on average in the 9mm² defect area on the histology slide (3mm in length of the gap times 3mm in diameter of the femur), the 3mm osteotomy model fits the definition of a critical size bone defect. This is defined as an osseous defect showing less than 10% healing of bone during the lifetime of an animal when under an untreated condition (Hollinger and Kleinschmidt 1990; Lindsey et al. 1998). Thus, implantation of bone marrow cells over expressing SDF-1 leads to improved new bone formation in the early bone repair in a critical size defect model. Also, SDF-1 improves the retention of the donor cells in the fracture area in the first three weeks and prevents bone loss in the peripheral bone area.

Many scientists have successfully used stem cells to regenerate bone in animal models, but its clinical application is limited because of the restriction of laboratory facilities and the timeframe required to obtain large numbers of stem cells. In tissue engineering and bone healing, the stem cell number is a crucial factor (Salgado et al. 2004; Kwong and Harris 2008). Others use SDF-1/CXCR4 interaction to mediate cell migration so that a large quantity of stem cells can be recruited in the repair site (Badillo et al. 2007). By blocking the SDF-1/CXCR4 interaction in the mice bone graft models, the recruitment of bone marrow stromal cells and the new bone formation were significantly reduced, indicating an important role of SDF-1/CXCR4 interaction in bone repair (Kitaori et al. 2009). In this rat fracture model, a relatively small number of cells was used, and it showed that the rBMC-SDF-1 group produced more new bone than the rBMCs group, indicating that the enhanced level of local SDF-1 can improve the bone healing. Also, the enhanced level of SDF-1 may be correlated to the prevention of bone loss in the adjacent bone area from the fracture healing processes.

Previous studies have suggested that immediate local expression of endogenous SDF-1 around the injured area was transiently up-regulated after myocardial infarction (MI) (Abbott et al. 2004; Askari et al. 2003), lung injury (Petty et al. 2007), retinal ischemia-reperfusion injury and inflamed human dental pulp (Lai et al. 2008; Jiang et al. 2008). Therefore, in this study, it is proposed that the femoral
defect model caused a transiently increased endogenous SDF-1 level, which facilitated the healing process in all groups. According to the six weeks experiment, all groups showed an increase in BMC during the first three weeks and a reduction during the second three weeks, which is in contrast with a typical bone healing procedure in a critical size bone defect model. By enhancing the local SDF-1 level, the rBMC-SDF-1 group showed significantly increased BMC and more new bone formation than the control and rBMCs groups at both three weeks and six weeks postoperatively. Interestingly, although control animals gained BMC at 3 weeks and lost that gain at 6 weeks, the rBMC-SDF-1 group gained the most and lost the least amount of BMC. Animals in the rBMCs group always showed intermediate gains and losses in BMC. This reflects a synergistic effect in the combination bone marrow cells and SDF-1 in improving bone mineral formation in the osteotomy gap. The rBMC-SDF-1 group also showed more new bone formation at both time points. Considering the results, I have shown in chapter 2 that SDF-1 plays an enhanced role when MSCs are cultured in the osteoinductive media. It has been reported that bone morphogenetic protein-2 (BMP-2), a dominant inducer of stem cell osteogenic differentiation, is elevated at the fracture site within 24 hours after fracture (Ai-Aql et al. 2008; Tsuji et al. 2006; Kidd et al. 2010). In another study, it has been shown that rat bone marrow-derived osteoblast progenitor cells (MOPCs) were mobilised from bone marrow to the BMP-2-pellet implantation site through the circulation system by chemo attraction of SDF-1 expressed in vascular endothelial cells and the de novo osteoblasts of the region (Otsuru et al. 2008). Thus, in my study, the increased bone healing may be due to the enhanced local SDF-1/CXCR4 interaction that led to the recruitment of larger numbers of the host’s stem cells from peripheral regions and distant sources through systemic circulation into the fracture site, combined with the enhanced osteoblastic differentiation of the osteoblastic progenitors by SDF-1. Moreover, FISH staining showed more donor cells in the compact new bone in the rBMC-SDF-1 group after three weeks, indicating higher stem cell maintenance which could be explained by the ability of SDF-1 to reduce the incidence of apoptosis (Otsuru et al. 2008; Tang et al. 2008b).

Significant reduction of BMD of the peripheral bone adjacent to a fracture site is a common finding after fracture or osteotomy treated with immobilisation (Eyres and
Kanis 1995; Karlsson et al. 2000; Schäfer et al. 2008). Eyres et al. reported up to an 50% BMD loss in the distal site to a tibial fracture at three months which persisted at six months, observed in five patients (Eyres and Kanis 1995). A similar observation was reported by Karlsson et al. that 35% of bone loss in the mid-diaphysis of the tibia took place at 9 months following osteotomy in 26 patients with localised medial arthritis of the knee (Karlsson et al. 2000). Reduction in BMD in patients with fracture also increases the risk for further fractures. In my study, the control group showed a rapidly decreased BMD in the peripheral bone from the first 3 weeks and reached a 30% decrease in six weeks time, which is consistent with the normal clinical observations. The rBMCs group showed a similar trend of bone loss to the control group and indicated no effect of donor cell in the bone loss after osteotomy. However, in both my short-term and long-term experiments, the rBMC-SDF-1 group showed a gain of BMD in the first 3 weeks. All groups in the long-term experiment showed a similar reduction of BMD during the second 3 weeks. After 6 weeks, a 20% less BMD decrease in the rBMC-SDF-1 group compared with the control and rBMCs groups was seen. Interestingly, the Ad-SDF-1 group had a similar trend to the rBMC-SDF-1 group during the 6 weeks, indicating that SDF-1 has some effect on preventing the bone loss when compared with the rBMCs group.

