# CXCR4-overexpressing Human Placenta-Derived Mesenchymal Stem Cells in Myocardial Regeneration

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# ABSTRACT

**Introduction:** Myocardial infarction (MI) persists as one of the leading causes of death worldwide. Recently, human Bone Marrow-Derived Mesenchymal Stem Cells (hBM-MSCs) autologous or allogeneic therapy has been extensively studied for regeneration and restoration of infarcted myocardium. However, early results from these clinical trials have produced mixed results often showing minor improvements which may be attributed to poor cell homing, inadequate cell migration, poor cell viability and low cell retention of hBM-MSCs in infarcted areas of the heart. In addition, obtaining autologous or allogeneic hBM-MSCs requires invasive procedures to harvest. These challenges necessitate the need for an alternative cell source, which has features comparable to hBM-MSCs. Placenta is a readily available medical waste and is a viable cell source of mesenchymal stem cells (hPD-MSCs). Stromal Cell-derived Factor-1α (SDF-1α) is a small pro-inflammatory chemoattractant cytokine. Interaction between SDF-1α and its cognate receptor C-X-C Chemokine Receptor Type 4 (CXCR4) is crucial for homing and migration of multiple stem cell types. In the current study, we have analyzed the SDF-1α /CXCR4-mediated cell migration and cell proliferation on both hPD-MSCs and hBM-MSCs. hPD-MSCs were compared to hBM-MSCs with respect to cellular response to glucose-induced and Cobalt Chloride ($CoCl_2$)-induced hypoxia.

**Methods:** Cell culture conditions for stimulation of CXCR4 expression, dosing of SDF-1α, glucose, and $CoCl_2$ were optimized using cell viability, cell proliferation, and cell migration assays. Cell surface localization of CXCR4 was detected and validated by immunofluorescence. Expression of CXCR4 was detected by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. To understand cell signaling, the expression of cell-signaling proteins CXCR4, HIF-1α, IL-6, IL-10, Akt, p-Akt, ERK, and p-ERK were analyzed by Western blot.

CXCR4-positive cells were sorted and analyzed by fluorescence-activated cell sorting (FACS) in optimized cell culture conditions.

**Results:** CXCR4 was expressed by both hBM-MSCs and hPD-MSCs at basal level. From cell migration assays, it was determined that hPD-MSCs had a significantly greater cell migration potential than hBM-MSCs towards SDF-1α in a dose dependent manner. Also, the expression of CXCR4 was found to be significantly higher ($P<0.05$) after exposure to $CoCl_2$-induced hypoxia than after treatment with SDF-1α or exposure to glucose-induced hypoxia in both hPD-MSCs and hBM-MSCs. In $CoCl_2$-induced hypoxic conditions, the expression of CXCR4 was significantly increased ($P<0.0001$) in hPD-MSCs, compared to hBM-MSCs. Also, the CXCR4/MEK/ERK signaling cascade was observed to be significantly activated ($P<0.05$) in hPD-MSCs, whereas, the CXCR4/PI3K/Akt cascade was significantly activated ($P<0.01$) in both cell types in $CoCl_2$-induced hypoxic conditions.

**Discussions:** Our data suggest that both hBM-MSCs and hPD-MSCs express CXCR4 at basal levels. In addition, their sensitivity to SDF-1α concentrations indicates that there might be other SDF-1α-mediated pathways involved in regulating the cell migration activities of both cell types. Complex downstream signaling cascades of SDF-1α/CXCR4 axis are activated, including PI3K/Akt and/or MEK/ERK/IKKαβ pathways in the $CoCl_2$-induced hypoxic condition. The CXCR4-dependent MEK/ERK pathway was activated dominantly in hPD-MSCs but not in hBM-MSCs, which might be responsible for the increased chemotactic response in hPD-MSCs in the cell proliferation. This hypothesis needs to be further validated. In line with exploring these detailed signaling cascades, more studies should be performed to see if they are being affected by placenta-specific factors such as placenta-induced growth factor (PIGF) - a cytokine, which enhances cell motility by mobilizing ERK phosphorylation and cytoskeletal

rearrangement in various kinds of cells and is innately present in cells of the placenta. Importantly, our results need to be explored and validated *in vivo* as well.

**Conclusions:** Based on the these findings, our data provide new insights into comparative molecular mechanisms that regulate the migration of mesenchymal stem cells derived from bone marrow and placenta. Also, based on SDF-1α/CXCR4-sensitivity in response to hypoxia, our data suggest that hPD-MSCs could represent an effective and efficient alternative to hBM-MSCs for translational studies in cellular repair.

# RÉSUMÉ

**Introduction:** L'infarctus du myocarde (IDM) est un des principales causes de décès au monde. Les cellules souches mésenchymateuses dérivées du placenta humain (hPD-MSCs) ont été étudié pour leurs applications aux thérapeutique cellulaires post-IDM. Cependant, les réactions des cellules à l'environnement local peuvent affecter l'efficacité du traitement : la viabilité, guidage et migration des cellules, ainsi que la sécrétion des cytokines et chimiokines sont tous des facteurs majeurs. Le potentiel thérapeutique des hPD-MSCs semble être meilleur que les cellules souches mésenchymateuses dérivées de la moelle osseuse (hBM-MSCs) : un autre type de cellule qui a beaucoup été étudié pour la régénération de l'infarctus. Autre leur transdifférenciation, les sécrétions des paracrines provenant des cytokines, comme les interleukines et facteurs de croissance des hPD-MSCs, ont aussi un rôle important pour la régénération de l'infarctus. De plus, les hPD-MSCs ont démontré une meilleure réactivité que les hBM-MSCs après avoir été exposé aux cytokines inflammatoires. Le facteur de stroma dérivés des cellules 1α (SDF-1α) et les récepteurs de chimiokine C-X-C type 4 (CXCR4) sont des pairs de ligand/récepteur ayant un rôle dans la circulation, migration et guidage des cellules souches mésenchymateuses. L'objectif de cette étude est de déterminer si les hPD-MSCs sont des candidats de cellules souches plus approprié que les hBM-MSCs pour les thérapeutiques cellulaires, mesuré par le guidage des cellules médiés par les SDF-1α/CXCR4 sur l'axe et leur sécrétion paracrine après le guidage. Dans l'étude présente, nous avons étudié la migration, inflammation et la prolifération des cellules médiées par SDF-1α/CXCR4 des hPD-MSCs et hBM-MSCs.

**Méthodes**: Le temps de la culture des cellules ainsi que les doses de SDF-1α, glucose, et $CoCl_2$

ont été optimisé pour l'expression de CXCR4, la viabilité des cellulaires, et la prolifération des cellules MTT. L'expression des gênes du CXCR4 a été détecté par RT-PCR. De plus, l'expression des protéines CXCR4, HIF-1α, IL-6, IL-10, Akt, p-Akt, ERK, et p-ERK ont été analysé par buvardage de Western. La localisation sur la surface des CXCR4 a été détectée et validé par immunofluorescence. Les cellules positives CXCR4 ont ensuite été tris et analysé par la méthode FACS (tri cellulaire activité par la fluorescence) pour des études in vivo.

**Résultats**: CXCR4 était naturellement exprimé chez les hBM-MSCs et hPD-MSCs. Les essais de migration cellulaire ont montré que l'interaction SDF-1α/CXCR4 est active dans les deux types de cellules; cependant, hPD-MSCs avait une meilleure réponse du SDF-1α que hBM-MSCs. En outre, l'expression de CXCR4 trouvé à été significativement plus élevé ($P<0.05$) après l'exposition au $CoCl_2$ induite en condition d'hypoxie qu'après traitement au SDF-1α ou l'exposition à l'hypoxie induite par le glucose dans les deux hPD-MSC et hBM-MSC. Dans des conditions l'induction du $CoCl_2$ en hypoxie, l'expression de CXCR4 a augmenté de façon significative ($P<0.0001$) chez hPD-MSC, comparativement à hBM-MSC. En outre, la cascade de signalisation CXCR4/MEK/ERK de CXCR4 a été significativement activé ($P<0.05$) dans hPD-MSC, alors que la cascade CXCR4/PI3K/Akt était significativement activé ($P<0.01$) dans les deux types de cellules après l'induction du $CoCl_2$ en condition d'hypoxie.

**Discussion:** Nos données suggèrent que les hBM-MSC et les hPD-MSC expriment CXCR4 à des niveaux de base. En plus leur sensibilité à la concentration de SDF-1α nous indique qu'il peut y avoir d'autres médiateurs de SDF-1α impliqué dans la régulation de l'activité de la migration cellulaires dans les deux types de cellules. De plus, suite à l'activation du complexe SDF-1α/CXCR4, différents genres de cascades de signalisation ont eu lieu, particulièrement les voies PI3K/Akt et/ou MEK/ERK/IKKαβ dans les conditions d'hypoxie. L'expression accrue de

la voie CXCR4/MEK/ERK est activement dominante chez hPD-MSCs que les hBM-MSCs. Ceci explique partiellement la réponse chimiotactique des hPD-MSCs dans les essais de la prolifération cellulaires. Toutefois, cette hypothèse aura besoin d'être validé en étudiant le changement de niveau des facteurs de transcription comme le NFκB. En plus avec l'exploration des cascades de signalisation détaillés, plus de détails devront être performé pour examiner si les facteurs spécifiques au placenta seraient affectés, comme le facteur de croissance pour placenta (PIGF), une cytokine qui améliore la motilité des cellules en phosphorylant ERK et réarrangeant le cytosquelette de différent genres de cellules nativement présent dans les cellules du placenta. Finalement, nos résultats devront être exploré et validé in vivo.

**Conclusions:** Basée sur ces résultats, nos données fournissent de nouvelles perspectives sur les mécanismes moléculaires qui régulent la comparaison de la migration des cellules souches mésenchymateuses dérivées de moelle osseuse et le placenta. Aussi, en se basant sur le sensibilité en réponse à l'hypoxie de SDF-1α/CXCR4, nos données suggèrent que hPD-MSC pourrait représenter une alternative efficace et une meilleur ressource que hBM-MSC pour les études translationnelles dans la réparation cellulaire.

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# LIST OF ABBREVIATIONS

Myocardial infarction (MI)

Human Placenta-Derived Mesenchymal Stem Cells (hPD-MSCs)

Human Bone Marrow Mesenchymal Stem Cells (hBM-MSCs)

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Cardiovascular disease (CVD)

Universal Definition Consensus Group (UDCG)

American Heart Association (AHA)

World Heart Federation (WHF)

Electrocardiographic (ECG)

Acute myocardial infarction (AMI)

Prior myocardial infarction (PMI)

Percutaneous coronary intervention (PCI)

Coronary artery bypass grafting (CABG)

Left anterior descending (LAD)

Cardiac troponin (cTn)

Upper reference limit (URL)

ST-segment-T wave (ST-T)

Left bundle branch block (LBBB)

ST elevation myocardial infarction (STEMI)

Alteplase (tPA)

Reteplase (rPA)

Tenecteplase (TNK)

Non–ST elevation myocardial infarction (NSTEMI)

Left ventricle (LV)

Mesenchymal stem cells (MSCs)

Electromechanical coupling (E-C)

Endothelial progenitor cells (EPC)

Heart failure (HF)

Human Mesenchymal Stem Cells (hMSCs)

International Society for Cellular Therapy (ISCT)

Umbilical cord blood (UCB)

Stromal cell-derived factor 1α (SDF-1α)

C-X-C motif chemokine 12 (CXCL12)

C-X-C chemokine receptor type 4 (CXCR4)

Cluster of differentiation 184 (CD184)

Cobalt chloride ($CoCl_2$)

Hypoxia induced factor-1-α (HIF-1α)

Protein kinase B (Akt)

Extracellular signal-regulated kinase (ERK)

Phosphorylated Akt (p-Akt)

Phosphorylated ERK (p-ERK)

Interleukin-6 (IL-6)

Fluorescence-activated cell sorting (FACS)

Phosphate-buffered saline (PBS)

Phosphoinositide-3 kinase (PI3K)

Nuclear factor of kappa beta (NFκB)

Mitogen-activated protein kinases (MAPK)

Radio immunoprecipitation buffer (RIPA buffer)

4', 6-diamidino-2-phenylindole (DAPI)

Vascular endothelial growth factor (VEGF)

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# CHAPTER 1. INTRODUCTION

Cardiovascular disease (CVD) is a major health problem and is one of the leading causes of death worldwide. CVD, unspecific coronary artery disease, and stroke contribute to approximately one-third of deaths in the world [1]. A primary manifestation of these diseases is the myocardial infarction (MI), which causes a great proportion of deaths and maintains a high morbidity rate every year [2]. In MI, there is an irreversible loss of billions of cardiomyocytes, which drastically reduces the contractility of the heart and ultimately leads to heart failure. Currently, no existing method of treatment could reverse the pathophysiological process and the damage caused by an MI, which includes damage to nature cardiomyocytes and the progression of necrosis in the infarcted heart, by replenishing lost cardiomyocytes. However, with the studies in the field of regenerative medicine in the past few decades, stem cell therapy has gained popularity as a potential treatment for various diseases, such as the treatment of leukemia [3]. It opens doors in developing treatment, which require the implantation of stem cells into an ischemic heart, thereby aiding in the process of repairing the damaged cardiac tissue. Recently, human Bone Marrow-Derived Mesenchymal Stem Cells (hBM-MSCs) based stem cell therapy has been extensively evaluated in clinical trials for regeneration and restoration of infarcted myocardium. However, from outcome studies, it has been clear that many challenges need to be overcome for successful and effective stem cell therapy, such as poor cell migration, cell retention [4, 5], choosing appropriate cell lines, which requires minimal surgical procedures and implanting methods [6-8]. It is still optimistic to expect that stem cell implantation with high safety and efficacy may turn to be a hopeful method of treatment for MI. Human placenta is a readily available medical waste and is a viable cell source of mesenchymal stem cells (hPD-

MSCs). As a potential alternative to hBM-MSCs, more and more cell therapy studies have been conducted to test the potency of hPD-MSCs for the treatment of MI [9, 10].

In this study, we have analyzed and compared SDF-1α/CXCR4-mediated cell migration, cell proliferation and their responsiveness to hypoxia condition of both hPD-MSCs and hBM-MSCs to test the appropriateness of hPD-MSCs as a potential and viable alternative to hBM-MSCs for the treatment of myocardial infarction.

# CHAPTER 2. LITERATURE REVIEW

## 2.1 Myocardial Infarction (MI)

A myocardial infarction (MI) occurs when there is an occlusion of a coronary artery, diminishing coronary flow reserve, which results in a deficiency of oxygen supply into the heart [11]. As myocardial demand increases, the supply/demand mismatch of oxygen requirements leads to the necrosis of cardiomyocytes [12].

The Universal Definition Consensus Group (UDCG), composed by American Heart Association (AHA) and the World Heart Federation (WHF), defines MI as a complicated syndrome [2]. MI can be recognized by the following clinical diagnoses: 1) electrocardiogram (ECG), 2) biochemical markers of myocardial necrosis (elevated values of biomarkers, such as cardiac troponin T and I), 3) imaging results (echocardiography), and 4) pathological analysis [13, 14].

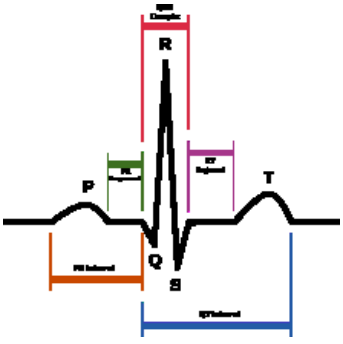
### 2.1.1 Definition of MI

As described in the *Third Universal Definition of Myocardial Infarction*, MI can be categorized into two categories: 1) acute myocardial infarction (AMI) and 2) prior myocardial infarction (PMI) [13]. In this study, we focus on evaluating an alternative cell source for stem cell therapy, which may be used to treat AMI. AHA and WHF in 2012 stated that: "The term acute myocardial infarction should be used when there is evidence of myocardial necrosis in a clinical setting consistent with acute myocardial ischemia. Under these conditions, any one of the following criteria meets the diagnosis for MI: (Table 1). [13]"

| | Criteria for acute myocardial infarction: |
|---|---|
| 1 | "Detection of a rise and/or fall of cardiac biomarker values [preferably cardiac troponin (cTn)] with at least one value above the 99th percentile Upper Reference Limit (URL) and with at least one of the following:<br>▪ Symptoms of ischemia<br>▪ New or presumed new significant ST-segment-T wave* (ST-T) changes or new left bundle branch block (LBBB)<br>▪ Development of pathological Q waves in the electrocardiographic (ECG)<br>▪ Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality<br>▪ Identification of an intracoronary thrombus by angiography or autopsy" [13] |
| 2 | "Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes or new LBBB, but death occurred before cardiac biomarkers were obtained, or before cardiac biomarker values would be increased." [13] |
| 3 | "Percutaneous coronary intervention (PCI) related MI is arbitrarily defined by elevation of cTn values (>5 × 99th percentile URL) in patients with normal baseline values (≤99th percentile URL) or a rise of cTn values >20% if the baseline values are elevated and are stable or falling. In addition, either (i) symptoms suggestive of myocardial ischemia or (ii) new ischemic ECG changes or (iii) angiographic findings consistent with a procedural complication or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required." [13] |
| 4 | "Stent thrombosis associated with MI when detected by coronary angiography or autopsy in the setting of myocardial ischemia and with a rise and/or fall of cardiac biomarker values with at least one value above the 99th percentile URL." [13] |
| 5 | "Coronary artery bypass grafting (CABG) related MI is arbitrarily defined by elevation of cardiac biomarker values (>10 × 99th percentile URL) in patients with normal baseline cTn values (≤ 99th percentile URL). In addition, either (i) new pathological Q waves or new LBBB, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality." [13] |

**Table 1. Definition of Myocardial Infarction.** [2, 13, 14]

* "The electrocardiograph (ECG) waveform consists of a P wave, a PR interval, the QRS complex, an ST segment, and T and U waves. The ST segment and T wave reflect repolarization of the ventricles" [15].