Bone mineral loss in the bone adjacent to the osteotomy site has been attributed to increased bone remodelling (Augat and Claes 2008). After immobilisation, disuse or injury, bone turnover is increased, but the amount of new bone laid down in erosion cavities is less than normal (Minaire et al. 1974). Bone remodelling is controlled by the dynamically balanced regulation between bone forming osteoblasts and bone resorbing osteoclasts. It is well known that osteoblasts can induce and activate the osteoclastogenesis by producing receptor activator of NF-κB ligand (RANKL) and macrophage-colony stimulating factor-1 (M-CSF), while mature osteoclasts secret bone morphogenetic proteins (BMPs) to induce osteoblastogenesis (Matsuo 2009). Osteoblasts can also inhibit osteoclast differentiation by secreting a decoy receptor for RANKL, known as osteoprotegerin (OPG). OPG blocks the interaction of RANKL and its receptor RANK on the surface of osteoclasts (Simonet et al. 1997). Therefore, through modulation of RANKL and OPG expression, osteoblasts can precisely regulate the formation of osteoclasts (Sims and Gooi 2008). Although the
predominant role of SDF-1 is to recruit CXCR4 positive hematopoietic cells, which are precursors of osteoclasts and, thus, may increase the bone remodelling and lead to more bone loss, SDF-1 has also been shown to recruit MSCs (Sordi et al. 2004). Earlier in this thesis (chapters 2 and 3), it showed that SDF-1 does affect both the migration and differentiation of MSCs. However, in this study, because the bone resorption is much faster than the bone formation during remodelling, the reduced bone loss in my results is less likely to be an improved osteoblastic differentiation of the stem cells but more as a negative effect on osteoclastogenesis, since less bone loss was observed from the early stages of healing. Thus, less bone loss in the SDF-1 experimental groups indicates that SDF-1, apart from the recruiting stem cells, may affect bone remodelling by other mechanisms. The signalling pathways of SDF-1 may be one of the possible explanations.

Many molecules and signalling pathways have been studied in the regulation of bone remodelling. Glass et al. reported the control of osteoclast differentiation by canonical Wnt signalling in differentiated osteoblasts (Glass et al. 2005). Wnt signalling pathway, by inactivating the glycogen synthase kinase 3β (GSK3β), prevents the cytoplasmic β-catenin from its ubiquitination and the following degradation, which leads to the interactions of β-catenin and down stream transcription factors and further regulations in cell adhesion, migration and transcription. In Glass et al.’s study, stabilisation of β-catenin in differentiated osteoblasts results in high bone mass in the gain-of-function mutant, while deletion of the β-catenin in differentiated osteoblasts leads to osteopenia. They further revealed that the stabilised β-catenin, together with T cell-specific transcription factor (TCF) proteins, raises the expression of OPG in osteoblasts, which inhibits the osteoclast differentiation. Thus, it indicates a negative regulation of Wnt signalling in osteoclastogenesis. Interestingly, there are several studies that revealed the interactions between SDF-1/CXCR4 and Wnt signalling pathways. Luo et al. reported that SDF-1/CXCR4 signalling activates β-catenin/TCF transcriptional activity in embryonic rat spinal cord neural progenitors (Luo et al. 2006). Liu et al. further reported that SDF-1 promotes the stabilisation of β-catenin by the activation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signalling.
cascade, resulting in the inhibition of GSK3β in pancreatic beta cells (Liu and Habener 2009). According to the studies above, high levels of SDF-1 at the osteotomy site in this experiment may promote the stabilised β-catenin in osteoblasts and further inhibit the osteoclastogenesis by enhanced OPG expression, thus reducing BMD loss in the adjacent bone. Apart from the improvement of new bone formation by SDF-1, this bone loss preventing effect of SDF-1 in fracture repair is also important to prevent the refracture, which normally happens due to the weaker bone structure caused by bone loss during fracture healing (Utrilla et al. 2005).

In this study, the rBMCs group did not show an improved bone healing effect, which indicates the possibility of an optimal cell number in the fracture site to initiate the improvement of bone healing. Kruyt et al. revealed the optimum of 8x10^6 bone marrow stromal cells (BMSCs)/cm^3 and the minimum of 8x10^4 BMSCs/cm^3 for new bone formation in a porous biphasic calcium phosphate scaffold implanted in goat muscle (Kruyt et al. 2008). This finding validates previous empirically derived seeding densities of 5x10^6 to 10x10^6/cm^3 in the majority of in vivo studies. In this study, the cell density used was about 1.2x10^6/cm^3, which is about five times less than the normal applied density. Although the cell density in this study was higher than the minimum density in Kruyt’s study, it indicates that a higher minimum cell density for bone defect healing is required than for ectopic bone formation, and, furthermore, it could be varied according to different defect sizes and the animal models applied. However, in a non-efficient cell density condition, the rBMC-SDF-1 group could still show significant improvements. This approves the attenuation of the minimum cell density required in bone fracture healing.

There is one more interesting finding in this study: at 6 weeks the effects of the rBMC-SDF-1 group and the Ad-SDF-1 group seemed to be dominant only during the first three weeks, and declined in the following three weeks. Considering that expression of adenoviral vector normally lasts for three to four weeks (Lanza et al. 2000) and the cell recruitment occurs in the early stage of injury repair, it is interesting to know whether a longer expression of SDF-1 in the fracture site will improve bone healing. To answer this question, in the next chapter I investigated the effect of a second injection of rBMC-SDF-1 at the third week in this model.
To summarise the results have been shown in this study, genetically modified rBMCs that overexpress SDF-1 appear to be more effective than the normal rBMCs to improve new bone formation and cell maintenance in the fracture site in the early bone healing stage and bone loss after the osteotomy. For this reason, it may be possible to utilise few cells to effect bone repair and regeneration in fracture.
Chapter Five

Application of Second Cell Transplantation in Rat Femoral Defect Model
5.1 INTRODUCTION

Transplantation of hematopoietic and mesenchymal stem cells has been giving promising outcomes. For instance, by destruction of the defective marrow and replacement with normal bone marrow, hematopoietic stem cells (HSCs) can reverse fetal diseases caused by congenitally defective stem cells, including severe combined immune deficiency (SCID), a disorder of the lymphoid stem cells, and osteopetrosis (Lanza et al. 2000). Mesenchymal stem cells (MSCs) have been used in bone repair (Lee et al. 2005), cartilage (Koga et al. 2009), heart (Paul et al. 2009), kidney (Imai et al. 2009). Stem cells have been shown to be a potential source of cells for tissue engineering of various connective tissues. However, most in vivo studies are based on the transplantation of single or multiple doses of cells in the early stages (Omori et al. 2008). The effect of multiple cell transplantations in a long-term interval has been rarely reported.