<https://en.wikipedia.org/wiki/ST_segment>

### 2.1.2 Universal classification of MI

The UDCG classifies MI under five categories [13, 16]: Type 1-Spontaneous MI; Type 2-MI secondary to an ischemic imbalance; Type 3-MI resulting in death when biomarker values are unavailable; Type 4a-MI related to percutaneous coronary intervention (PCI); Type 4b-MI related to stent thrombosis; Type 5-MI related to coronary artery bypass grafting (CABG), and described their clinical features (Table 2).

Among these five types of MI, Type 1 and Type 2 are easily confused. Therefore, type 1 & 2-MI are more commonly discussed and distinguished with each other's differences in the aspect of the damaged condition of the infarcted coronary arteries [13]. Plaque rupture with thrombus belongs to Type 1 MI, and vasospasm or endothelial dysfunction, fixed atherosclerosis/supply-demand imbalance, and supply-demand imbalance belong to Type 2 MI (Figure 1). [13]

### 2.1.3 Arteries and Cardiac areas affected by MI

Three main coronary arteries may be occluded in MI: 1) left anterior descending (LAD), 2) right coronary, and 3) left circumflex (Table 3). The most frequent types of infarction in the heart are: 1) anterior infarction, 2) anterolateral infarction, 3) true posterior infarction, and 4) diaphragmatic or inferior infarction [17] (Figure 2). [13]

| Type | Name | Description and clinical features |
|---|---|---|
| 1 | Spontaneous myocardial infarction | "Spontaneous myocardial infarction related to atherosclerotic plaque rupture, ulceration, Assuring, erosion, or dissection with resulting intraluminal thrombus in one or more of the coronary arteries leading to decreased myocardial blood flow or distal platelet emboli with ensuing myocyte necrosis. The patient may have underlying severe coronary artery disease but on occasion non-obstructive or no coronary artery disease." [13] |
| 2 | Myocardial infarction secondary to an ischemic imbalance | "In instances of myocardial injury with necrosis where a condition other than coronary artery disease contributes to an imbalance between myocardial oxygen supply and/or demand, e.g. coronary endothelial dysfunction, coronary artery spasm, coronary embolism, tachy-/brady-arrhythmias, anemia, respiratory failure, hypotension, and hypertension with or without left ventricular hypertrophy (LVH)." [13] |
| 3 | Myocardial infarction resulting in death when biomarker values are unavailable | "Cardiac death with symptoms suggestive of myocardial ischemia and presumed new ischemic ECG changes or new LBBB, but death occurring before blood samples could be obtained, before cardiac biomarker could rise, or in rare cases cardiac biomarkers were not collected." [13] |
| 4a | Myocardial infarction related to percutaneous coronary intervention (PCI) | "Myocardial infarction associated with PCI is arbitrarily defined by elevation of cTn values >5 × 99th percentile URL in patients with normal baseline values (< 99th percentile URL) or a rise of cTn values > 20% if the baseline values are elevated and are stable or falling. In addition, either (i) symptoms suggestive of myocardial ischemia, or (ii) new ischemic ECG changes or new LBBB, or (iii) angiographic loss of patency of a major coronary artery or a side branch or persistent slow- or no-flow or embolization, or (iv) imaging demonstration of new loss of viable myocardium or new regional wall motion abnormality are required." [13] |
| 4b | Myocardial infarction related to stent thrombosis | "Myocardial infarction associated with stent thrombosis is detected by coronary angiography or autopsy in the setting of myocardial ischemia and with a rise and/ or fall of cardiac biomarkers values with at least one value above the 99th percentile URL." [13] |
| 5 | Myocardial infarction related to coronary artery bypass grafting (CABG) | "Myocardial infarction associated with CABG is arbitrarily defined by elevation of cardiac biomarker values > 10 × 99th percentile URL in patients with normal baseline cTn values (< 99th percentile URL). In addition, either (i) new pathological Q waves or new LBBB, or (ii) angiographic documented new graft or new native coronary artery occlusion, or (iii) imaging evidence of new loss of viable myocardium or new regional wall motion abnormality." [13] |

**Table 2. Universal Classification of Myocardial Infarction.** [2, 13]

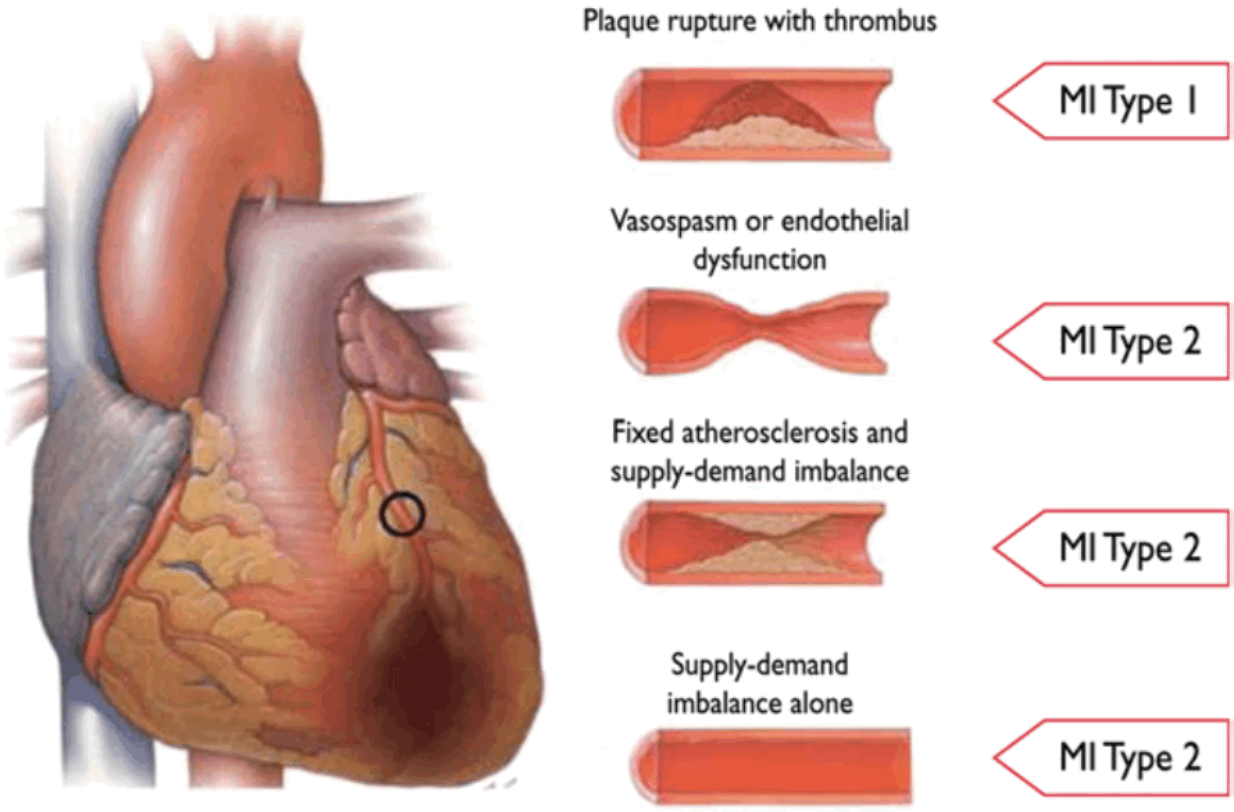



**Figure 1. Differentiation between myocardial infarction (MI) types 1 and 2 according to the condition of the coronary arteries [13].** Plaque rupture with thrombus belongs to Type 1 MI. Vasospasm or endothelial dysfunction, fixed atherosclerosis/supply-demand imbalance, and supply-demand imbalance belong to Type 2 MI.

| Artery and Area Affected by MI | |
|---|---|
| **Artery occluded** | **Frequency and affected area** |
| LAD | 40–50%; affects anterior and apical left ventricle and anterior two thirds of interventricular septum (IVS) |
| Right coronary | 30–40%; affects posterior wall of left ventricle, posterior one third of IVS (if right-dominant coronary circulation) |
| Left circumflex | 15–20%; affects lateral wall of left ventricle (can also affect posterior wall if left dominant coronary circulation) |

**Table 3. Artery and area affected by MI [17].** The most frequently occluded arteries that result in MI are 1) left anterior descending (LAD), 40-50%; 2) right coronary, 30-40%; and 3) left circumflex, 15-20%.

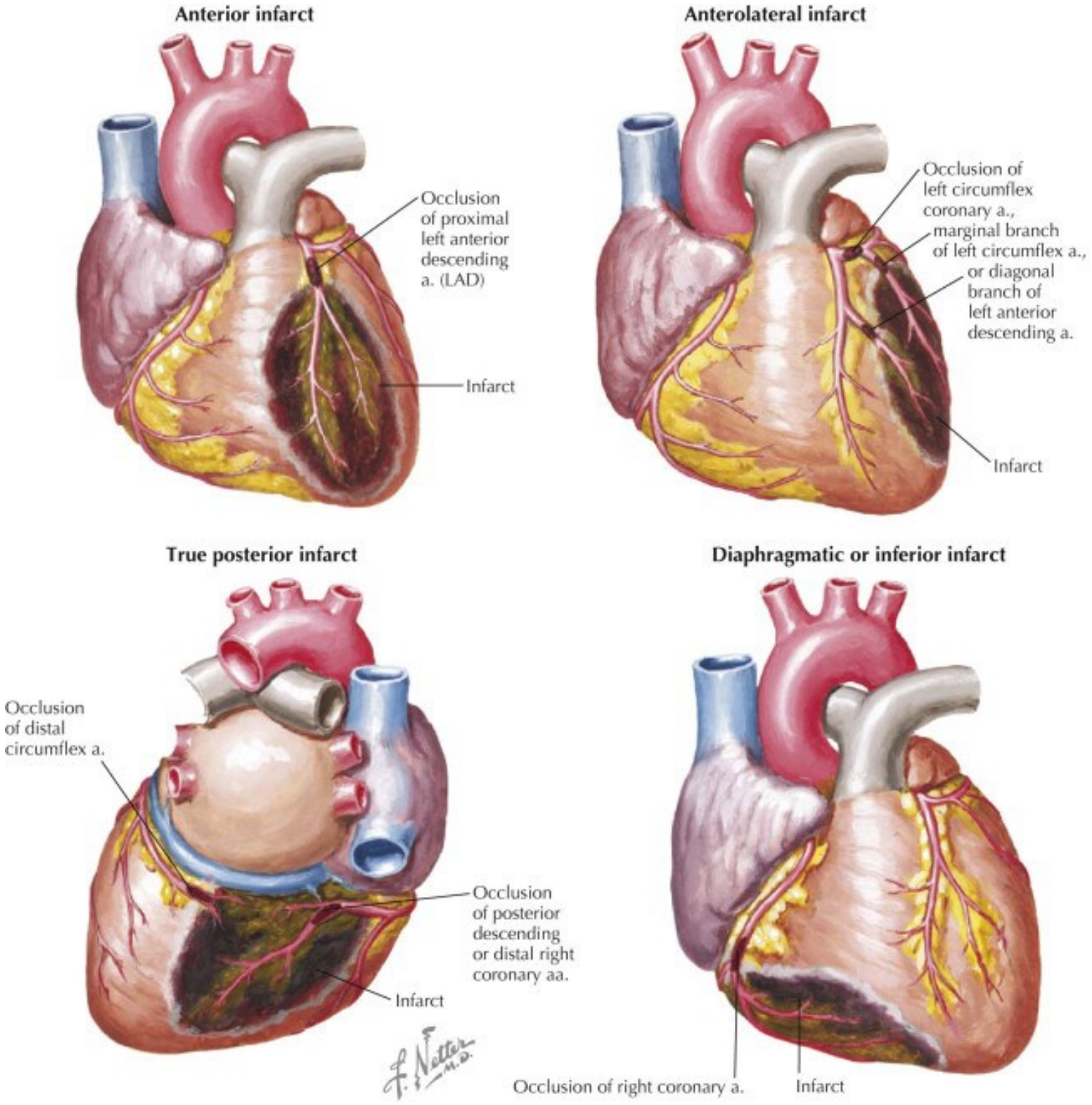



**Figure 2. Artery and area affected by MI [17].** "Myocardial infarction (MI) is a major cause of death. Coronary artery atherosclerosis and thrombosis, the major causes of MI, precipitate local ischemia and necrosis of a defined myocardial area. Necrosis usually occurs approximately 20 to 30 minutes after coronary artery occlusion. Usually, MI begins in the subendocardium because this region is the most poorly perfused part of the ventricular wall." [17] The most frequently

caused infarctions are 1) anterior infarction, 2) anterolateral infarction, 3) true posterior infarction, and 4) diaphragmatic or inferior infarction.

### 2.1.4 Management and therapy of MI

#### 2.1.4.1 ST-elevation myocardial infarction (STEMI)

Immediate reperfusion therapy is administered to patients who suffer from ST elevation (Table 1 "*") or new left bundle branch block (LBBB), in order to restore coronary blood flow. The increase of coronary flow reserve can be achieved by both: 1) pharmacologically with thrombolytic medications and 2) mechanically with percutaneous coronary intervention (PCI) [18].

Intravenous streptokinase and recombinant tissue plasminogen activators are commonly used thrombolytic agents in clinics. Streptokinase is a relatively inexpensive medication that is commonly used worldwide. The recombinant tissue plasminogen activators include tPA (alteplase), rPA (reteplase), and TNK (tenecteplase) [18, 19].

Primary PCI of the infarcted artery is a superior approach to thrombolysis in terms of increasing efficiency by reducing mortality and enhancing safety by reducing bleeding complications. (Figure 3 & 4) However, the timely access to qualified catheterization facilities and the availability of doctors limit its use [18, 19].

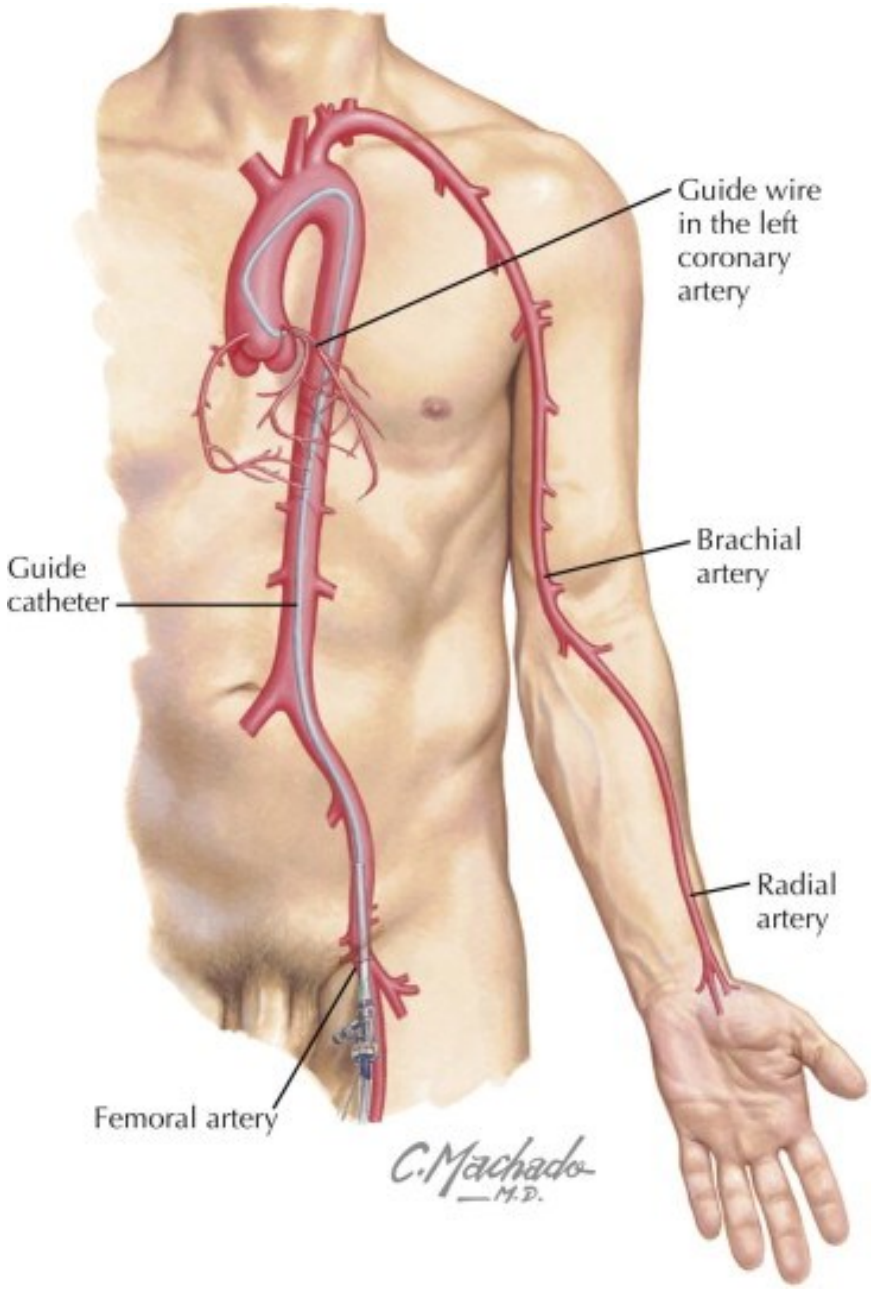



**Figure 3. Percutaneous coronary intervention: vascular access [20].** PCI is performed via the femoral, radial, or brachial artery access with the same radiographic equipment used for diagnostic coronary arteriography in cardiac catheterization laboratories. The most frequently chosen artery access is the femoral artery, and it is the preferred approach applied in most clinics [20].