According to the results in chapter 4, I have shown that rat bone marrow cells over-expressing SDF-1 can improve bone healing. This may be important in a tissue engineering context as healing by MSCs maybe more efficient, which means that either the time required for the defect may be reduced or the number of the cells may be less due to the increased efficiency. One of the limiting factors is producing enough cells in a timely manner to repair bone defects. Considering the expression and survival time of the adenoviral vector and the results that indicated the effects only appeared in the early stages of bone healing soon after the addition of MSCs, it leads to the question, will multiple cell transplantations have further and additive improvement in the femoral defect model. To answer this question, a second cell injection was applied in this study. The hypothesis of this study is that a second injection of bone marrow cells over-expressing SDF-1 will improve further bone healing.
5.2 MATERIALS AND METHODS

5.2.1 Cell culture and adenoviral infection
Young male Wistar rat bone marrow cells (rBMCs) were harvested and cultured as described previously in chapter 3. Passage 5 rBMCs were used in this study. rBMCs over-expressing SDF-1 (rBMC-SDF-1) were engineered by infection of Ad-SDF-1 at MOI 500. Procedures of infection were as described in chapter 3.

5.2.2 Experiment design
To investigate whether multi-implantation of rBMCs and rBMC-SDF-1 will further improve bone healing, a second injection of the cells was applied in this study. All experimental conditions are the same as the conditions in chapter 4. Briefly, 18 adult female Wistar rats were equally separated into three groups: (1) rBMC-SDF-1, (2) rBMCs and (3) control. Femoral defect model with a 3mm gap was performed as described previously. Two hundred and forty thousand rBMC-SDF-1 or rBMCs were suspended in 200 μl culture medium and seeded on the collagen sponge (4mm x 4mm x 7mm) (Helistat, COLLA-TEC, NJ) and transplanted into the gap. For the control group, sponges soaked in 200μl culture medium without cells were used.

Considering that the expression of adenoviral vectors normally lasts for 3 to 4 weeks and the early effects of SDF-1 in bone healing shown in chapter 4, a second injection of cells was applied three weeks after the operation. The same amount of 2.4 x 10^5 rBMC-SDF-1 or rBMCs was suspended in 100μl culture medium and injected in the middle of the osteotomy gap by using a 23 gauge needle. During the operation, the pins and the needles were previously marked at the location, which was equally distant away from their points, to ensure the right position of the needle point in the middle of the osteotomy gap when the two marks were lined up to each other during performing of the second injection. Radiographs of the osteotomy site were taken six weeks postoperatively. Bone mineral content (BMC) in the osteotomy gap and bone mineral density (BMD) of the bone adjacent to the gap were measured by dual-energy X-ray absorptiometry (DXA) scanning at the first, third, and sixth week after operation. Rats were sacrificed at the sixth week. The area of new bone formation was then measured using histomorphometry on Hematoxylin and Eosin stained...
sections and quantified using an imaging analysis system. All protocols of the measurements were the same as described in chapter 4.

5.2.3 Statistics
Results were analysed using a one-way analysis of variance (ANOVA) test at the level of significance of $p \leq 0.05$. For ANOVAs with significant F tests, a Tukey’s post-hoc procedure was performed to compare the significance between two groups.
5.3 RESULTS

5.3.1 Radiographs of the osteotomy
Radiographs of the osteotomy site at the sixth week showed new bone in all three groups (Figure 5-1). rBMC-SDF-1 group had more new bone formation within the gap.

![Radiographs of osteotomy gap after six weeks. Scale bars, 5mm.](image)

5.3.2 Bone mineralization during the six weeks
BMC in the gap and BMD in the adjacent bone were measured every three weeks during the experiment. In the first three weeks, the rBMC-SDF-1 group showed more increased BMC than the control and the rBMCs group without significant difference (Figure 5-2). During the second three weeks, although all groups showed decreased BMC, the rBMC-SDF-1 group had significantly less BMC decrease than the control group (p= 0.009), while the rBMCs group was in between (Figure 5-3). A significantly higher BMC level was recorded in the rBMC-SDF-1 group compared to the control group (p= 0.006) six weeks after the operation (Figure 5-4).

BMD change in the first three weeks showed no difference in any of the groups (Figure 5-5). After the second injection of the cells (for both rBMCs and rBMC-SDF-1) at the third week, the rBMCs and rBMC-SDF-1 groups showed an increased density in the adjacent bone area during the second three weeks, while the control group had a decreased BMD, respectively (p= 0.008 for rBMCs and control groups) (Figure 5-6). The overall BMD change after six weeks showed that the rBMCs group had significantly less BMD loss than the control group (p= 0.0035) while the rBMC-SDF-1 group had less decreased BMD compared with the control group (p= 0.089) (Figure 5-7).
Figure 5-2. Total BMC change within the osteotomy gap from the first week to the third week (mean ± S.D.).

Figure 5-3. Total BMC change within the osteotomy gap from the fourth week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p ≤ 0.05).
Figure 5-4. Total BMC change within the osteotomy gap from the first week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

Figure 5-5. BMD change of the original bone area adjacent to the osteotomy gap from the first week to the third week (mean ± S.D.).
Figure 5-6. BMD change of the original bone area adjacent to the osteotomy gap from the fourth week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).

Figure 5-7. BMD change of the original bone area adjacent to the osteotomy gap from the first week to the sixth week (mean ± S.D.). Groups have * are significantly different to each other (p≤0.05).
5.3.3 New bone formation

Hematoxylin and Eosin staining of the osteotomy gap showed the new bone formed after six weeks (Figure 5-8). The rBMC-SDF-1 group had more new bone formation than the other two groups on average without significant difference (Figure 5-9).

![Figure 5-8. H&E staining of new bone formation after six weeks (arrows). Scale bars, 200µm.](image)
Figure 5-9. New bone area in the osteotomy gap after six weeks (mean ± S.D.).
5.4 DISCUSSION

In this study, a second injection of rBMCs and rBMC-SDF-1 was applied at the third week. The rBMC-SDF-1 group showed an improved new bone formation in the osteotomy site and less BMD loss in the adjacent bone area after the six weeks experiment, which is similar to the trend shown in chapter 4.

Although the rBMC-SDF-1 group in this study showed significantly improved BMC than the control group six weeks postoperatively, when compared with the results of a single injection in chapter 4, both the second injection of rBMCs and rBMC-SDF-1 did not enhance the BMC from the third week to the sixth week (Figure 5-10). This result indicates that the second injection of cells did not have a further enhancing effect in fracture healing in this study. The possible explanation is the engraftment of the secondary implanted cells, which may fail to maintain the donor cells within the fracture site. Scaffolds in tissue engineering provide several characters to facilitate the clinical usage of cells, including allowing cell attachment, proliferation, and differentiation (Hutmacher 2000). The second cell injection, in which cells were suspended in culture medium, therefore, may spread out from the osteotomy gap and result in less cells within the gap compared to the way the cells were introduced during the first application. Non-attachment of the cells to a suitable scaffold may also effect the SDF-1 expression of the rBMC-SDF-1 group. However, use of a suitable scaffold such as fibrin glue to retain the cells at the site at the osteotomy site may have a detrimental affect as it may displace the repairing tissue.

Another possible explanation is the changes of the microenvironment in the osteotomy site during the fracture healing. The pattern of molecules expressed in different stages in fracture healing is varying. Many of the osteoinductive factors, including transforming growth factor-beta (TGF-β) and bone morphogenetic proteins (BMPs), were up-regulated during early fracture healing and were found to be reduced after three weeks (Bostrom et al. 1995; Cho et al. 2002; Gerstenfeld et al. 2003; Dimitriou et al. 2005). This reduced expression of osteoinductive factors, for example, BMP-2, could attenuate the enhancing effect of SDF-1, which relies on the activated osteogenic differentiation reported in chapter 2.
Figure 5-10. Comparison of BMC change within the osteotomy gap from the third week to the sixth week between the single and double injection of cells (mean ± S.D.). Data were analysed by Student t-test within the same group.

Interestingly, the second injection of cells made a significant improvement in preventing bone loss in the bone area adjacent to the osteotomy site from the third week to the sixth week compared with the result of a single injection of cells in chapter 4 (Figure 5-11). All groups of the single injection showed about 15% decreased BMD during the second three weeks, while the rBMCs and rBMC-SDF-1 groups of the double injection increased the BMD (p=0.020 for rBMCs and p=0.074 for rBMC-SDF-1), which indicates that the second implantation of cells can still effect bone loss in the adjacent bone area. The increased BMD could also be related to lodging of the diffused cells from the osteotomy gap. In the control groups, the second injection of the culture medium made a slight increase in BMD when compared with the single injection. This difference may be due to the performance of the injection that gave the repairing site physical information or due to the fresh
culture medium. Further study is needed to investigate the effect of physical information in bone fracture healing.

Figure 5-11. Comparison of BMD change of the original bone area adjacent to the osteotomy gap from the third week to the sixth week between the single and double injection of cells (mean ± S.D.). Data were analysed by Student t-test within the same group. Data have * are significantly different to each other (p<0.05).

There have only been a few studies reported about multiple stem cell transplantation in cell therapy (Omori et al. 2008; Abdelkefi et al. 2008). Omori et al. reported that a single high dose of stem cell transplanted intravenously gave better results than a multiple low dose stem cell transplantation in a rat cerebral ischemia model. Considering the transient increase of the local chemo-attractive cytokines, for example SDF-1, in the early stage after injury, the results of the multiple low cell transplantation could be affected by the weakened recruitment of the transplanted cells. In my study, although the second injection of cells did not further improve the new bone formation and BMC in the osteotomy gap, it still improved the bone loss in
the adjacent bone area. Therefore, to optimise the protocol of second cell transplantation in the bone defect model, an alternative cell delivery system which can keep the cells in the gap and improve the cell attachment, should be studied. Also, the time point for secondary cell injection should be considered.
Chapter Six
General Discussion
The aim of this thesis is to improve bone healing treated with bone marrow cells over-expressing SDF-1. The general hypothesis of this work was that fracture healing will be improved by transplanting SDF-1 over-expressing bone marrow cells using a low cell number.

In bone tissue engineering, osteoinductive factors play key roles in bone healing to stimulate osteoprogenitor cell differentiation and synthetic activity (Reddi 2001; Chen et al. 2004; Lind 1998). Many studies have revealed the multiple function of SDF-1/CXCR4 interaction in the development and regeneration of tissues (Claps et al. 2005), stem cell homing (Chavakis et al. 2008), angiogenesis (Salcedo and Oppenheim 2003), cancer cell metathesis (Gelmini et al. 2008) and tissue repairing (Zaruba and Franz 2010). Most of the studies focus on the effect of SDF-1/CXCR4 interaction on cell recruitment, but the effects on cell differentiation have rarely been reported. Chapter 2 of this thesis demonstrated that SDF-1 could enhance the early osteogenic differentiation of stem cells under osteoinductive conditions. This finding is consistent with another study (Hosogane et al. 2010), which revealed that blocking of the SDF-1/CXCR4 signal axis or adding SDF-1 protein to MSCs significantly affected BMP-2-induced alkaline phosphatase (ALP) activity and osteocalcin (OCN) synthesis in mature osteoblasts. It has shown that the effect of SDF-1 is to intensify osteogenic differentiation and discussed the possible signalling pathway (Figure 2-5 and Figure 6-1) in chapter 2. This conditional enhancing effect of SDF-1 relies on the presence of an osteoinductive environment, which leads to the enhanced level of Runx2 and may further result in the activation of inactivated Runx2 by SDF-1. Therefore SDF-1 expressing cells may enhance bone repair by recruiting more stem cells and may also enhance osteogenic differentiation of the recruited cells. Because of the effect of SDF-1 in osteogenic differentiation, one interesting question is whether SDF-1 is involved in other types of stem cell differentiation under different conditions. Understanding if SDF-1 can play a role in other types of differentiation under particular conditions may be helpful for the use of stem cells in different clinical applications.
Critical size bone defects caused by trauma or cancer are the greatest clinical challenge in bone tissue engineering (Pelled et al. 2010). The most common tissue engineering concept of treating a bone defect is to combine a scaffold-matrix, living cells and/or biologically active molecules into an implantable construct (Lee et al. 2009). Many studies utilized the transplantation of mesenchymal stem cells (MSCs) or genetically modified MSC (for example, expressing of BMP-2) and these have shown promising outcomes (Niemeyer et al. 2010; Zhang et al. 2010; Kumar et al. 2010). In applying stem cell transplantation, it is critical to attain a population of cells that is large enough to seed on/into a scaffold which can effectively contribute to repair and healing of the major defect (Zaky and Cancedda 2009). With the low
cell numbers of MSCs isolated from bone marrow, where about 1 in 100,000 cells are progenitors (Cui et al. 2007; Mirabet et al. 2008), it is laborious and time-consuming to achieve the required cell number after cell sorting and \textit{ex vivo} expansion of the stem cell. This limitation also weakens the feasibility of stem cell transplantation in clinical therapy. In chapter 4, transplantation of rBMC-SDF-1 improved bone healing even when the cell number was five times less than the normal applied cell density, which is $5 \times 10^6$ to $10 \times 10^6$/cm$^3$ (Kruyt et al. 2008). These results support the hypothesis that the number of SDF-1 expressing cells required to effect repair of the defect can be lower than if none genetically modified cells were used. Although these SDF-1 expressing cells are more efficient than normal cells in the bone healing processes, further investigation is still needed to establish whether bone healing can be improved when a normal cell density is applied (for example, $5 \times 10^6$/cm$^3$) and also to establish the optimal cell density for these SDF-1 expressing cells applied in bone repair therapy. A similar concept, which applies the cell recruitment ability of SDF-1, was used in treatments for myocardial infarction (Tang et al. 2009), heart transplantation (Zhao et al. 2009) and wound healing (Rabbany et al. 2009). In bone tissue engineering, the role of SDF-1/CXCR4 interaction in bone healing has only recently been investigated (Kitaori et al. 2009). According to the results presented in my thesis, increased new bone formation and bone mineral content, together with the effect on osteogenic differentiation under osteoinductive conditions, SDF-1 administration could be a potential strategy in musculoskeletal tissue healing.