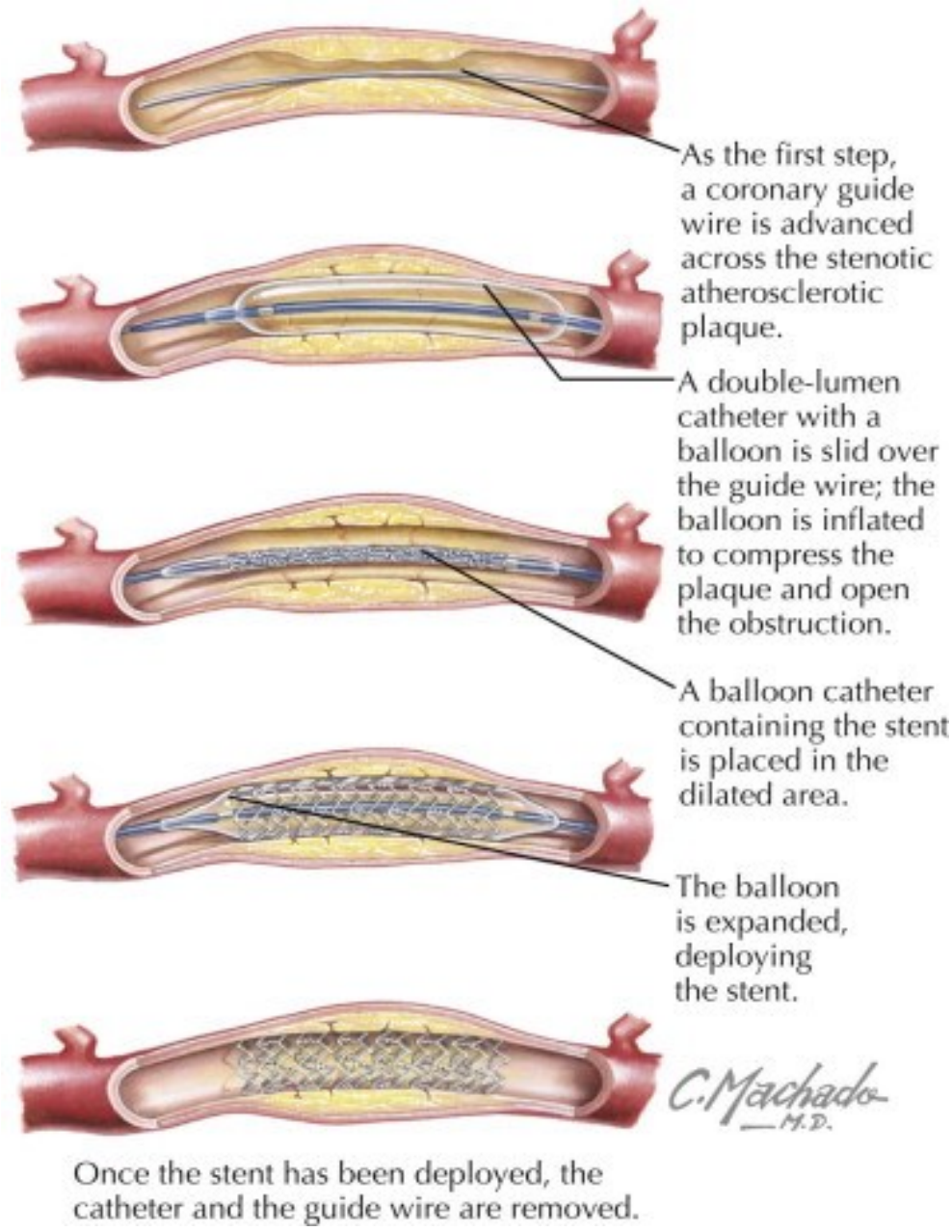



**Figure 4. The performance of percutaneous coronary intervention-stent deployment [20].** "Interventional guide catheters are slightly larger than diagnostic catheters so as to accommodate balloons, stents, and other devices. After visualization of the coronary artery and target lesion via arteriography, a coronary guide wire is advanced across the lesion and positioned in the distal vessel. A small double-lumen catheter with a distal balloon is passed over the guidewire and positioned at the lesion. An inflation device is used to expand the balloon and open the obstruction by fracturing and compressing plaque. Today, coronary stenting is an integral part of virtually all angioplasty procedures. The undeployed stent is mounted on a second balloon catheter that is passed over the guide wire to the area initially dilated. Balloon inflation expands

and deploys the stent. A high-pressure balloon catheter is then used to fully expand the stent. With continued improvements in devices, it is increasingly common to insert and fully expand the stent using a single-balloon catheter without pre-dilatation." [20]

#### 2.1.4.2 Non–ST elevation myocardial infarction (NSTEMI)

Although patients experiencing STEMI are often prioritized in terms of accessing treatments at early stages, it has been shown that early cardiac catheterization (either PCI or surgical bypass grafting) (Figure 5), helps achieve appropriate revascularization, reducing MI mortality and morbidity in patients experiencing NSTEMI [21]. This finding is emphasized in patients with NSTEMI, who also experience the following symptoms: 1) recurrent ischemia, 2) elevated cardiac troponin, 3) heart failure symptoms, and 4) new ST-segment changes on ECG. In addition, antiplatelet and antithrombotic medications remain as the cornerstones of NSTEMI therapy pharmacologically [21].

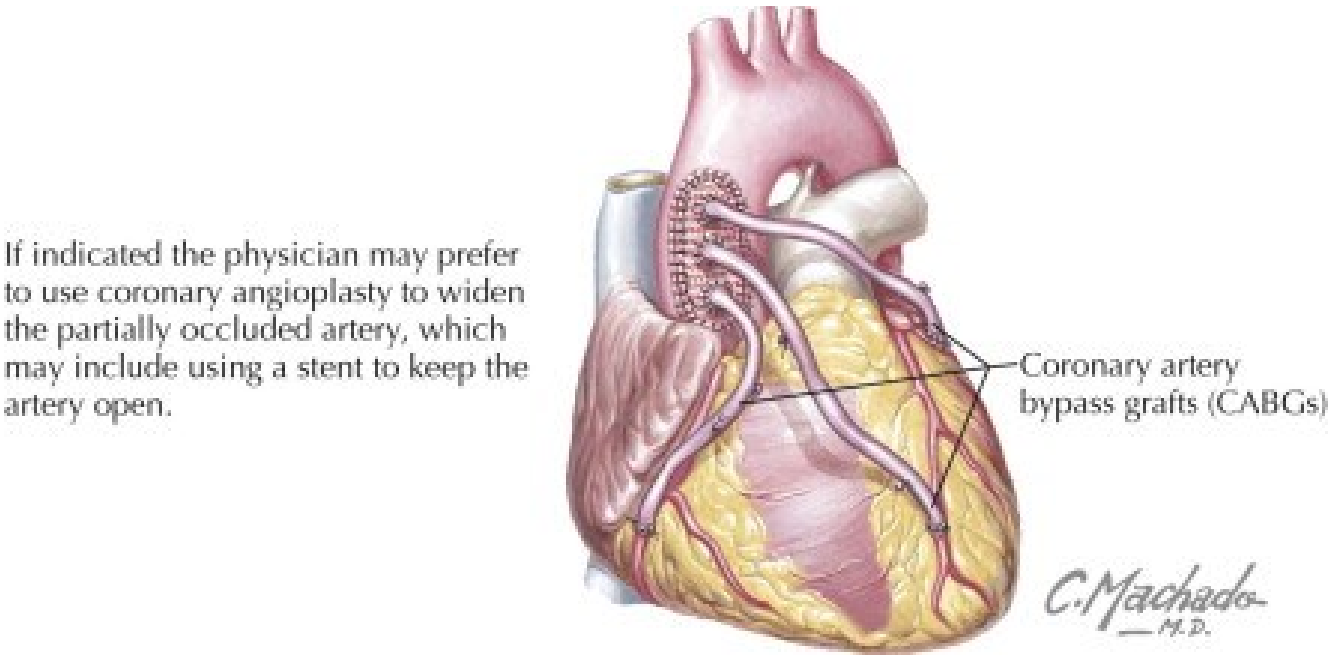



**Figure 5. Coronary bypass [17].** A surgical approach for revascularization is the coronary artery bypass graft (CABG), which is also called 'the cabbage procedure'. CABG is an approach that improves blood supply through grafting veins or arteries to the patient's coronary arteries. Veins or arteries are from elsewhere in the patient's body, such as 1) the great saphenous vein harvested from the patient's lower limb, 2) internal thoracic artery, and 3) radial artery [17].

### 2.1.5 Post-MI

The death of apoptotic and necrotic cells post-MI leads to cardiac tissue damage and loss, which are unable to be reconstituted for by the remaining cardiomyocytes, thereby deteriorating the functionality of the heart with time. This is because the damaged cardiac muscles are replaced by fibrous scar, which does not support cardiac functions. Although timely reperfusion and optimal drug- and device-based therapies are traditional therapeutic strategies, which have been commonly employed in current treatments and have reduced MI-associated mortality [22, 23], they suffer from limitations. Additionally, the main and primary focus of those current treatments is limiting disease progression rather than repairing and restoring the damaged cardiac tissue and function. Therefore, alternative and additional long-term therapeutic approaches have been investigated to overcome the limited efficacy and co-morbidity of these current treatments and to explore more options for personalized treatment [24]. In particular, new approaches are needed for patients who are diagnosed with left ventricular (LV) dysfunction [23, 25] and whose cardiac functions cannot be improved effectively by current treatments. Accordingly, many auxiliary medical technologies are currently being investigated, among which one class is cell-based therapy. Stem cells are used to repopulate infarcted regions of ischemic cardiomyopathic hearts and have emerged as a promising treatment strategy [26]. (Table 4)

<table>
<tr><th colspan="2">Traditional treatments<br><br>(Limiting disease progression)</th><th>New treatments<br><br>(Repairing cardiac tissue and functions)</th></tr>
<tr><th>STEMI</th><th>NSTEMI</th><td rowspan="2"><ul><li>Stem cell therapy: “administrating stem cells into infarcted cardiac tissues” [8, 23, 27, 28].</li><li>Tissue engineering [24]:<ul><li>“Utilizing polymeric scaffolding material as a support matrix</li><li>Incorporating cardiac myocytes within biodegradable gels</li><li>Utilizing polymeric temperature-sensitive surfaces”</li></ul></li></ul></td></tr>
<tr><td><ul><li>Immediate reperfusion</li><li>Thrombolytic medications</li></ul></td><td><ul><li>PCI</li><li>Surgical bypass grafting</li><li>Antiplatelet and antithrombotic medications</li></ul></td></tr>
</table>

**Table 4. Summary of the treatments of MI.** Traditional treatments of MI focus on limiting disease progression. For STEMI treatments, immediate reperfusion and the thrombolytic medications are the most frequently used methods in clinics. For NSTEMI treatments, PCI, surgical bypass grafting, and antiplatelet and antithrombotic medications are the most frequently employed methods in clinics. New treatments, which are focusing on repairing cardiac tissues and functions, have been being investigated. Two main new methods are stem cell therapy and tissue engineering approaches, such as scaffolds, biodegradable gels, and temperature-sensitive surfaces.

## 2.2 Stem cell therapy

Stem cell therapy has been extensively studied as therapeutic modality for the treatment of post-MI [3]. Indeed, recent studies have suggested that stem cell engraftment into newly infarcted myocardium can lead to improved cardiac function [29].

Stem cell therapy widens the possibilities for methods of restoring tissue properties by 1) allowing stem cells to integrate with the target tissue as participants or 2) allowing stem cells to deliver complex signals to a target tissue as vehicles, without the direct integration of the target tissue [30]. It was reported that in cardiac ischemic injury mesenchymal stem cells (MSCs) do not appear to incorporate into the native tissue, which offers a strategy of using stem cells as a platform for delivering "drug-like effects" – slowly delivering complex signals. However, controversy about their use has been engendered because of their lack of incorporation and the complexity and difficulty of measuring an improved heart function/cardiac tissue performance [30]. We still see the optimistic aspects of stem cell therapy through the number of studies in the literature indicating its short-term beneficial effects, which cannot and should not be dismissed.

### 2.2.1 Goals of stem cell therapy

There are three main goals of stem cell-based therapies after MI: 1) to minimize the apoptotic and necrotic cell death of cardiomyocytes, 2) to facilitate and promote left ventricle (LV) remodeling, and 3) to repair and regenerate myocardial structures (blood vessels and cardiomyocytes) [29].

### 2.2.2 Stem cells are superior to all cell types in cell therapy

There are many challenges remaining to be overcome to achieve a successful cell therapeutic method for MI, which begins with choosing a suitable cell type. Many different cell types, each with its own theoretical advantages and disadvantages, are being investigated (Figure 6) [24]. Purification of a single cell type and its sufficient quantities of the certain cells need to be made available, in order to provide an "off-the-shelf" efficacy [31]. Cell manufacturing is likely to be the most costly and time-consuming aspect that limits cell therapies [31]. Meanwhile, criteria of the ideal cell type for this therapy is strict: high safety, high efficacy, the ability of regenerating healthy and functional cardiac muscles, convenience of harvest, free of immune reaction/rejection, and must be extracted without any ethical issues [3]. Multiple cell types have been used in the setting of MI, but unfortunately, ending with inconsistent efficacy [23, 32-35] and modest results were achieved overall [23, 36]. Among various types of cell sources, mesenchymal stem/stromal cells (MSCs) are the most prospective and promising candidates, due to their potential to differentiate into cardiomyocytes, vascular smooth muscle cells, and endothelial cells [37]. In addition, MSCs produce angiogenic factors through paracrine mechanisms [37], which promote the myocardial regeneration.

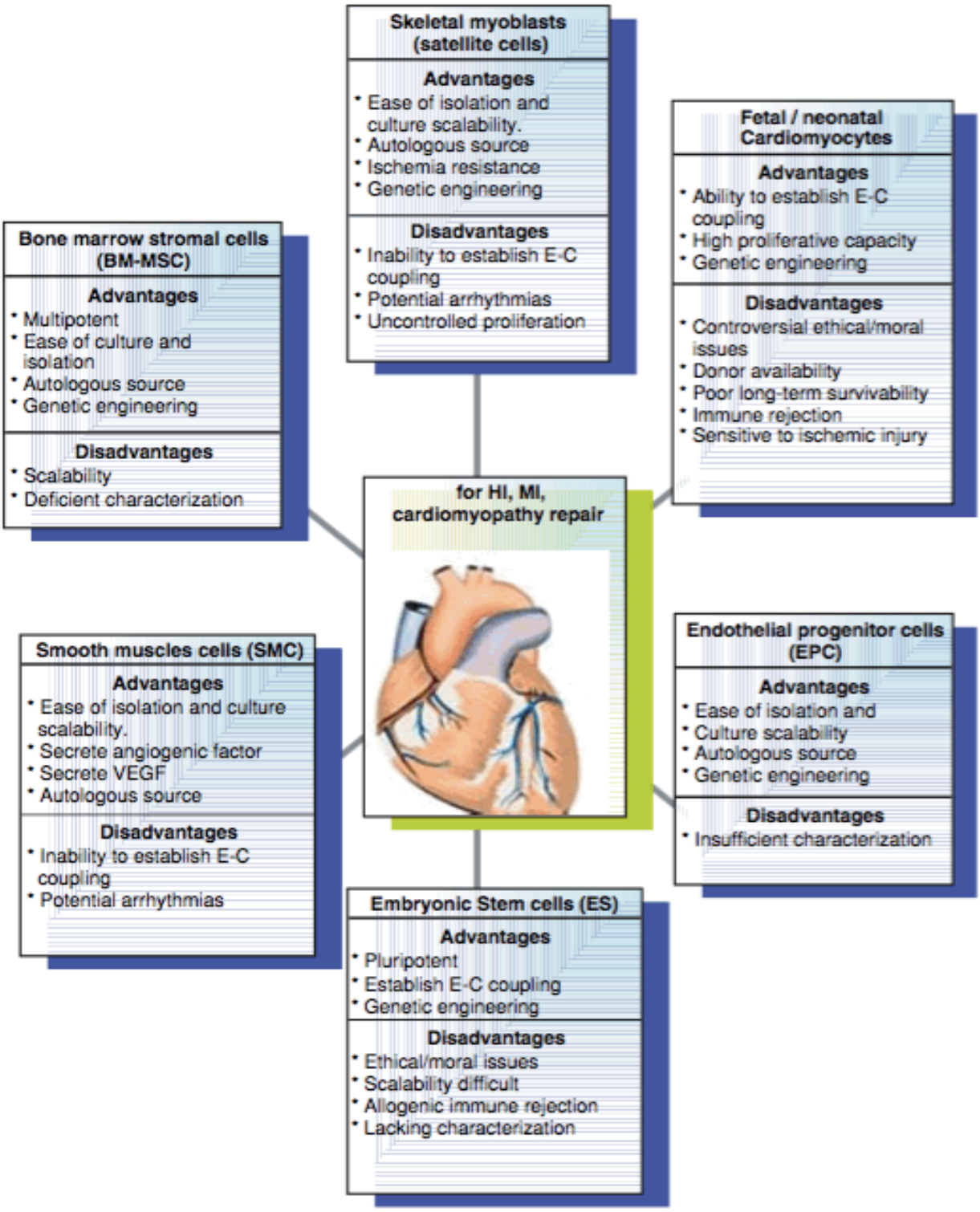



**Figure 6. Potential cell types for cell-based therapies for the heart.** (E-C: electromechanical coupling; EPC: Endothelial progenitor cells; MI: myocardial infarction; HF: heart failure) [24].

## 2.3 Human Mesenchymal stem/stromal cells (hMSCs)

Cells from bone marrow, cord blood, or adipose tissue, which show adherent growth in culture, are regarded as mesenchymal stem cells (MSCs) [38], also called mesenchymal stromal cells [24]. MSCs are non-hematopoietic stromal cells that are capable of multi-lineage differentiation. They are able to differentiate into mesenchymal tissues such as bone, cartilage, muscle, ligament, tendon, and adipose while contributing to the regeneration of those tissues. Besides the ability of self-renew, MSCs are able to proliferate and expand by many folds in culture. They are able to retain their multi-lineage potential while keeping abundant growth. Bone marrow MSC population represents approximately 1 out of 10,000 nucleated cells [39]. MSCs are identified by the expression of many surface molecules (cell markers).