Interestingly, in chapter 4, SDF-1 showed prevented bone loss in the bone area adjacent to the osteotomy site. Post-traumatic osteopenia around a fracture has been reported and it has been shown that it can amount to almost 30% loss in the same bone (Findlay et al. 2002; Veitch et al. 2006) and bone mass reduction may persist for many years (Eyres and Kanis 1995; Karlsson et al. 1993). This bone loss in the area adjacent to the fracture site, which is indicative of high bone remodelling activity (Augat and Claes 2008), will increase the risk of re-fracture of the affected bone and could reduce the holding power of bone screws, which would impair implant fixation and cause migration, cutout and deformity (Utrilla et al. 2005; Goldhahn et al. 2005, 2008). For example, this type of bone loss may have been the
cause of aseptic loosening of the implant in total hip replacement (THR), which led to a 70-94% revision rate in a particular design of THR (Choplin et al. 2008). Thus, the administration of SDF-1 could be used to prevent bone loss. In revision THR, the interaction between the implant, graft and original bone is one of the most important parameters. New bone ingrowth from the surrounding bone area into the graft and to the surface of the implant is the ultimate goal to achieve the best incorporation. Many studies using modified bone graft, for example, supplemented with BMP-7 (Tägil et al. 2000) or seeded by MSCs (Korda et al. 2010), achieved improved new bone formation within the graft. The application of MSCs expressing SDF-1 in bone graft may not only enhance ingrowth of the new bone, but also reduce the bone loss in the surrounding area, which may lead to a better and faster bone incorporation. On the other hand, SDF-1/CXCR4 interaction may also play a role in osteoporosis, which is caused by the imbalance of bone resorption and formation during remodelling (Galliera et al. 2008). A recent study where intravenous injection of mice MSCs, which over-express CXCR4 and Runx2, in an osteoporotic mouse model, has reported a return to normal bone stiffness and strength (Lien et al. 2009). This study, together with the finding of the potential regulatory role of SDF-1 in bone remodelling in my thesis, indicates the possibility of using augmented SDF-1/CXCR4 interaction and osteoinductive factors in the therapy for osteoporosis.

Chapter 5 considers the strategy of a secondary injection of rBMC-SDF-1. Although this did not improve further new bone formation within the osteotomy gap, it did have an effect on preventing bone loss or increasing bone mass in the adjacent bone area. This result indicates that SDF-1 may have a role in bone remodelling, and it is interesting to study the possibility of SDF-1 as a long-term gene therapy to prevent bone loss from the area around the implant. Considering the expression period of adenoviral vector, which is about three to four weeks (Lanza et al. 2000), long-term expression of SDF-1 can be achieved by applying either multiple administrations of adenovirally infected MSCs or a single dose of retrovirally infected MSCs. The former offers a predictable and controllable gene expression over time and could be suitable for a temporary treatment such as fracture healing. The latter, by its permanent gene expression, could benefit the long-term treatment of chronic diseases such as osteoporosis. However, retroviral vectors may randomly incorporate into the
host’s genome and at the present time should be considered as risky as this could promote mutagenesis in a long-term treatment.

Results in this thesis revealed the potential of SDF-1 application in bone tissue engineering *in vivo* and also indicate the requirement to further elucidate the strategy of SDF-1 usage. One alteration that could improve the outcome reported in chapter 5 is to change the scaffold. Although the collagen sponge used for primary cell transplantation provides good support for cell attachment, proliferation and migration, the secondary cell injection, where the cells were suspended in culture medium, could not provide proper cell attachment. It is likely that these suspended cells flowed out of the osteotomy gap and led to an attenuated cell density in the healing area. Thus, to avoid invasive cell transplantation, an injectable hydrogel of collagen, which also permits homogeneous cell distribution throughout the scaffold, should be considered (Nöth *et al*. 2010).

It would also be interesting to know whether there is a dose dependant effect of the SDF-1 on the improved bone healing. Kruyt *et al.* had reported the dose dependant effect on transplanted cell number in new bone formation (Kruyt *et al*. 2008). Identifying the best combination for the expression of SDF-1, which involves optimisation of the MOI of the infection, may be important.

To extend the idea based on my findings, where it is necessary to use an osteoinductive environment for optimisation of the SDF-1 effect, a combination of SDF-1 with osteoinductive factors may be beneficial. Among the various osteoinductive factors that have been studied, BMP-2 has been the most promising and widely used factor. Healing of long bone critical-sized defects by BMP-2 has been demonstrated in species including rats, rabbits, dogs, sheep and non-human primates (Murakami *et al*. 2002). Adenoviral infected MSCs expressing BMP-2 also improved the healing ability in bone defects (Chang *et al*. 2003). To date, BMP-2 has been approved by the United States Food and Drug Administration (FDA) for use in open long-bone fracture and for use inside titanium cages for anterior spinal fusion (Lee *et al*. 2009). Encouragingly, BMP-2 together with SDF-1 has demonstrated a synergistic effect on C2C12 cell *in vitro* osteogenic differentiation compared with
BMP-2 alone (Hosogane et al. 2010). This finding further confirms the potential of the combination of BMP-2 and SDF-1 to achieve better results in fracture healing than when each is used separately.

During the fracture healing process, inflammatory cytokines involved in fracture repair, for example, interleukins-1 and -6 (IL-1 and IL-6), are believed to play a role in initiating the repair cascade following injury (Al-Aql et al. 2008). BMP-2 with an early expression pattern during fracture healing (Cho et al. 2002; Gerstenfeld et al. 2003; Dimitriou et al. 2005) has been revealed as a necessary component of the signalling cascade that governs and initiates fracture repair (Tsuji et al. 2006). As the expression of BMP-2 reduced after early inflammation in fracture repair, a second cell transplantation with expression of SDF-1 and BMP-2 may re-initiate the repairing process, leading to further improvement.

In summary, this thesis has shown that SDF-1 can enhance early stage stem cell osteogenic differentiation when in osteoinductive conditions. Transplantation of genetically modified rat bone marrow cells, which overexpress SDF-1, can improve new bone formation and prevent bone loss at the early stage of fracture healing. Moreover, second transplantation of the SDF-1 expressing cells can further improve bone loss of the adjacent bone area of the fracture.
References


Fox JM, Chamberlain G, Ashton BA, Middleton J. Recent advances into the


Hattori K, Heissig B, Tashiro K, Honjo T, Tateno M, Shieh JH, Hackett NR, Quitoriano MS, Crystal RG, Rafii S, Moore MA. Plasma elevation of stromal cell-


Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. Stem Cells. 2004; 22 (3): 415-27.


Lai P, Li T, Yang J, Xie C, Zhu X, Xie H, Ding X, Lin S, Tang S. Upregulation of stromal cell-derived factor 1 (SDF-1) expression in microvasculature endothelial...


Lien CY, Chih-Yuan Ho K, Lee OK, Blunn GW, Su Y. Restoration of bone mass and strength in glucocorticoid-treated mice by systemic transplantation of CXCR4


Mastrogiacomo M, Muraglia A, Komlev V, Peyrin F, Rustichelli F, Crovace A,


Quarto R, Mastrogiacomo M, Cancedda R, Kutepov SM, Mukhachev V, Lavroukov


Tse WT, Pendleton JD, Beyer WM, Egalka MC, Guinan EC. Suppression of


Zhang H, Bradley A. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development. 1996; 122 (10): 2977-86.


Appendix I

Adenovirus titer measurement — Plague assay (Stratagene, Catalog No. 240010)

The following protocol may be used to determine the titer (pfu/ml) of a viral stock.

Preparing Viral Stock Dilutions
1. Plate AD-293 cells at a density of $5 \times 10^5$ per well of 6-well tissue culture plates.
2. Incubate overnight at 37°C.
3. Dilute viral stocks in 1-ml volumes over a 10-fold series from $10^{-5}$ to $10^{-9}$ in growth medium. Carry dilutions in duplicate.
4. Add 1 ml of each dilution to a separate well of the 6-well plate. Leave one well “medium only” (no virus added) as a control.
5. Incubate at 37°C for 2 hours. Gentle rocking during the incubation is beneficial but not required.
6. Proceed to Overlaying the Infected Cells with Agarose.

Overlaying the Infected Cells with Agarose

The agarose overlay should be applied so that it spreads fast enough to cover the plate before solidifying but not so fast that the cells are disrupted. It may be necessary to practice the overlaying technique on uninfected cells prior to performing the plaque assay. Prior to the addition of the agarose overlay, inspect the plates containing the cells to ensure adequate adherence.

1. Prepare a solution of 5% SeaPlaque® agarose (Stratagene, CA) in sterile PBS, autoclave, and store in 10-ml aliquots at 4°C in 50-ml sterile conical tubes.
2. Prior to use, melt the agarose by placing the tube in a beaker of boiling water. Do not microwave the tube of agarose.
3. Once melted, cool the agarose to 45°C.
4. Add 30 ml of growth medium previously equilibrated to 37°C and mix. This makes the final agarose concentration 1.25%. Proceed immediately to the next step.
5. Completely remove the growth medium from the wells that will receive the overlay.

6. Gently pipet 3 ml of agarose/growth medium mix very gently along the side of the well and allow it to completely cover the bottom of the well.

7. Incubate the plate at 37°C. Plaques, having the appearance of small white spots, should be visible to the naked eye within 12–21 days. During that time, if the agarose/growth medium overlay becomes yellow, pour additional overlays at a volume of 1.5 ml per addition.

8. To determine titer, count plaques from wells where isolated plaques are clearly visible and countable. Average the counts from duplicate wells and multiply that number by the dilution factor to estimate pfu/ml.
SDF-1 ELISA assay (R&D systems, Catalog No. DSA00)

Reagent preparation
(Bring all reagents to room temperature before use)

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to prepare 500 mL of Wash Buffer.

Substrate Solution - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 200 μL of the resultant mixture is required per well.

SDF-1 Standard - Reconstitute the SDF-1α Standard with 1.0 mL of deionized or distilled water. This reconstitution produces a stock solution of 100,000 pg/mL. Mix the standard gently to ensure complete reconstitution and allow the standard to sit for a minimum of 30 minutes with gentle agitation prior to making dilutions.