As previously reported [39-45], "Phenotypically, MSCs express a number of markers, none of which, unfortunately, are specific to MSCs. It is generally agreed that adult human MSCs do not express the hematopoietic markers CD45, CD34, CD14, or CD11. They also do not express the co-stimulatory molecules CD80, CD86, or CD40 or the adhesion molecules CD31 (platelet/endothelial cell adhesion molecule [PECAM]-1), CD18 (leukocyte function associated antigen-1 [LFA-1]), or CD56 (neuronal cell adhesion molecule-1). MSCs can express CD105 (SH2), CD73 (SH3/4), CD44, CD90 (Thy-1), CD71, and Stro-1 as well as the adhesion molecules CD106 (vascular cell adhesion molecule [VCAM]-1), CD166 (activated leukocyte cell adhesion molecule [ALCAM]), intercellular adhesion molecule (ICAM)-1, and CD29" (Table 5). However, MSCs from other species do not express all the same molecules as human MSCs (hMSCs) express. Therefore, some researchers [39, 46] assumed that "the differences in cell surface expression of many markers may be influenced by factors secreted by accessory cells in the initial passages, and that the *in vitro* expression of some markers by MSCs do not always

correlate with their expression patterns *in vivo*". These assumptions are reasonable and important to bench works.

| | Positive | Negative |
|---|---|---|
| Immunophenotype of hMSCs | CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120a, CD124, CD166, Stro-1, ICAM-1, MHCI | CD11, CD14, CD18, CD31, CD34, CD40, CD45, CD56, CD80, CD86, MHCII |

**Table 5 Markers used to identify *in situ* and/or to isolate MSCs [39, 42, 47, 48].**

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) proposed 3 minimal criteria to define hMSC: "1) the ability to adhere to plastic when maintained in culture; 2) presence of >95% expression of hMSC-specific antigen markers (such as CD105, CD73 and CD90) and >95% absence of hematopoietic/endothelial marker expression (like CD45, CD34) and markers for monocytes, macrophages and B cells; and 3) the differentiation of the hMSCs at least into osteoblasts, adipocytes, and chondroblasts under standard in vitro differentiating conditions" [49, 50]. This definition and the concept of positive/negative-MSC-markers are primarily based on the scientific studies performed by a pharmaceutical company named Osiris Therapeutics. In particular, hMSC markers are mainly adopted from their *in vitro* expansion and differentiation assays [51]. In addition, it was reported that hMSCs express a unique set of chemokine receptors. Honczarenko et al. [52] have found three CC receptors (CCR1, CCR7 and CCR9) and three CXC receptors (CXCR4, CXCR5 and CXCR6) expressed by hMSCs. hMSCs also secrete several of the following ligands: CCL2, CCL4, CCL5, CCL20, CXCL12, and CXCL8 [50].

Importantly, MSCs are immunologically privileged cells, which allows them to be generally transplanted across major histocompatibility complex (MHC) barriers without the need for immune suppression. This beneficial characteristic is demonstrated even in large outbred animals, making MSCs appear to be superior to many other cell types for cell therapy [53]. The hypothesis behind the ability of MSCs to circumvent the immune response is that an active process leads to suppression of T-cell functions, which has important implications for the therapeutic application of MSCs. Although the mechanism of this process remains unclear, it is still promising to achieve "off-the-shelf" efficacy [31], if MSCs derived from healthy unrelated volunteer donors can be cryopreserved. It will be ideal to make them available in a timely manner in clinic, in order to help patients in both acute and chronic clinical settings. Successful treatments of MHC-identical, MHC-haploidentical, and MHC-unmatched MSC have already been achieved in the clinic [53]. The properties of MSCs make them potentially ideal candidates for myocardial regeneration.

### 2.3.1 Human Bone Marrow-Derived Mesenchymal Stem Cells (hBM-MSCs)

There are still debates and challenges remaining in the field of hBM-MSC characterization, due to the contradicting reports of MSC markers from different researchers [54-56]. It is agreed that hBM-MSCs may generally reside in three distinct niches. With different immunophenotypic features within the bone (namely endosteal), stromal, and perivascular niches [57], the characterization and lineage markers of hBM-MSCs cannot be easily summarized. Therefore, there is still no consensus on the properties of hBM-MSCs. One of the possibilities is that the lineage hierarchy of hBM-MSCs causes and represents its heterogeneous populations [58]. However, it was reported that besides sharing the common characteristics to hMSCs, hBM-

MSCs (maybe even a heterogeneous population) have their own set of unique phenotypic characteristics, such as lacking the expression of CD133 [59, 60].

The clinical and experimental applications of hBM-MSCs have their pros and cons. It is ethically acceptable if one is accessing the use of the patient's own cells for regeneration purposes (of such as cardiac muscles). Even though hBM-MSCs are from cross donors, the treatment still has the advantage of the unnecessary immunosuppression. Moreover, the use of unmanipulated hBM-MSCs has potential benefits such as 1) ready availability and 2) no *in vitro* cell culture preparation is required [24]. However, two major factors limit the use of hBM-MSCs in treatment in stem cell therapy: 1) the limited quantity of accessible hBM-MSCs [38, 39] and 2) the increasing donor age, which negatively affects hBM-MSCs in different aspects, such as cell numbers, proliferation, and differentiation capacity [61].

### 2.3.2 Human Placenta-Derived Mesenchymal Stem Cells (hPD-MSCs)

The invasive nature of hBM-MSC aspiration has limited its status as the primary source of hMSCs, and hence, the demand for finding better or equivalent cell candidates which can meet clinical need and accessibility, still remains. Therefore, explorations and efforts are underway to identify new hMSC sources, which are abundant and reliable for stem cell therapy in clinical uses. The umbilical cord blood (UCB) [62, 63], amnion and amniotic fluid [64], and placental tissues [65-67] have been reported as new sources of hMSCs. hMSCs isolated from these peripheral sources are called human placenta derived MSCs that display similar immunophenotypes to hBM-MSCs and also display multipotent properties [47]. Anker et al. [64] have demonstrated the expansion potential of hPD-MSCs to be greater than hBM-MSCs.

Phenotypically, "hPD-MSCs are positive for CD29, CD44, CD166 and CD105 and negative for CD14, CD34, CD45, and CD117. Meanwhile, they are also positive for HLA-ABC but negative for HLA-DR. Similar to hBM-MSCs, hPD-MSCs are moderately positive (in the range 33.5% – 44.6%) for SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and Oct-4." [68] (Table 6) Some of these markers are mainly expressed in pluripotent stem cells, such as embryonic stem cells, which may be a function of their *in vivo* tissue milieu [69]. hPD-MSCs also exhibit specific phenotypic difference, for instance, they express Oct-4, which is a transcription factor associated with pluripotent stem cells [59, 70]. The above findings suggest that hPD-MSCs are primitive cells and have multi-potency for differentiation.

| | Positive | Moderately positive | Negative |
|---|---|---|---|
| Immunophenotype of hPD-MSCs | CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120a, CD124, CD166, Stro-1, ICAM-1, MHCI, HLA-ABC | SSEA-1, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81 and Oct-4 | CD11, CD14, CD18, CD31, CD34, CD40, CD45, CD56, CD80, CD86, CD117, MHCII, HLA-DR |

**Table 6 Immunophenotipic characters of hPD-MSCs [39, 42, 47, 48, 68, 70].**

Moreover, hPD-MSCs have other advantages: 1) hPD-MSCs are abundantly available from maternity wards. 2) Invasive procedures can be avoided to obtain hPD-MSCs. The morphology of the cells is not compromised when isolated and expanded *in vitro*. 3) hPD-MSCs treatment does not cause massive rejection but can enhance angiogenesis [71]. Therefore, with fewer ethical restriction, greater availability as a medical waste, and their similar biological properties to hBM-MSCs, hPD-MSCs may be an ideal alternative to hBM-MSCs for stem cell therapy in myocardial regeneration [68].

## 2.4 Limitations of stem cell therapy in MI

Conceptually, after being administrated into the infarcted cardiac regions, hMSCs could 1) repair and replace dead cells by transdifferentiating into cardiomyocytes, 2) positively interact with host cells in ischemic areas and fuse with the niche, 3) contribute to rebuilding vascular structures by differentiating into vascular cells, 4) promote angiogenesis by releasing trophic factors and cytokines and reduce fibrosis and inflammation in the cardiac ischemic regions, 5) propagate and activate cells in peri-infarcted areas, such as cardiac resident stem cells and cardiac progenitor cells, which stimulates endogenous regeneration [72]. Hence, transplanted MSCs could repair and regenerate infarcted myocardium by either one or more ways.

Intravenous administration is one of the easiest routes and one of the most common methods of cell delivery [24]. However, the number of injected cells that will successfully flow through the heart is only a small fraction of the total amount. (Table 7) Moreover, cells migrate to other organs through blood circulation, thus reducing the number of donor stem cells that could populate the target areas [24]. For instance, these injected cells may retain in the lungs during the first passage after their delivery [73]. Reports showed that more than 80% of administered hMSCs accumulated immediately in the lungs after cell injection, which were later cleared and had a half-life of 24 hours [74-76]. Thus the main disadvantage is the cell administration technique into the infarcted myocardium. Besides the rapid and massive loss of cells after engraftment, another major bottleneck of stem cell therapy in MI is the low survival rate of transplanted cells in the ischemic and peri-infarcted region [77]. High mortality of implanted cells limits the potential effect of this therapy, since most transplanted donor cells die in the first day after implantation [78].

| | | |
|---|---|---|
| Time after intravenous injection | | The percentages of injected cells that successfully flew through the heart |
| | [76] | |
| | 1 h | < 1% |
| | 24 h | ~ 0% |
| | 7 days | ~ 0% |
| | 30 days | ~ 0% |
| | [79] | |
| | 5 days | ~ 0% |
| | 10 days | ~ 0% |
| | 20 days | ~ 0% |
| | 30 days | ~ 0% |

**Table 7 Representative studies describing the *in vivo* distribution of MSCs upon systemic administration [47].**

Increasing injected donor cell amount may not be able to overcome the current limitation. Indeed, higher doses of cells were reported as an ineffective strategy to improve engraftment by the early qualitative studies for bone regeneration [47]. Previous works revealed that it might be because of the saturated engraftment of osteoprogenitor cells that limited the outcome [47, 80]. In addition, this parameter (administered cell amount) alone may not capture the actual activity of this complex therapy. Therefore, it is important to include other significant parameters such as those are relevant to increasing cell homing (tissue-specific homing in particular) and cell retention after cell recruitment.

## 2.5 Cell homing

Mobilization of myocardial-ischemia-caused hMSC at the site of injury occurs through a process called homing, which is induced due to production of cytokines, soluble receptors, and adhesive molecules [81, 82]. It indicates a response of the administered hMSCs to the injured

cardiac tissue (tissue-specific homing) [39, 83]. A report showing that MSCs are capable of engrafting to the site of injury in a mouse model of myocardial infarction [47], suggested that MSCs possess migratory capacities [39]. Additionally, the immunohistochemistry results have shown that the MSCs are able to differentiate into cardiomyocyte-like cells with typical cardiomyocyte markers [47, 84]. It was also shown that, in particular, binding of SDF-1α to CXCR4 involved in cell trafficking of hMSCs [59, 85]. With all these processes simultaneously occurring, SDF-1α/CXCR4-mediated tissue-specific homing could be a promising strategy to increase the number of injected cells successfully flowing through the heart in a long term and may overcome the current limitation of cell flow.

### 2.5.1 SDF-1α

The stromal cell-derived factor 1 (SDF-1) is also known as C-X-C motif chemokine 12 (CXCL12), which is a chemokine protein belonging to the CXC subfamily [86]. In humans, it is encoded by the *CXCL12* gene [87]. SDF-1 is produced in two isoforms: 1-alpha (1α) and 1-beta (1β), whose members activate leukocytes and are often induced by pro-inflammatory stimuli, such as lipopolysaccharide, TNF, or IL-1 [88]. Its isoform 1α (SDF-1α) is a powerful chemoattractant cytokine that stimulates directional migration of many types of cells: both hematopoietic and non-hematopoietic cells (such as MSCs) [88, 89]. It has been also reported that the migration and invasion of MSCs can increase after stimulated by SDF-1α *in vitro* conditions [29, 87, 90].

After acute myocardial infarction (AMI), SDF-1α is generated by damaged cardiomyocytes [88]. However, the SDF-1α expression level severely fluctuates after MI: it elevates at 1 hour

right after MI, returns to the baseline by the 7th day and remains through the following 30 days post-MI [91].

The receptor for SDF-1α is CXCR4, which was previously called LESTR or fusin [92]. And it is well known that SDF-1α is the sole endogenous ligand for CXCR4 [93].

### 2.5.2 CXCR4

C-X-C chemokine receptor type 4 (CXCR4), also known as fusin or CD184 (cluster of differentiation 184), is a G protein-coupled receptor [93]. It is a protein encoded by the *CXCR4* gene in humans [94]. CXCR4 is an alpha-chemokine receptor located on the MSC cell surface, which is specific for SDF-1α. SDF-1α and CXCR4 are believed to be a relatively monogamous ligand-receptor pair [93, 95]. SDF-1α and CXCR4 are unlike other promiscuous chemokines that tend to use several different chemokine receptors [96]. The CXCR4 receptor, which has been reported in animal [97] and human [91] studies to be a very efficient inducer of mesenchymal stem cell mobilization, can be directly blocked [98] by plerixafor (AMD3100). AMD3100 is a drug that recently approved for routine clinical use [99], which is a specific CXCR4 antagonist.

In the last three years, it has been shown that high levels of CXCR4 expression on MSCs correlate with their higher engraftment at the injury site [100].

### 2.5.3 The SDF-1α/CXCR4-mediated cell homing

During inflammation or injury after MI, the damaged cardiac tissue releases various signaling molecules in ischemic zones, initiating the stem cell mobilization process. Among all of them, it is significant to discuss the SDF-1α/CXCR4 axis in terms of hMSC function. Several

investigations reported that BM-MSCs are chemoattracted towards the cardiac SDF-1α gradient after being released into the peripheral blood in an SDF-1α-dependent manner [88, 101]. Therefore, the hMSC infusion time frame, which allows them to target the injured cardiac tissue, is critical for efficient hMSC homing following an MI. This is especially critical since it is known that myocardial SDF-1α expression is increased only in the early phase post-infarction [102, 103].

In multiple studies, MSCs were shown to engraft into the myocardium at higher rates one day after MI, as compared to a 14-days-experiment in an acute MI (AMI) model post-MI [59, 104]. This naturally occurs because CXCR4 expression begins 24 hours after AMI [29]. Furthermore, this upregulation is sustained for the ensuing to 36 to 48 hours [91]. The peak of CXCR4 expression was seen at 96 hours. In contrast, very low levels (if not nearly absent) of SDF-1α was seen in infarcted areas, causing a time mismatch [105], which leads to an insufficiency of MSC homing and recruitment in natural conditions.

In addition, the CXCR4 surface expression of MSCs can be declined in cell culture *in vitro*. This could happen after MSCs are being expanded for only a few cell passages [100, 106, 107]. However, the loss of CXCR4 expression on plasma membrane can be re-induced by the pre-treatment of MSCs with a cytokine cocktail, which can restore MSC's homing ability [59, 108]. Therefore, it is critical to investigate the unique role of the SDF-1α/CXCR4-mediated cell homing capabilities in hPD-MSCs (the target cell type in our study), since the SDF-1α/CXCR4-dependent signaling differs between cell types and between malignant and normal counterparts [109].

Altogether, implanting hPD-MSCs overexpressing CXCR4 could be used as a strategy to overcome the limitation of the time mismatch between SDF-1α and CXCR4, which may increase cell recruitment into cardiac ischemic regions post-MI.

### 2.5.4 CXCR4 overexpression

Many researchers [87, 108, 110-112] have demonstrated that overexpression of CXCR4 enhanced MSCs migration/homing potential towards SDF-1α. There are many methods discussed in the literature regarding CXCR4 overexpression: 1) CXCR4 expression can be upregulated by 100 nM SDF-1α in multiple myeloma cell line. When exposed in 30 μM AMD3100, CXCR4 expression can be inhibited and declined, even though with the presence of stimulated SDF-1α [109]. This suggested that, as a ligand, SDF-1α impacted the expression of its receptor CXCR4. 2) Pharmaceutical cobalt chloride ($CoCl_2$) leads to chemical hypoxia, due to its prevention of the oxygen signals [113, 114], which elevates hypoxia-induced factor 1α (HIF-1α). Simultaneously, the expression of the CXCR4 receptor on progenitor cells is induced. This helps to guide these cells towards SDF-1α gradients in ischemic tissues [88, 97, 115]. 3) High glucose has also been demonstrated to enhance the expression of the transcription factor HIF-1α [113, 116], as well as affecting the expression of CXCR4 in several cells [117-121]. Therefore, in our study SDF-1α, $CoCl_2$, and high glucose are employed to induce the expression of CXCR4 in hPD-MSCs.

## 2.6 Parameters of interest post cell homing

Reports from several studies showed that the SDF-1α/CXCR4 axis exerts multiple downstream signaling cascades in the following pathways [122, 123], the MEK/ERK (MAPK p42/44)-ELK-1 and the PI3K-Akt-NFκB pathways [88]. Both pathways are seen to regulate

various biological effects in target cells, including cell motility, cell survival, cell proliferation, chemotactic responses, and cell adhesion, etc. [88, 124].

Protein kinase B (Akt) and extracellular signal-regulated kinase (ERK) are activated via their phosphorylation after MI, due to myocardial-infarction-induced myocardial hypertrophy or hypertension [125]. Several investigators have reported their impact in the disease progression. Phosphorylated Akt (p-Akt) is involved in multiple aspects concerning the genesis of cardiac remodeling, which are induced by various humoral factors [126-128]: p-Akt induces cardiac hypertrophy [129], initiates cell proliferation [130], and prevents apoptotic cell death [131]. Phosphorylated ERK (p-ERK) is considered to be an important cell marker in cardiomyocytes [132] and cardiac fibroblasts [133, 134]. It is related to intracellular signaling for the induction of hypertrophy in cardiomyocytes. Additionally, it could also be indicative of proliferative activity of cardiac fibroblasts [124]. These two aspects lead to cardiac remodeling during the pathophysiological process and could affect engrafted hMSC functions in infarcted cardiac tissues during the development of heart failure.