Use polypropylene tubes. Pipette 900 μL of Calibrator Diluent RD6Q into the 10,000 pg/mL tube. Pipette 500 μL of Calibrator Diluent RD6Q into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 10,000 pg/mL standard serves as the high standard. Calibrator Diluent RD6Q serves as the zero standard (0 pg/mL).

Assay procedure
(Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and controls be assayed in duplicate)

1. Prepare all reagents, working standards, and samples as directed in the previous sections.
2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.
3. Add 100 μL Assay Diluent RD1-55 to each well.
4. Add 100 μL of Standard, sample*, or control per well. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12” orbit) set at 500 rpm. A plate layout is provided to record standards and samples assayed.
5. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (400 μL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 200 μL of SDF-1α Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature on the shaker.

7. Repeat the aspiration/wash as in step 5.

8. Add 200 μL of Substrate Solution to each well. Incubate for 30 minutes at room temperature on the benchtop. Protect from light.

9. Add 50 μL of Stop Solution to each well. The color in the wells should change from blue to yellow. If the color in the wells is green or the color change does not appear uniform, gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
**Cell staining — Tolidine blue**

**Reagent preparation**

1. Toluidine blue stock solution — Mix 1 gm of Toluidine blue with 100 ml 70% alcohol.
2. 1% Sodium chloride — Mix 0.5 gm sodium chloride with 50 ml distilled water.
3. Toluidine blue working solution — Mix 5 ml Toluidine blue stock solution with 1% Sodium chloride.

**Assay procedure**

1. Fix the cells remained on the trans-well membrane with 4% formaldehyde for 10 minutes at room temperature.
2. Gently scratch off the cells on the inner trans-well membrane (the site that the cells were seeded on) by using cotton bud.
3. Stain the cells with Toluidine blue working solution for 1-2 minutes.
4. Rinse in distilled water.
5. Count the cell number remained on the trans-well membrane under light microscope.
Appendix II

Numerical data of the figures in the thesis

Figure 2-2. ALP activity of hMSCs was measured in weekly intervals during osteoinductive treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Absorbance at 405nm (medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days Group 1</td>
<td>0.416</td>
</tr>
<tr>
<td>7 days Group 2</td>
<td>0.172</td>
</tr>
<tr>
<td>7 days Group 3</td>
<td>0.13</td>
</tr>
<tr>
<td>7 days Group 4</td>
<td>0.504</td>
</tr>
<tr>
<td>14 days Group 1</td>
<td>0.645</td>
</tr>
<tr>
<td>14 days Group 2</td>
<td>0.329</td>
</tr>
<tr>
<td>14 days Group 3</td>
<td>0.271</td>
</tr>
<tr>
<td>14 days Group 4</td>
<td>0.954</td>
</tr>
<tr>
<td>21 days Group 1</td>
<td>0.449</td>
</tr>
<tr>
<td>21 days Group 2</td>
<td>0.075</td>
</tr>
<tr>
<td>21 days Group 3</td>
<td>0.032</td>
</tr>
<tr>
<td>21 days Group 4</td>
<td>1.379</td>
</tr>
<tr>
<td>28 days Group 1</td>
<td>0.844</td>
</tr>
<tr>
<td>28 days Group 2</td>
<td>0.184</td>
</tr>
<tr>
<td>28 days Group 3</td>
<td>0.155</td>
</tr>
<tr>
<td>28 days Group 4</td>
<td>1.008</td>
</tr>
</tbody>
</table>

Figure 2-4. Cell proliferation was measured by MTS assay in weekly intervals during the osteoinductive treatment.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Absorbance at 490nm (medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 days Group 1</td>
<td>0.748</td>
</tr>
<tr>
<td>7 days Group 2</td>
<td>0.436</td>
</tr>
<tr>
<td>7 days Group 3</td>
<td>0.412</td>
</tr>
<tr>
<td>7 days Group 4</td>
<td>0.752</td>
</tr>
<tr>
<td>14 days Group 1</td>
<td>0.862</td>
</tr>
<tr>
<td>14 days Group 2</td>
<td>0.412</td>
</tr>
<tr>
<td>14 days Group 3</td>
<td>0.391</td>
</tr>
<tr>
<td>14 days Group 4</td>
<td>0.877</td>
</tr>
<tr>
<td>21 days Group 1</td>
<td>0.492</td>
</tr>
<tr>
<td>21 days Group 2</td>
<td>0.338</td>
</tr>
<tr>
<td>21 days Group 3</td>
<td>0.398</td>
</tr>
<tr>
<td>21 days Group 4</td>
<td>0.511</td>
</tr>
<tr>
<td>28 days Group 1</td>
<td>0.443</td>
</tr>
<tr>
<td>28 days Group 2</td>
<td>0.165</td>
</tr>
<tr>
<td>28 days Group 3</td>
<td>0.232</td>
</tr>
<tr>
<td>28 days Group 4</td>
<td>0.495</td>
</tr>
</tbody>
</table>
Figure 3-11. **SDF-1 expression of hMSCs and rBMCs 5 days after Ad-SDF-1 infection with different MOI.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>SDF-1 expression (pg/ml) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs MOI 0</td>
<td>2384 ± 734</td>
</tr>
<tr>
<td>hMSCs MOI 25</td>
<td>7170 ± 1480</td>
</tr>
<tr>
<td>hMSCs MOI 50</td>
<td>10570 ± 823</td>
</tr>
<tr>
<td>hMSCs MOI 125</td>
<td>14530 ± 1647</td>
</tr>
<tr>
<td>hMSCs MOI 250</td>
<td>17090 ± 976</td>
</tr>
<tr>
<td>hMSCs MOI 500</td>
<td>16837 ± 1344</td>
</tr>
<tr>
<td>rBMCs MOI 0</td>
<td>6993 ± 3924</td>
</tr>
<tr>
<td>rBMCs MOI 25</td>
<td>4893 ± 1563</td>
</tr>
<tr>
<td>rBMCs MOI 50</td>
<td>5693 ± 1601</td>
</tr>
<tr>
<td>rBMCs MOI 125</td>
<td>12560 ± 1743</td>
</tr>
<tr>
<td>rBMCs MOI 250</td>
<td>24660 ± 1900</td>
</tr>
<tr>
<td>rBMCs MOI 500</td>
<td>36293 ± 1159</td>
</tr>
</tbody>
</table>