It is also known that the SDF-1α/CXCR4 interaction can mediate the induction of interleukin 6 (IL-6) [89]. IL-6 is considered as a pro-inflammatory factor and normally involved in the regulation of the immune response. This multifunctional cytokine, IL-6, also plays a role in apoptosis, cell proliferation, and survival [135]. Therefore, the survival and maintenance of migrated hMSCs in the infarcted areas could also be affected by their secretion of IL-6.

Here, we explored the following significant indicators of hMSC functions post-MI: p-Akt, p-ERK, and IL-6. Collectively, they are involved in cell migration, proliferation, inflammation, vascular remodeling, and angiogenesis [135-138].

# CHAPTER 3. OBJECTIVES

Stem cell therapy is developed as an auxiliary medical approach post-MI for patients whose cardiac functions cannot be effectively recovered or maintained by traditional treatments. Human PD-MSCs are primitive cells and have multipotency for differentiation. The therapeutic potential of hPD-MSCs has been reported to be greater than that of hBM-MSCs, indicating that hPD-MSCs could likely be an alternative cell source to hBM-MSCs for stem cell therapy in the treatments post-MI. Our study focuses on the potential of the stromal cell-derived factor 1α (SDF-1α)/ C-X-C chemokine receptor type 4 (CXCR4)-mediated cell homing of hPD-MSCs and its other characteristics, such as cell survival and proliferation potentials.

Therefore, the objective of this study was to compare the potentials of hPD-MSCs and hBM-MSCs with respect to cell homing, cell survival, and cell proliferation *in vitro* for myocardial regeneration.

# CHAPER 4. MATERIALS AND METHODS

## 4.1. Cells and reagents

Human PD-MSCs were a kind gift from Dr. Huang Yen from the Institute of Cellular and System Medicine (Zhunan, Taiwan). Human BM-MSCs were purchased from Lonza (Allendale, NJ, USA). Recombinant human SDF-1α (CXCL12) was purchased from Peprotech (Rocky Hill, USA). AMD3100 was purchased from Abcam (Toronto, CA). Glucose and $CoCl_2$ were purchased from Sigma (Oakville, ON, CA). Antibodies for beta-actin, CXCR4, HIF-1α, IL-6, and IL-10 detection were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibody for Akt, p-Akt, ERK, p-ERK, and α-tubulin detection were purchased from Cell Signalling Technology (Danvers, Massachusetts, USA). Antibody for CXCR4 immunofluorescence detection was purchased from Abcam (Toronto, CA).

## 4.2. Immunofluorescence

Human PD-MSCs (1 $10^4$ cells) and hBM-MSCs (1 $10^4$ cells) were seeded on 24×55 mm microscope cover glasses (Fisher Scientific) in 6 cm cell culture dishes. After removing culture media, the cells were fixed with ice-cold acetone for 3 minutes. After blocking with 2% BSA in room temperature for 1 hour, the slides were incubated with the primary antibody – rabbit-anti-CXCR4 (1:250, Abcam, Toronto, CA) – at 4 °C overnight. These were then incubated with Alexa Fluor 488 (green) labeled donkey-anti-rabbit antibody (1:250, Abcam, Toronto, CA) for 2 hours at room temperature, and then observed under the fluorescence microscope (Olympus, BX40, 40× magnification). Nuclei were stained using 4', 6-diamidino-2-phenylindole (DAPI).

## 4.3. Cell migration assay

The transwell cell migration assay was performed in a 24-well plate (Costar; Corning, Acton, MA) using the 8.0 μm pore size cell culture insert with PET track-etched membrane (Becton Dickinson and Company, 353097) as described by the manufacturer. Both hPD-MSCs and hBM-MSCs were suspended separately in DMEM and plated ($1\times10^5$ cells per well) in the upper chambers of the transwell plates with serial concentrations of SDF-1α (in a dose range of 0 to 800 ng/mL) in the lower chambers in 1 mL of DMEM. AMD3100 (500 ng), a CXCR4 inhibitor, was added to the SDF-1α-treated group. Cells cultured in 500 ng/mL of AMD3100 represented the negative control. After 12 hours of incubation at 37°C, DAPI was employed to stain the nucleus. Five random spots were chosen on the membranes and taken pictures through the microscope (Olympus, CKX41, 10× magnification). Cells that migrated to the lower chambers were counted by Image J software (Wayne Rasband). The amount of migrated cell from each sample was calculated as the average number of the cells at five random selected spots. All experiments were done in triplicates, and the means and standard deviations were calculated.

## 4.4. Cell viability assay

The survival of hMSCs was measured with the cell viability assay. hPD-MSCs and hBM-MSCs were seeded in 24-well plates (Costar; Corning, Acton, MA). A total of $1\times10^5$ cells were seeded in each well in the presence of SDF-1α (0 - 400 ng/mL), glucose (0 - 800 mM), and $CoCl_2$ (0 - 800 μM) and incubated at 37°C incubator for 24 hours. After washing with phosphate-buffered saline (PBS), cells were resuspended, and 1/4 volume of 0.4% solution of trypan blue (Thermo Fisher Scientific, ON, CA) was added, and then examined by a microscope

(cells were passed to a microscopic haemocytometer and gently covered with a cover slip). Percentages of living (non-stained) and dead (blue-stained) cells were assessed by three independent observers under a light microscope (Olympus, CH-2, 10× magnification).

Cell viability was calculated by dividing the number of viable cells by the total number of cells determined experimentally [139] using the following formula:

Cell viability (%) = (the number of viable cells / the total number of cells) × 100%

Optimized doses (concentrations that are less toxic and resulted in the maximum cell migration efficiency) of each chemical (SDF-1α, glucose, and $CoCl_2$) were chosen from this assay for further treatments.

## 4.5. MTT cell proliferation assay

$1\times10^4$ cells were seeded into each well of a 96-well plate with the phenol-red free medium for 6 hours before the treatments. After this, the optimized concentrations from cell viability assay of three reagents (100 ng/mL SDF-1α, 50 mM glucose, and 400 μM $CoCl_2$) were added to the medium in the different treatment groups. Each treatment was performed at five different time points: 1, 2, 6, 12, and 24 hours. 10 μL of MTT (12 mM) solution (Life Technologies, Burlington, ON, CA) was added to each well following the designated incubation periods. Plates were then incubated for another 4 hours at 37°C. 100 μL of SDS-HCl (10%SDS in 0.01M HCl) was added to each well and mixed by pipetting. Plates were incubated at 37°C overnight and analyzed for absorbance at 570 nm using a plate-reader (TECAN, Infinite M200 Pro). Optimized cell culture times for each treatment (SDF-1α, glucose, and $CoCl_2$) were chosen for later treatment.

## 4.6. Cell culture and treatments

Both kinds of hMSCs (hBM-MSCs and hPD-MSCs) were cultured in DMEM medium supplemented with 10% FBS and 5% penicillin (complete medium, Invitrogen, Carlsbad, CA, USA). Cells at confluence were harvested and seeded onto 10 cm cell culture dishes (Nest Biotechnology Co., LTD, China). Cells were treated under different conditions: SDF-1α (100 ng/mL), glucose (50 mM), and $CoCl_2$ (400 μM) for 12 hours followed by incubation at 37°C in humidified 5% $CO_2$ conditions.

## 4.7. RNA isolation and RT-PCR

Total RNA was isolated with the RNeasy Plus Mini Kit (QIAGEN, Toronto, CA). Gene expressions were detected by reverse transcription-polymerase chain reaction (RT-PCR) assay. Sequences of forward and reverse primer pairs are as follows: Human CXCR4 Forward: 5-GGTGGTCTATGTTGGCGTCT-3; reverse: 5-TGGAGTGTGACAGCTTGGAG-3 Human GAPDH Forward: 5-ATGTTCGTCATGGGTGTGAACC-3; reverse: 5-GGCAGGTTTTTCTAGACGGCAG-3 (Bio Corp DNA Inc., QC, CA).

## 4.8. Western blot analysis

Cells were washed twice with ice-cold PBS and lysed in radio immunoprecipitation (RIPA) buffer (Tris-HCl pH 7.4, 50 mM, Triton X-100 1%, Sodium deoxycholate 1%, SDS 0.1%, EDTA, 1 mM, NaCl 150 mM at pH 7.0) containing a cocktail of protease inhibitors (Sigma, Oakville, ON, CA) and NaF (Sigma, Oakville, ON, CA) for 30 minutes on ice. Supernatants of cell lysates were obtained after centrifugation at 10,000 rpm for 10 min, denatured with laemmli buffer, and loaded onto SDS-polyacrylamide gels (10%). Proteins were transferred onto a PVDF

membrane (Bio-Rad, Hercules, CA, USA) and blocked for 1 hour in skimmed milk powder (5%) dissolved in TBST (150 mM NaCl, 50mM Tris-HCl, pH 7.5, 10% Tween 20) at room temperature.

The blotting membranes were incubated overnight at 4 °C, with the following primary antibodies: CXCR4 (1:1,000), HIF-1α (1:1,000), IL-6 (1:1,000), IL-10 (1:1,000) (Santa Cruz Biotechnology, Dallas, TX, USA); ERK (1:1,000), p-ERK (1:1,000), Akt (1:1,000), and p-Akt (1:1,000) (Cell Signalling Technology, Danvers, Massachusetts, USA). Then these membranes were extensively washed in TBST for 25 minutes (5 times, 5 minutes each), incubated with HRP-conjugated anti-mouse secondary antibody (1:2,000) (Santa Cruz Biotechnology, Dallas, TX, USA) or HRP-conjugated anti-rabbit (1:2,000) (Cell Signalling Technology, Danvers, Massachusetts, USA) for 1 hour at room temperature, and washed again with TBST. The blotting membranes were developed with a chemiluminescent reagent, Western Lightning Plus-ECL (PerkinElmer, Inc.)**.** To assure equal protein loading between lanes (20 μg), beta-actin or alpha-tubulin (1: 5,000) were used as loading controls.

### 4.9. Fluorescence-activated cell sorting (FACS)

FACS analysis was performed to detect CXCR4 expression in hPD -MSCs and hBM-MSCs in either normoxic or $CoCl_2$-induced hypoxic conditions. For each sample, $8\times10^6$ cells were washed in phosphate-buffered saline (PBS) and incubated with antibodies for 1 hour at room temperature. The primary antibody used was rabbit-anti-CXCR4 (1:250, Abcam, Toronto, CA), and the secondary antibody used was donkey-anti-rabbit (1:250, Abcam, Toronto, CA). Cell sorting was set up so that cells in the top 15% of CXCR4 expression in each group were isolated. Data was analyzed using a BD FACSAria analyzer fitted with BD FACSDiva software.

## 4.10. Statistical analysis

All experiments were performed at least three times, and all the samples were in triplicate. Data are expressed as mean ± standard deviation (SD). Student's t-test was used for comparison between two groups and ANOVA with a Tukey test was used for multiple comparisons of more than two groups. $P<0.05$ was considered statistically significant. Graphs were plotted using GraphPad Prism 6.0 version for Mac (GraphPad Software, San Diego, CA, USA). In figures, '*' represents $P<0.05$, '**' represents $P<0.01$, '***' represents $P<0.001$, and '****' represents $P<0.0001$.

# CHAPTER 5. RESULTS

## 5.1. Expression of CXCR4 in hPD-MSCs and hBM-MSCs

In order to determine the expression of CXCR4 at basal level in hPD-MSCs and hBM-MSCs (control), RT-PCR and immunofluorescence staining were performed. As shown in Figure 7A, both hPD-MSCs and hBM-MSCs expressed CXCR4 (cDNA) at basal level in normoxia cell culture condition (12 hours, at 37°C, 5% $CO_2$ with regular DMEM medium supplemented with 10% FBS and 5% penicillin). HeLa cells were used as positive control, and DMEM medium (without cells) was used as negative control. As shown in Figure 7B, both hPD-MSCs and hBM-MSCs exhibited an elongated spindle-like (fibroblast-like) morphology under a light microscope (20×). We also determined the expression of CXCR4 at the protein level using immunofluorescence. In Figure 7C, the green color represents CXCR4, the blue color represents the counterstaining with DAPI for nuclei. We further confirmed the cell surface expression of CXCR4 in both hPD-MSCs and hBM-MSCs by co-localization with endoglin (CD105), which is a hMSC specific cell surface marker (Figure 7D). The red color represents the CD105.

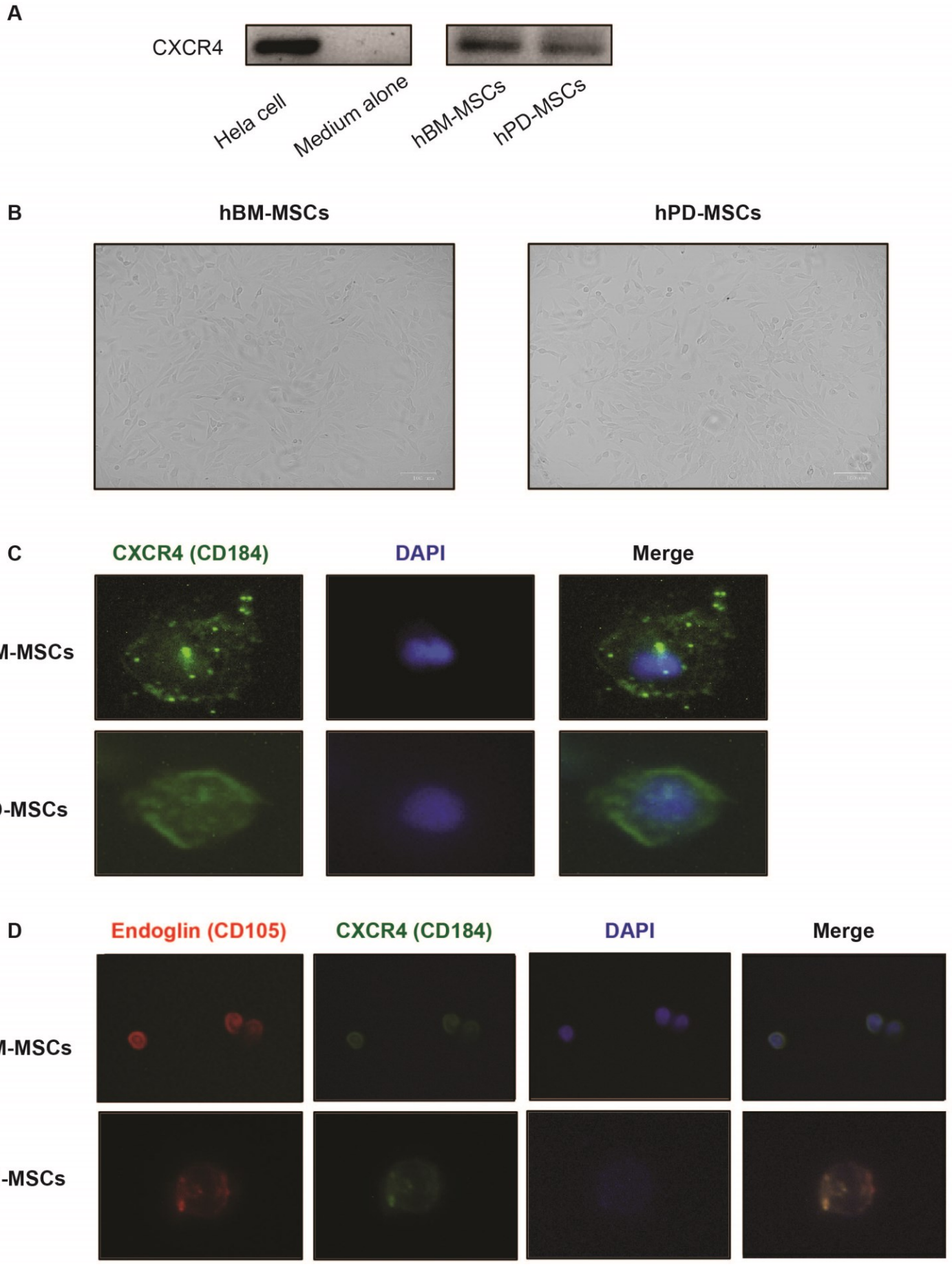

A
CXCR4
Hela cell
Medium alone
hBM-MSCs
hPD-MSCs
B
hBM-MSCs
hPD-MSCs
C
CXCR4 (CD184)
DAPI
Merge
hBM-MSCs
hPD-MSCs
D
Endoglin (CD105)
CXCR4 (CD184)
DAPI
Merge
hBM-MSCs
hPD-MSCs

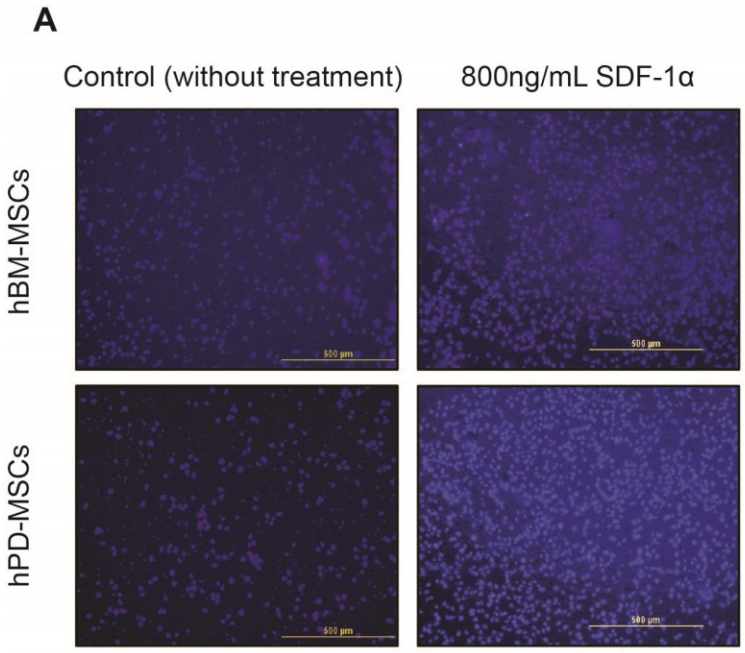
**Figure 7. Expression of CXCR4 in hPD-MSCs and hBM-MSCs.** (A) Cells were grown up to 70% confluence, RNA was extracted and reverse transcribed using high capacity cDNA reverse transcription kits (Applied Biosystems, California, USA). The expression level of CXCR4 was analyzed by semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) assay. Representative Ethidium bromide gel shows the expression of CXCR4. HeLa cells were used as positive control, and DMEM medium was used as negative control. (B) Phase-contrast microscopic images of MSCs cultured for 24 hours as monolayers. Both hPD-MSCs and hBM-MSCs exhibited elongated spindle-shaped (fibroblast-like) morphology (Magnification ×20). (C) hPD-MSCs ($1\times10^4$ cells) and hBM-MSCs ($1\times10^4$ cells) were plated on coverslips and allowed to grow. 24 hours after plating, cells were fixed and stained for CXCR4 (1:250, Abcam, Toronto, CA). Expression of CXCR4 is shown in green. The nucleus was stained using 4', 6-diamidino-2-phenylindole (DAPI) as shown in blue. Scale bar is 100 μm. Original magnification was 40×. (D) As also observed by fluorescence microscopy (40×), CXCR4 cell surface expression was determined in both non-permeablized hPD-MSCs and non-permeablized hBM-MSCs by being compared to the expression of a co-localization reference endoglin (CD105), which is an hMSC specific cell surface marker. The green color (Alexa Fluor 488) represents CXCR4, the red color represents CD105, and the blue color represents nucleus.

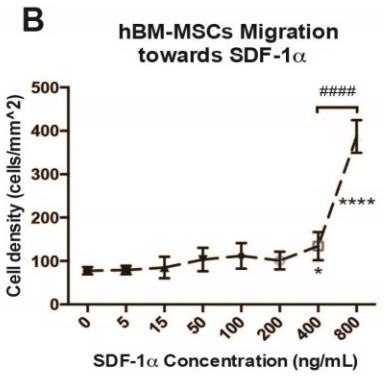
5.2. hPD-MSCs has higher migration potential towards SDF-1α

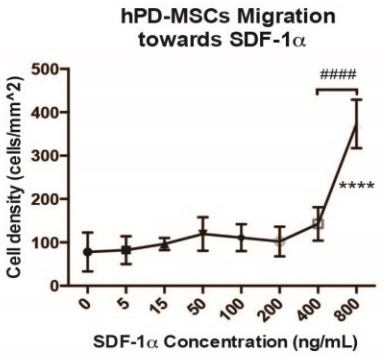
The SDF-1α/CXCR4 axis is involved in cell trafficking activity in many cell lines. In order to determine the chemotactic activity of both hPD-MSCs and hBM-MSCs in response to the SDF-1α/CXCR4 axis, cell migration assays were performed. As shown in Figure 8A, the number of migrated cells significantly increased in the SDF-1α-induced group than that in the control group (regular DMEM medium-induced group) in both hPD-MSCs and hBM-MSCs. The number of migrated cells were counted and plotted as a graph (Figure 8B), which shows that SDF-1α significantly induced the cell migration of both hPD-MSCs and hBM-MSCs in a dose-dependent manner.

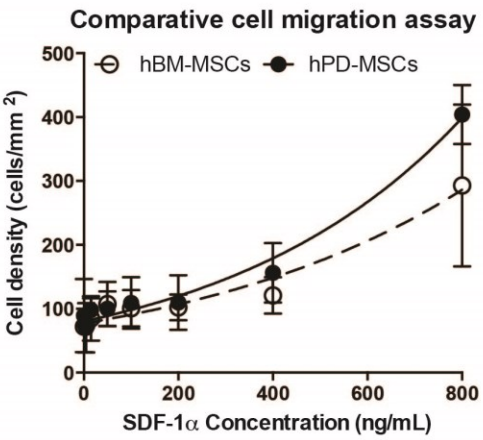
Although both these cell types showed increased migration in response to SDF-1α, hPD-MSCs ($Y_0 = 80.40 \pm 7.89$; $R^2 = 0.8866$) showed higher migration potential than hBM-MSCs ($Y_0 = 77.13 \pm 9.99$; $R^2 = 0.6949$) towards SDF-1α in a dose-dependent manner (Figure 8C). In order to determine the role of CXCR4 in migration, we used an antagonist AMD3100 for inhibiting CXCR4. Pre-treatment of both hPD-MSCs and hBM-MSCs with the CXCR4 specific antagonist AMD3100 blocked their basal migration as seen in Figure 8D. The dependency on the SDF-1α/CXCR4 axis was determined by comparing groups treated with AMD3100 (500 ng/mL), SDF-1α (800 ng/mL), and AMD3100+SDF-1α. However, in terms of cell density (cells/$mm^2$), the effect of AMD3100 was more pronounced at a basal level in hPD-MSCs (142 ± 17.18 to 16.29 ± 2.55) than in hBM-MSCs (114 ± 12.41 to 3.63 ± 1.02) (Figure 8E). The increased migration of cells in response to SDF-1α in the presence of AMD3100 indicates that there are additional pathways mediated by SDF-1α besides the CXCR4 axis, which are involved in the migration of these cells.

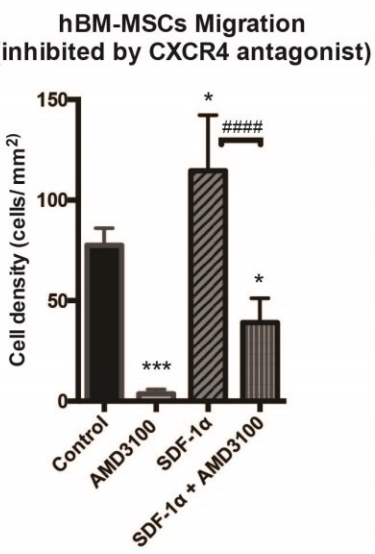
Altogether, the data confirms that the SDF-1α/CXCR4 axis was expressed at basal level in both hPD-MSCs and hBM-MSCs.

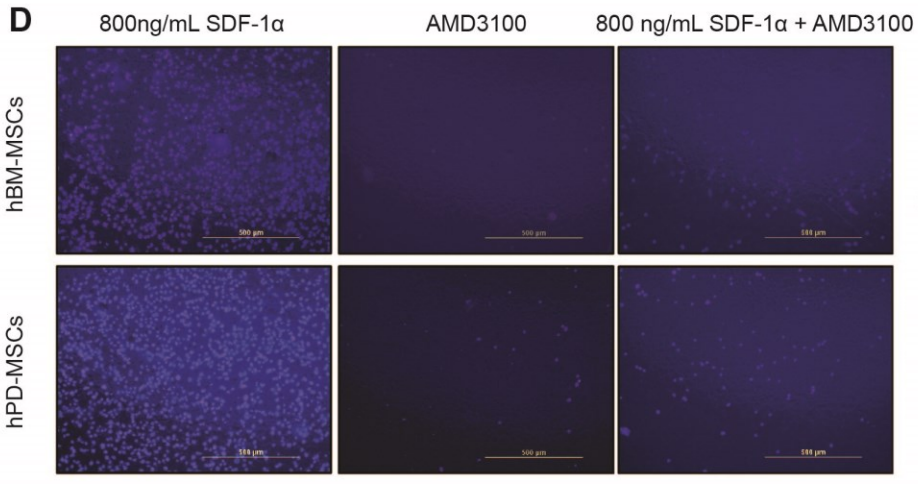

A
Control (without treatment)
800ng/mL SDF-1α
hBM-MSCs
hPD-MSCs


B
hBM-MSCs Migration towards SDF-1α
Cell density (cells/mm^2)
SDF-1α Concentration (ng/mL)
0
5
15
50
100
200
400
800
####
****
*


hPD-MSCs Migration towards SDF-1α
Cell density (cells/mm^2)
SDF-1α Concentration (ng/mL)
####
****


C
Comparative cell migration assay
hBM-MSCs
hPD-MSCs
Cell density (cells/mm 2)
SDF-1α Concentration (ng/mL)


E
hBM-MSCs Migration (inhibited by CXCR4 antagonist)
Cell density (cells/ mm2)
Control
AMD3100
SDF-1α
SDF-1α + AMD3100
***
####


D
800ng/mL SDF-1α
AMD3100
800 ng/mL SDF-1α + AMD3100
hBM-MSCs
hPD-MSCs

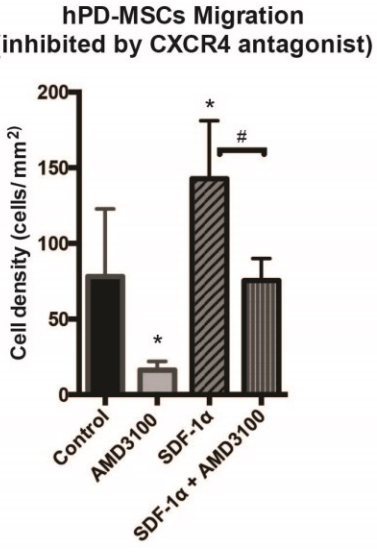

hPD-MSCs Migration (inhibited by CXCR4 antagonist)
Cell density (cells/ mm2)
Control
AMD3100
SDF-1α
SDF-1α + AMD3100
#

**Figure 8. hPD-MSCs had higher migration potential towards SDF-1α.** (A) The transwell cell migration assay was performed using cell culture insert with PET track-etched membrane as described by the manufacturer. $1\times10^5$ cells were plated in the upper chambers of the transwell plates with serial concentrations of SDF-1α (in a dose range of 0 to 800 ng/mL) in the lower chambers in 1 mL of DMEM. AMD3100 (500 ng), a CXCR4 inhibitor, was added to the SDF-1α-treated group. Cells cultured in 500 ng/mL of AMD3100 represented the negative control. After 12 hours of incubation at 37°C, counterstaining (with DAPI) was performed to show the nuclei. Five random spots were chosen on the membranes and taken pictures through a microscope (Olympus, CKX41, 10×). Cells that migrated to the lower chambers were counted by Image J software. The numbers of migrated cells of each sample were calculated as the average of the number of cells at five random selected spots. All experiments were done in triplicates, and the means and standard deviations were calculated. (B) Cell migration data was plotted, which shows an increase in SDF-1α mediated migration in both hBM-MSCs and hPD-MSCs. (C) Cell migration data was plotted on a non-liner fit curve using the exponential growth equations. hPD-MSCs showed higher migration potential than hBM-MSCs towards SDF-1α in a dose-dependent manner. (D) Transwell migration assays were carried out in the presence of a CXCR4 inhibitor - AMD3100 (500ng/ml) - in response to SDF-1α (800ng/ml). (E) The representative graph shows the number of cells migrated in each treatment group as indicated. Error bars represent standard deviation. In Figure 8B, **** denotes a statistically significant difference ($P<0.0001$) vs. 0 ng/mL SDF-1α and #### denotes a statistically significant difference ($P<0.0001$) vs. other indicated experimental groups. In Figure 8E, # denotes a statistically significant difference ($P<0.05$) vs. indicated experimental groups and * denotes a statistically significant difference ($P<0.05$) vs. Control.

5.3. Dose optimization of SDF-1α, $CoCl_2$, and Glucose for increased CXCR4 expression

Previous reports have shown the role of glucose, $CoCl_2$ and SDF-1α in the induction of CXCR4 in various cell types. So, we determined the optimum dose of these factors for increased CXCR4 expression. During 24 hours of treatment, hBM-MSCs remained 100% alive in all range of SDF-1α concentrations (Figure 9A). Human PD-MSC showed a significant decrease in viability after treatment with 100 ng/mL SDF-1α for 24 hours (Figure 9B). After 24 hours of treatment with 50 mM (and higher) glucose, cell viability of both hBM-MSCs (0.7869 ± 0.0149) and hPD-MSCs (0.8026 ± 0.0494) were significantly decreased (Figure 9C and 9D). After 24 hours of treatment with 400 μM $CoCl_2$, hBM-MSCs showed a slight decrease in cell viability (0.9040 ± 0.0283) (Figure 9E). As showed in Figure 9F, 400 μM $CoCl_2$ slightly lowered the cell viability of hPD-MSCs to 0.9503 ± 0.0285 after 24 hours, whereas 800 μM $CoCl_2$ caused a significant inhibition of cell viability (0.8116 ± 0.0204). Therefore, the optimized concentrations for *in vitro* cell culture were chosen as 100 ng/mL SDF-1α, 50 mM glucose and 400 μM $CoCl_2$ for both hPD-MSCs and hBM-MSCs.

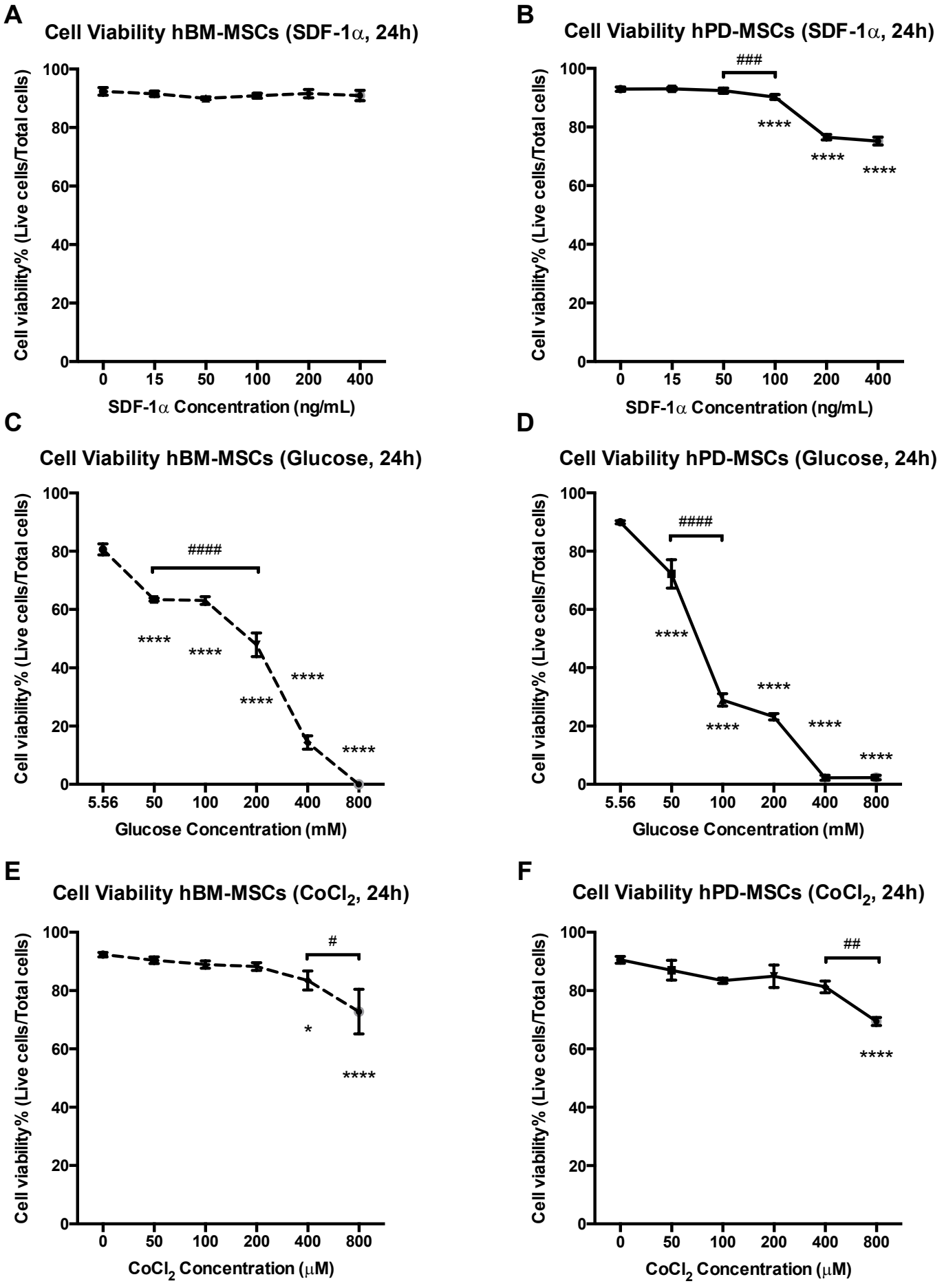

A
Cell Viability hBM-MSCs (SDF-1α, 24h)
Cell viability% (Live cells/Total cells)
SDF-1α Concentration (ng/mL)
0
15
50
100
200
400
B
Cell Viability hPD-MSCs (SDF-1α, 24h)
###
****
****
****
C
Cell Viability hBM-MSCs (Glucose, 24h)
####
5.56
50
100
200
400
800
Glucose Concentration (mM)
D
Cell Viability hPD-MSCs (Glucose, 24h)
E
Cell Viability hBM-MSCs (CoCl2, 24h)
#
*
CoCl2 Concentration (μM)
F
Cell Viability hPD-MSCs (CoCl2, 24h)
##

**Figure 9. Dose optimization of SDF-1α, $CoCl_2$, and Glucose for increased CXCR4 expression (based on cell viability assay).** In order to optimum concentrations of SDF-1α, glucose, and $CoCl_2$ for cell culture, $1\times10^5$ cells were seeded in the presence of SDF-1α (A, B), glucose (C, D), and $CoCl_2$ (E, F) in a dose dependant manner. After 24 hours of treatment cells were stained with trypan blue and percentage of living (non-stained) and dead (blue-stained) cells were assessed. Data were shown as mean ± SD of three experiments. In Figure 9, error bars represent standard deviation. * denotes a statistically significant difference ($P<0.05$) vs. control group (without treatments), # denotes a statistically significant difference ($P<0.05$) vs. other indicated experimental groups. **** denotes a statistically significant difference ($P<0.0001$) vs. control group (without treatments), and #### denotes a statistically significant difference ($P<0.0001$) vs. other indicated experimental groups.