Figure 3-12. **Cell migration of the non-infected (normal) hMSCs and rBMCs due to the secreted SDF-1 in Ad-SDF-1 infected hMSCs and rBMCs after 6 hours.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell number (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hMSCs MOI 0</td>
<td>26.2 ± 6.51</td>
</tr>
<tr>
<td>hMSCs MOI 125</td>
<td>48.4 ± 11.42</td>
</tr>
<tr>
<td>hMSCs MOI 250</td>
<td>54.1 ± 4.52</td>
</tr>
<tr>
<td>rBMCs MOI 0</td>
<td>16.2 ± 9.02</td>
</tr>
<tr>
<td>rBMCs MOI 250</td>
<td>41.6 ± 9.40</td>
</tr>
<tr>
<td>rBMCs MOI 500</td>
<td>57.7 ± 14.86</td>
</tr>
</tbody>
</table>

Figure 4-8. **Total BMC change within the osteotomy gap after three weeks.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00136 ± 0.00137</td>
</tr>
<tr>
<td>rBMCs</td>
<td>0.00252 ± 0.00058</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>0.00425 ± 0.00096</td>
</tr>
</tbody>
</table>

Figure 4-9. **BMD change of the original bone area adjacent to the osteotomy gap after three weeks.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-9.966 ± 9.384</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-1.873 ± 18.293</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-1.344 ± 9.407</td>
</tr>
</tbody>
</table>

Figure 4-11. **New bone area in the osteotomy gap after three weeks.**

<table>
<thead>
<tr>
<th>Groups</th>
<th>New bone formation (mm²) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.445 ± 0.15</td>
</tr>
<tr>
<td>rBMCs</td>
<td>0.44 ± 0.095</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>0.823 ± 0.354</td>
</tr>
</tbody>
</table>
Figure 4-12. FiSH staining of donor cells.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ratio of cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>rBMCs</td>
<td>1</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>2.625</td>
</tr>
</tbody>
</table>

Figure 4-14. Total BMC change within the osteotomy gap from the first week to the third week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.00146 ± 0.00077</td>
</tr>
<tr>
<td>rBMCs</td>
<td>0.00246 ± 0.0003</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>0.00435 ± 0.00098</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>0.0031 ± 0.00110</td>
</tr>
</tbody>
</table>

Figure 4-15. Total BMC change within the osteotomy gap from the fourth week to the sixth week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.0061 ± 0.00199</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-0.003 ± 0.0016</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-0.00212 ± 0.0008</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>-0.00408 ± 0.00243</td>
</tr>
</tbody>
</table>

Figure 4-16. Total BMC change within the osteotomy gap after six weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.00463 ± 0.00276</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-0.00053 ± 0.0017</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>0.00222 ± 0.00029</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>-0.00098 ± 0.00221</td>
</tr>
</tbody>
</table>

Figure 4-17. BMD change of the original bone area adjacent to the osteotomy gap from the first week to the third week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-17.745 ± 19.835</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-19.212 ± 14.188</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>7.975 ± 14.614</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>5.100 ± 16.113</td>
</tr>
</tbody>
</table>
Figure 4-18. BMD change of the original bone area adjacent to the osteotomy gap from the fourth week to the sixth week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-14.777 ± 18.994</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-13.959 ± 15.324</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-17.181 ± 15.03</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>-23.569 ± 11.475</td>
</tr>
</tbody>
</table>

Figure 4-19. BMD change of the original bone area adjacent to the osteotomy gap after six weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-32.41 ± 3.414</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-31.592 ± 7.590</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-12.073 ± 6.065</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>-19.240 ± 18.206</td>
</tr>
</tbody>
</table>

Figure 4-21. New bone area in the osteotomy gap after six weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>New bone formation (mm²) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.393 ± 0.15</td>
</tr>
<tr>
<td>rBMCs</td>
<td>0.645 ± 0.39</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>1.041 ± 0.167</td>
</tr>
<tr>
<td>Ad-SDF-1</td>
<td>0.558 ± 0.27</td>
</tr>
</tbody>
</table>

Figure 5-2. Total BMC change within the osteotomy gap from the first week to the third week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0018 ± 0.00073</td>
</tr>
<tr>
<td>rBMCs</td>
<td>0.00178 ± 0.00033</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>0.00263 ± 0.00084</td>
</tr>
</tbody>
</table>

Figure 5-3. Total BMC change within the osteotomy gap from the fourth week to the sixth week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.00582 ± 0.00122</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-0.00264 ± 0.00302</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-0.00081 ± 0.00169</td>
</tr>
</tbody>
</table>

Figure 5-4. Total BMC change within the osteotomy gap after six weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total BMC change (grames) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.00402 ± 0.00106</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-0.00086 ± 0.00306</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>0.00181 ± 0.00218</td>
</tr>
</tbody>
</table>
Figure 5-5. BMD change of the original bone area adjacent to the osteotomy gap from the first week to the third week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-34.85 ± 8.283</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-38.354 ± 8.363</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-28.562 ± 10.458</td>
</tr>
</tbody>
</table>

Figure 5-6. BMD change of the original bone area adjacent to the osteotomy gap from the fourth week to the sixth week.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-10.424 ± 11.77</td>
</tr>
<tr>
<td>rBMCs</td>
<td>26.328 ± 13.52</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>3.454 ± 15.212</td>
</tr>
</tbody>
</table>

Figure 5-7. BMD change of the original bone area adjacent to the osteotomy gap after six weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>BMD change (%) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-42.045 ± 6.563</td>
</tr>
<tr>
<td>rBMCs</td>
<td>-22.57 ± 9.253</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>-26.843 ± 10.465</td>
</tr>
</tbody>
</table>

Figure 5-9. New bone area in the osteotomy gap after six weeks.

<table>
<thead>
<tr>
<th>Groups</th>
<th>New bone formation (mm²) (mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.67 ± 0.254</td>
</tr>
<tr>
<td>rBMCs</td>
<td>0.705 ± 0.112</td>
</tr>
<tr>
<td>rBMC-SDF-1</td>
<td>1.067 ± 0.505</td>
</tr>
</tbody>
</table>