## 5.4. Optimized duration of treatment

In order to optimize the duration of treatment to achieve high CXCR4 expression, MTT cell proliferation assays were performed. Both hPD-MSCs and hBM-MSCs were cultured in 100 ng/mL SDF-1α, 50 mM glucose and 400 μM $CoCl_2$ respectively. As shown in Figure 10A, few hBM-MSCs survived after treatment with 100 ng/mL SDF-1α for 1, 2, and 6 hours, as SDF-1α inhibited the cell metabolic activity and showed toxic effects causing severe cell death. However, at 12 hours, there was significant increase in the metabolic activity of SDF-1α-treated hBM-MSCs (0.7477 ± 0.0317). This was also observed in SDF-1α-treated hPD-MSCs, which showed an absorbance of 0.7471 ± 0.0029 at 12 hours (Figure 10B). Possibly, when newly exposed to chemical stress, cells were unable to cope with it and died, but after they had got accustomed to the stress, cells were able to grow again and proliferate. Similar observations were made in both hPD-MSCs and hBM-MSCs with the treatments of 50 mM glucose and 400 μM $CoCl_2$. hBM-MSCs showed high metabolic activity at 12 hours in both glucose- (0.6583 ± 0.0044) (Figure 10C) and $CoCl_2$- (0.7819 ± 0.0046) (Figure 10E) treated groups. The metabolic activity of hPD-MSCs was also high at 12 hours both in glucose- (0.6148 ± 0.0198) (Figure 10D) and $CoCl_2$- (0.7451 ± 0.0391) (Figure 10F) treated groups. Taken together, the optimized duration of treatments was 12 hours for both hPD-MSCs and hBM-MSCs.

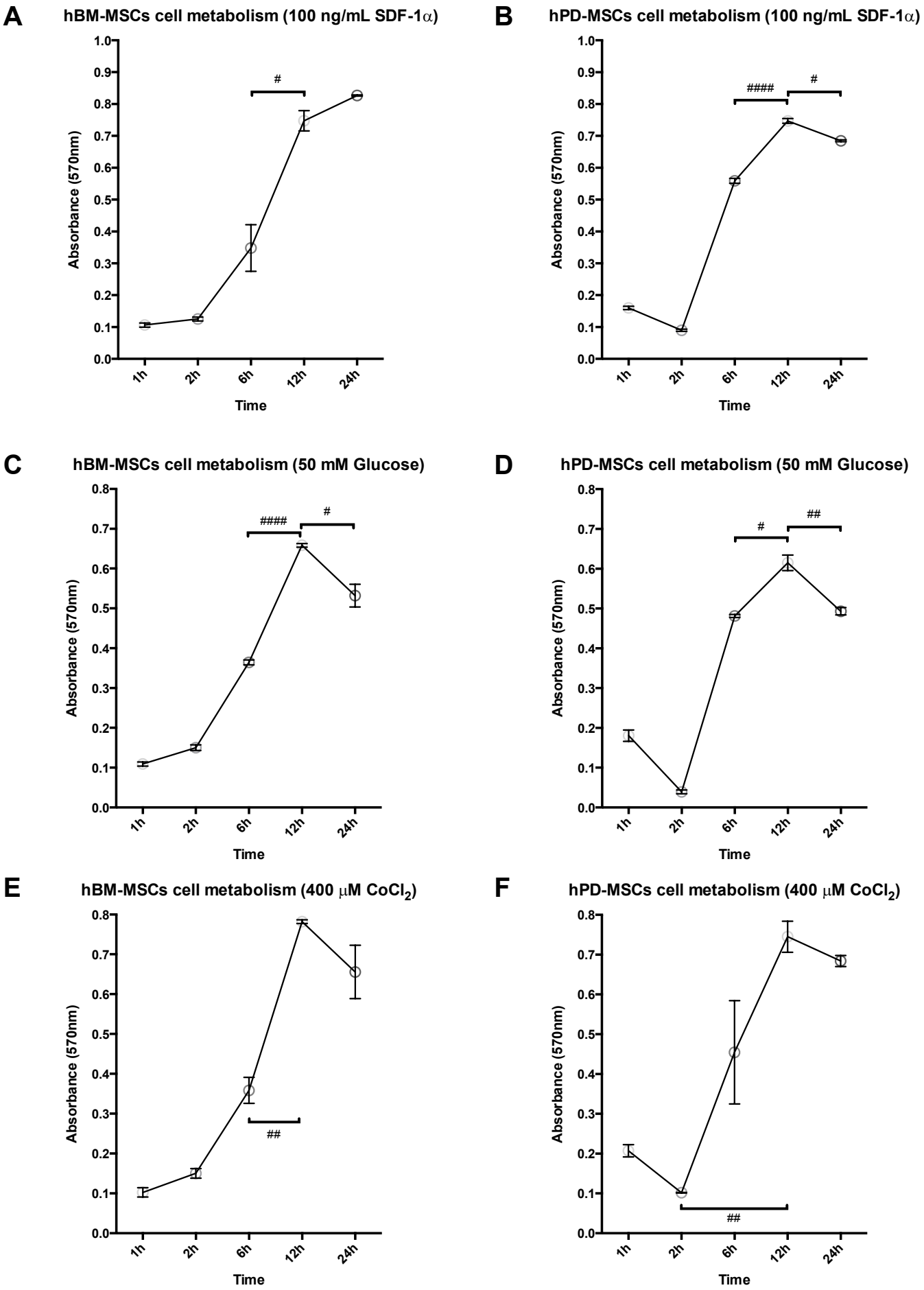

A
hBM-MSCs cell metabolism (100 ng/mL SDF-1α)
B
hPD-MSCs cell metabolism (100 ng/mL SDF-1α)
C
hBM-MSCs cell metabolism (50 mM Glucose)
D
hPD-MSCs cell metabolism (50 mM Glucose)
E
hBM-MSCs cell metabolism (400 μM CoCl2)
F
hPD-MSCs cell metabolism (400 μM CoCl2)
Absorbance (570nm)
Time
1h
2h
6h
12h
24h
#
##
####

**Figure 10. Optimized duration of treatment (based on MTT cell proliferation assay).** $1\times10^4$ cells were seeded into each well of a 96-well plate. Cells were treated with 100 ng/mL SDF-1α (A, B), 50 mM glucose (C, D), and 400 μM $CoCl_2$ (E, F) for 1, 2, 6, 12 and 24 hours. After treatment, MTT reagent was added to each well. The absorbance was measured at 570 nm and plotted in the form of a graph. Data were shown as mean ± SD of three experiments. In Figure 10, error bars represent standard deviation. # denotes a statistically significant difference ($P<0.05$), ## denotes a statistically significant difference ($P<0.01$), and #### denotes a statistically significant difference ($P<0.0001$) vs. indicated experimental groups.

## 5.5. Effects of SDF-1α, Glucose, and $CoCl_2$ on the expression of CXCR4

In order to determine the best treatment for CXCR4 overexpression, both hPD-MSCs and hBM-MSCs were treated with SDF-1α, Glucose, and $CoCl_2$, and CXCR4 expression was analyzed by immunoblotting. As shown in Figure 11A and 11B, glucose treatment had no significant effect on CXCR4 expression in either cell, whereas CXCR4 expression was increased ($P<0.05$) under SDF-1α treatment in hPD-MSCs (from 1.13 ± 0.08 to 1.41 ± 0.03 folds) compared to that in hBM-MSCs (from 1 to 1.03 ± 0.07 folds). CXCR4 expression was also found to increase in response to the $CoCl_2$-induced hypoxic condition in hBM-MSCs (from 1 to 1.21 ± 0.012 folds) and in hPD-MSCs (from 0.95 ± 0.08 to 1.65 ± 0.05 folds) ($P<0.05$). Therefore, $CoCl_2$ was found to be the best treatment for CXCR4 overexpression. Furthermore, $CoCl_2$-induced hypoxic conditions can mimic the hypoxic environment in infarcted regions in the heart post-MI, therefore it is also beneficial to keep a relatively constant environment for hMSCs both *in vitro* and *in vivo* (for future studies). CXCR4 expression was further confirmed by immunofluorescence imaging, as shown in Figure 11C, where the green color (Alexa Fluor 488) represents CXCR4, and blue color represents counterstaining (DAPI) of nuclei. $CoCl_2$-induced hypoxia was chosen to be the final treatment for inducing CXCR4 overexpression.

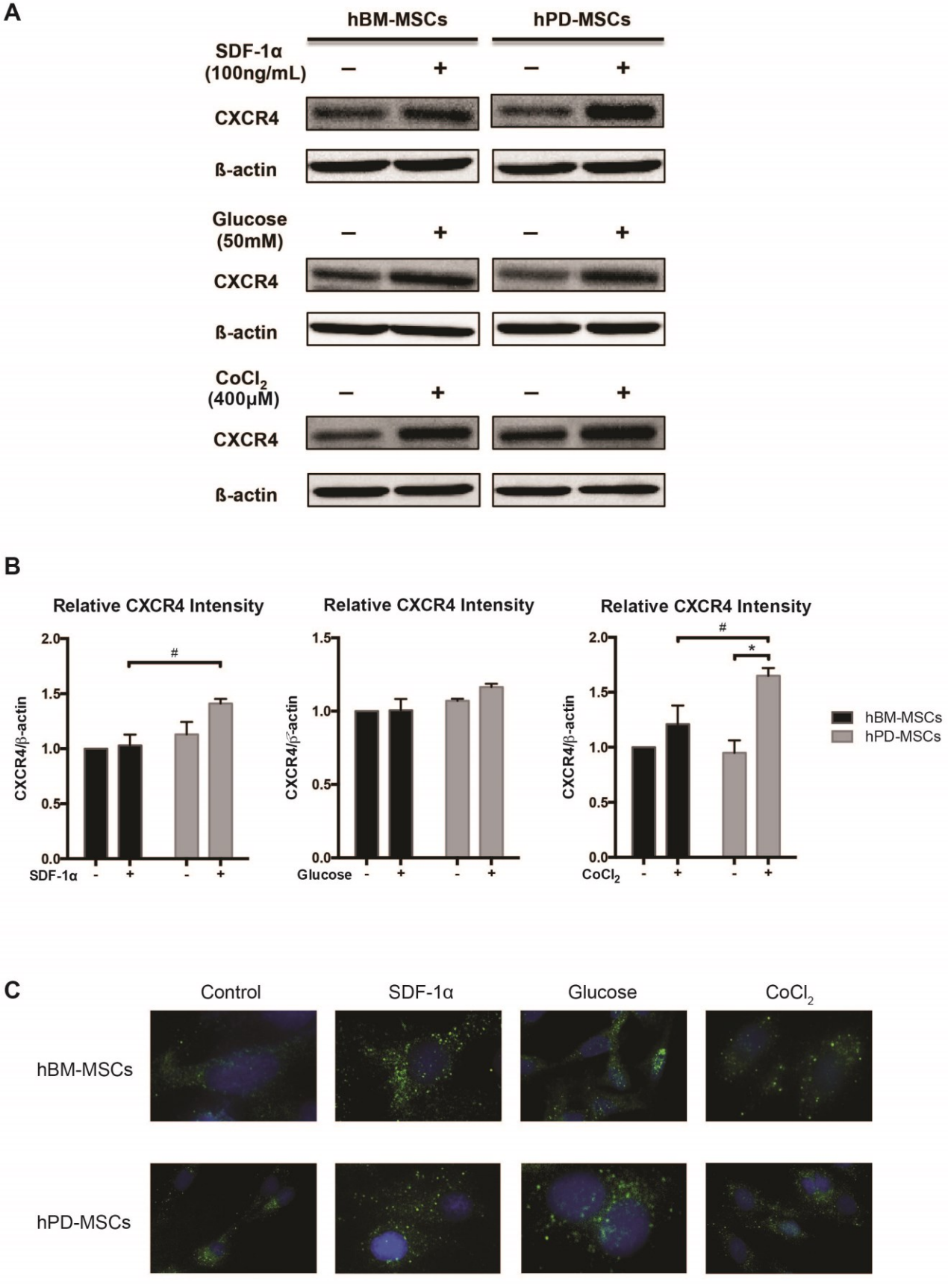

A
hBM-MSCs
hPD-MSCs
SDF-1α (100ng/mL)
CXCR4
ß-actin
Glucose (50mM)
CXCR4
ß-actin
CoCl2 (400μM)
CXCR4
ß-actin
B
Relative CXCR4 Intensity
CXCR4/β-actin
SDF-1α
Relative CXCR4 Intensity
CXCR4/β-actin
Glucose
Relative CXCR4 Intensity
CXCR4/β-actin
CoCl2
hBM-MSCs
hPD-MSCs
C
Control
SDF-1α
Glucose
CoCl2
hBM-MSCs
hPD-MSCs

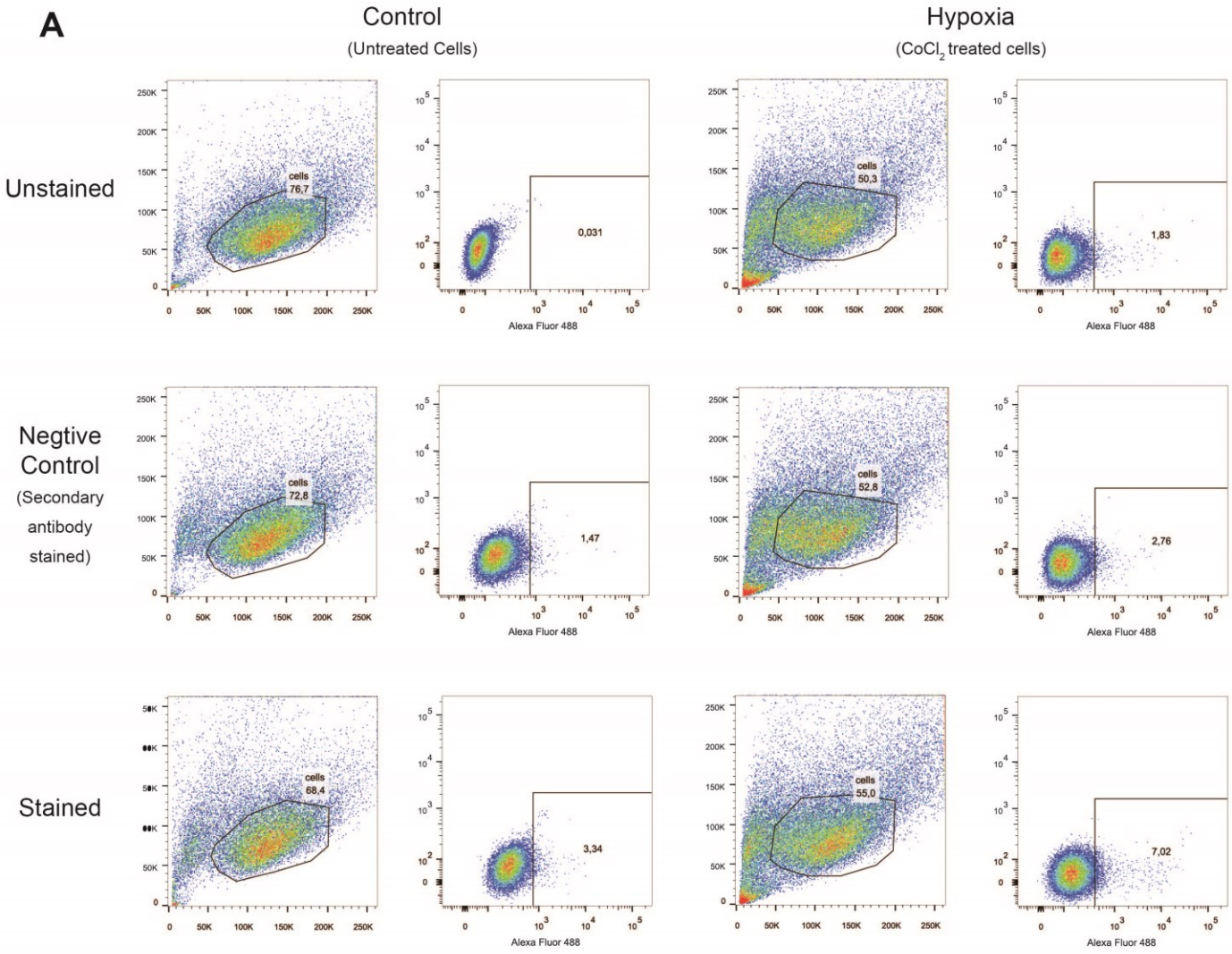
**Figure 11. Expression of CXCR4 in different conditions (Control, SDF-1α, Glucose, $CoCl_2$-induced hypoxia).** (A) Representative immunoblots showing CXCR4 expression in hBM-MSC and hPD-MSC cells after treatment with SDF-1α, Glucose, and $CoCl_2$. Immunoblot for β-actin served as loading control. (B) The density of specific bands was quantified using Image J software. The relative band density was calculated as a ratio of sample to β-actin and plotted. There was a significant increase in CXCR4 expression under $CoCl_2$-induced hypoxic conditions. (C) CXCR4 expression was further confirmed by immunofluorescence imaging, where the green color (Alexa Fluor 488) represents CXCR4, and blue color represents counterstaining (DAPI) of nuclei. In Figure 11B, error bars represent standard deviation. * denotes a statistically significant difference ($P<0.05$) vs. control group (without treatments), and # denotes a statistically significant difference ($P<0.05$) vs. other indicated experimental groups.

## 5.6. Hypoxia-induced increase in $CXCR4^+$ hMSC population

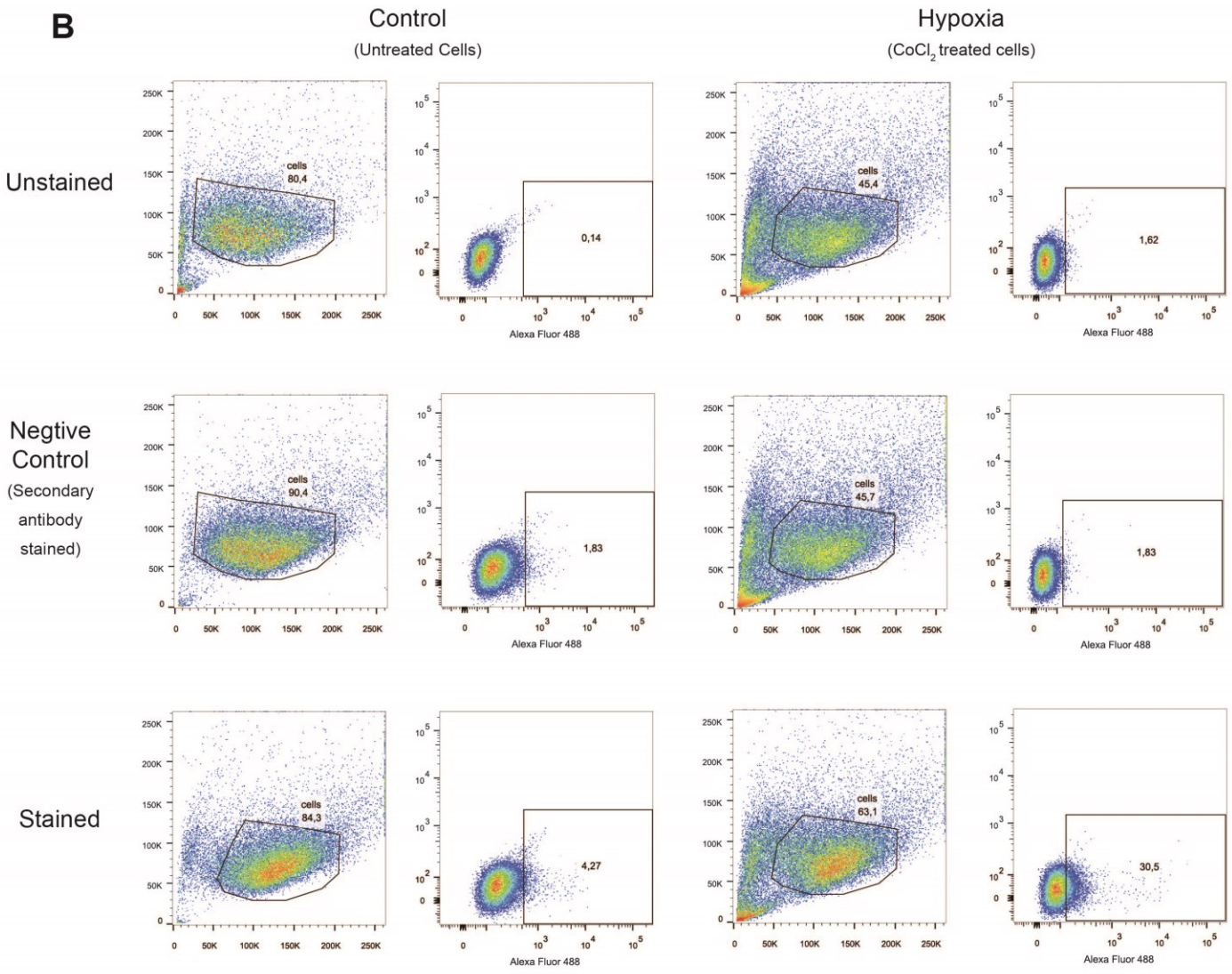
After $CoCl_2$-induced hypoxia treatment, FACS was done using anti-CXCR4 antibody in order to sort $CXCR4^+$ cells (Figure 12). A total of 3.34% ± 0.47 of hBM-MSCs were sorted to be $CXCR4^+$ in normoxia group, whereas 7.02% ± 0.69 of hBM-MSCs were sorted to be $CXCR4^+$ in the hypoxia group, as shown in Figure 12A. A total of 4.27% ± 0.68 of hPD-MSCs were sorted to be $CXCR4^+$ in normoxia group, whereas 30.50% ± 1.77 of hPD-MSCs were sorted to be $CXCR4^+$ in the hypoxia group, as shown in Figure 12B. Unstained samples were set to calibrate the instrument and samples stained with only secondary antibodies were set as negative controls to deduct the background signals. The results were plotted in the form of a graph (Figure 12C), which shows that in $CoCl_2$-induced hypoxic condition, the $CXCR4^+$ hBM-MSC population (% of total cells) increases by ~2-fold (from 3.00 ± 0.33 to 7.50 ± 0.48), whereas $CXCR4^+$ hPD-MSC population increases by ~8-fold (from 3.79 ± 0.48 to 31.75 ± 1.25) ($P<0.0001$).

Our data suggested that hPD-MSCs had a higher level of expression of CXCR4 than hBM-MSCs in $CoCl_2$-induced hypoxic conditions, which indicated that hPD-MSCs could be more responsive to the SDF-1α/CXCR4-mediated cell homing.

hBM-MSCs


A
Control
(Untreated Cells)
Hypoxia
(CoCl2 treated cells)
Unstained
Negtive Control
(Secondary antibody stained)
Stained
cells 76,7
0,031
cells 50,3
1,83
cells 72,8
1,47
cells 52,8
2,76
cells 68,4
3,34
cells 55,0
7,02
Alexa Fluor 488

hPD-MSCs


B
Control
(Untreated Cells)
Hypoxia
(CoCl2 treated cells)
Unstained
Negtive Control
(Secondary antibody stained)
Stained
cells 80,4
0,14
cells 45,4
1,62
cells 90,4
1,83
cells 45,7
1,83
cells 84,3
4,27
cells 63,1
30,5
Alexa Fluor 488

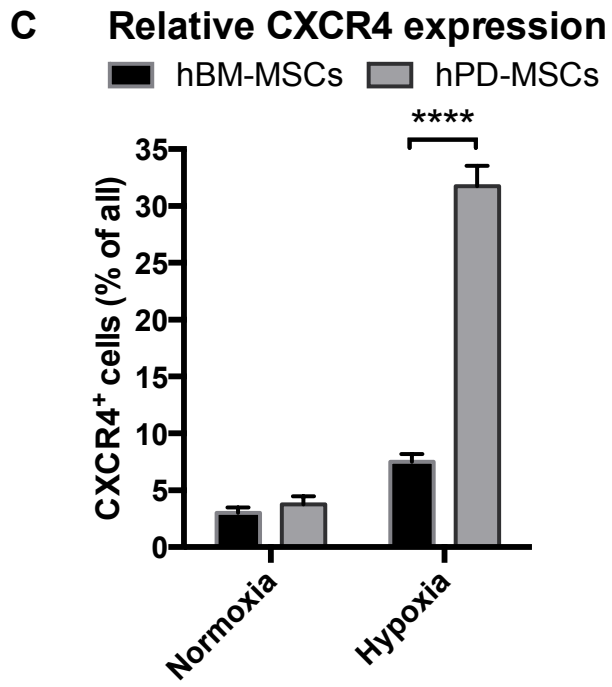



**Figure 12. Hypoxia-induced increase in CXCR4$^+$ hMSC population sorted by FACS.** (A and B) FACS analysis using anti-CXCR4 antibody (0.2 mg/mL) (Abcam, Toronto, CA) confirmed the surface expression of CXCR4 on both hPD-MSCs and hBM-MSCs. Expressions of CXCR4 receptor on both hBM-MSCs and hPD-MSCs were unaltered on treating the cells with $CoCl_2$. (Unstained samples were set to calibrate the instrument and samples stained with only secondary antibodies were set as negative controls to deduct the background signals.) (A) A total of 3.34% ± 0.47 CXCR4$^+$ cells were sorted in hBM-MSCs normoxia group, whereas 7.02% ± 0.69 CXCR4$^+$ cells were sorted in the hypoxia group. (B) A total of 4.27% ± 0.68 CXCR4$^+$ cells were sorted in hPD-MSCs normoxia group, whereas 30.5% ± 1.77 CXCR4$^+$ cells were sorted in the hypoxia group. (C) In $CoCl_2$-induced hypoxic conditioning, expression of CXCR4 was highly significant ($P<0.0001$) in the cell population of hPD-MSCs (~8-fold) versus its normoxia counterpart, compared to the difference between cell population of hBM-MSCs in hypoxia condition (~2-fold) versus its normoxia counterpart.

## 5.7. hPD-MSCs have high cell survival and proliferation potential and reduced inflammatory response

In order to detect and compare the potential of cell survival, cell proliferation, and the inflammatory responses of hPD-MSCs and hBM-MSCs, p-Akt, p-ERK and IL-6 were investigated. HIF-1α levels were increased in both hPD-MSCs and hBM-MSCs under the $CoCl_2$-induced hypoxic condition (Figure 13A). Also, the level of p-Akt was significantly higher in hBM-MSCs (from 1 to 3.53 ± 0.25 folds) and hPD-MSCs (from 1.64 ± 0.40 to 3.09 ± 0.52 folds) from normoxic to $CoCl_2$-induced hypoxic conditions (Figure 13B). As shown in Figure 13C, there was an increase in p-ERK levels ($P<0.05$) in hPD-MSCs (from 0.923 ± 0.024 to 1.124 ± 0.24 folds) under $CoCl_2$-induced hypoxic conditions, however, in hBM-MSCs the p-ERK levels remained the same. This suggested that hPD-MSCs might have higher proliferation potential than hBM-MSCs in $CoCl_2$-induced hypoxic conditions in the myocardial infarcted regions.

As shown in Figure 13D and 13E, the expression of IL-6 was significantly decreased in hPD-MSCs ($P<0.001$), but not in hBM-MSCs, which was decreased ($P<0.05$), after the exposure to $CoCl_2$-induced hypoxic conditions. Moreover, there was no IL-10 being detected (data not shown). This suggested that hPD-MSCs might cause less inflammation in the healing process for myocardial regeneration.

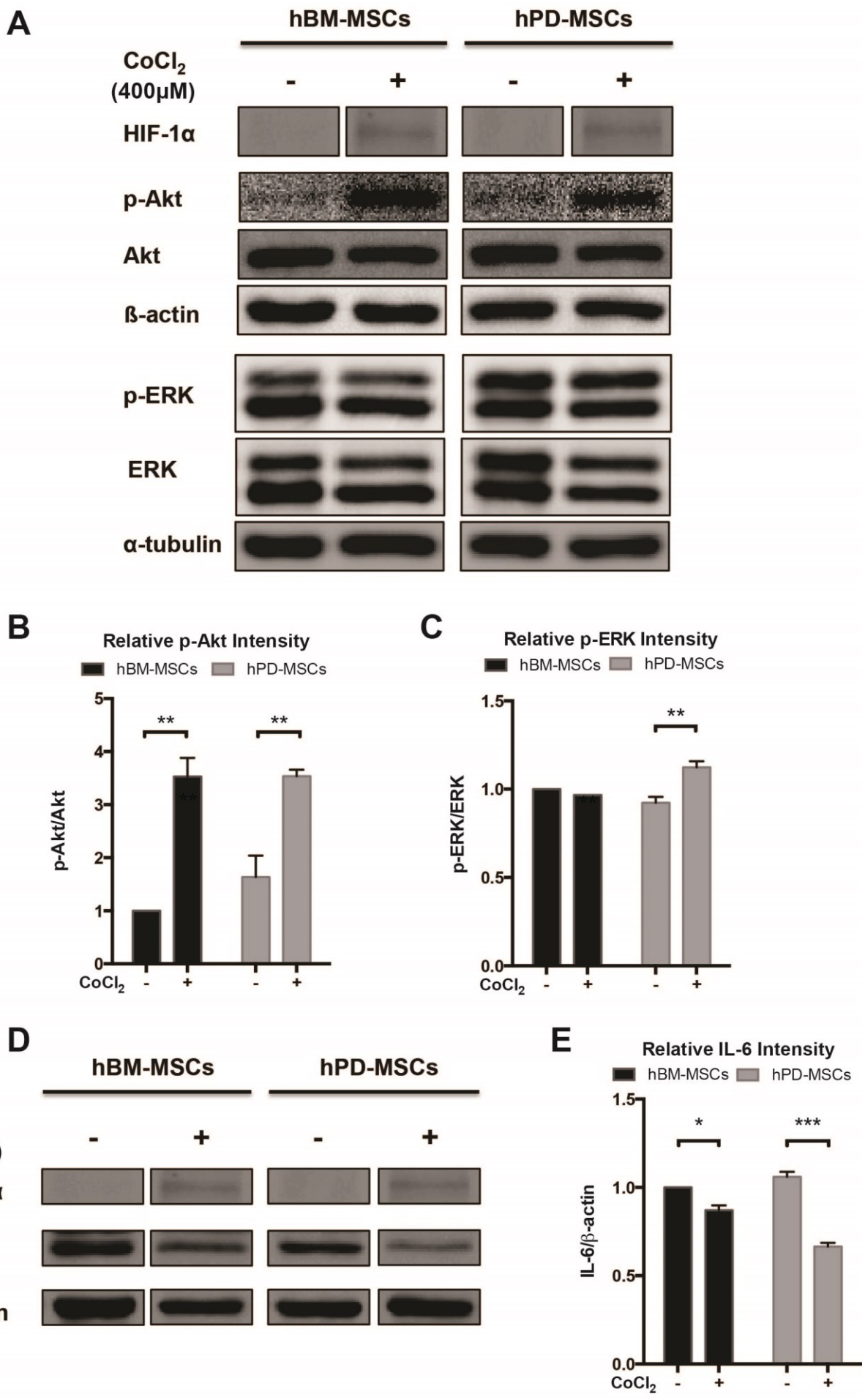

A
hBM-MSCs
hPD-MSCs
CoCl2 (400μM)
-
+
-
+
HIF-1α
p-Akt
Akt
ß-actin
p-ERK
ERK
α-tubulin
B
Relative p-Akt Intensity
hBM-MSCs
hPD-MSCs
p-Akt/Akt
**
**
**
CoCl2
C
Relative p-ERK Intensity
hBM-MSCs
hPD-MSCs
p-ERK/ERK
**
**
CoCl2
D
hBM-MSCs
hPD-MSCs
CoCl2 (400μM)
HIF-1α
IL-6
ß-actin
E
Relative IL-6 Intensity
hBM-MSCs
hPD-MSCs
IL-6/β-actin
*
***
CoCl2

**Figure 13. hPD-MSCs have high cell survival and proliferation potential and reduced inflammatory response.** (A) Representative blots showing levels of HIF-1α, p-AKT and p-ERK in hBM-MSC and hPD-MSC under $CoCl_2$-induced hypoxic conditions. Total AKT, Total ERK, β-actin, and α-tubulin were used as loading control. Expression values were quantitated, and the graph (B and C) represents the ratio of phosphorylated protein to total protein. (D) Representative immunoblot showing HIF-1α and IL-6 levels in both hBM-MSC and hPD-MSC cells in $CoCl_2$-induced hypoxic condition. β-actin serves as a loading control. Expression values were quantitated, and the graph (E) represents the ratio of IL-6 to β-actin. In Figure 13B, 13C and 13E, error bars represent standard deviation. * denotes a statistically significant difference ($P<0.05$), ** denotes a statistically significant difference ($P<0.01$), and *** denotes a statistically significant difference ($P<0.001$) vs. control group (without treatment).

# CHAPTER 6. DISCUSSION

Coronary occlusion leads to myocardial hypoxia, because of the lack of blood and oxygen supply and eventually myocardial infarction (MI), if blood flow is not restored within a short-time frame through thrombolysis, PCI or surgical intervention. Here we hypothesized that cell homing of CXCR4 positive cells will be accelerated, since the ischemic myocardium begin to secrete SDF-1α within 1 hour after ischemia. Our data showed that hPD-MSCs had a significantly greater cell migration potential than hBM-MSCs towards SDF-1α in a dose dependent manner. Also, the expression of CXCR4 was higher ($p<0.05$) after exposure to $CoCl_2$-induced hypoxia than after treatment with SDF-1α or exposure to glucose-induced hypoxia in both hPD-MSCs and hBM-MSCs. In $CoCl_2$-induced hypoxic conditions, the expression of CXCR4 was significantly increased ($P<0.0001$) in hPD-MSCs, compared to hBM-MSCs. Also, the CXCR4/MEK/ERK signaling cascade was activated ($P<0.05$) in hPD-MSCs, whereas, the CXCR4/PI3K/Akt cascade was significantly activated ($P<0.01$) in both cell types in $CoCl_2$-induced hypoxic conditions. Therefore, our data suggest that hPD-MSCs could represent an effective and efficient alternative to hBM-MSCs for translational studies in cellular repair.

## 6.1. hPD-MSC homing could be regulated by multiple SDF-1α mediated pathways

Our study demonstrated the biological advantage and potential beneficial effects of hPD-MSCs in stem cell-based therapy in MI. However, two essential requirements need to be met for stem cell facilitated myocardial repair [88]: "1) ample signaling to achieve the recruitment of sufficient amounts of cells and 2) their proper engraftment to ischemic tissue". During cell homing process post-MI, paracrine signals dynamically regulate and play significant roles in

various activities of the candidate cells, such as mobilization, adhesion, survival, proliferation and differentiation [88]. Our data determined that CXCR4 was constitutively expressed by both hBM-MSCs and hPD-MSCs. In addition, we observed that the SDF-1α/CXCR4 axis and other SDF-1α-mediated pathways are involved in regulating the cell homing activities in both cell types. This alludes to the high homing potential of both hPD-MSCs and hBM-MSCs toward higher SDF-1α concentrations in infarcted cardiac tissues.

### 6.2. HIF-1α may be a fundamental regulator in the SDF-1α/CXCR4 axis mediated stem cell migration

The oxygen tension is reported as one of the developmentally important stimuli that regulate stem cell functions, since stem cells are particularly sensitive to their environment, because of their plasticity. It plays a role in the intricate balance between cell proliferation and commitment towards differentiation [88]. In vitro, hMSCs are usually cultured under normoxic conditions (21% oxygen); however, this is different from their physiological niches *in vivo*, which has a much lower oxygen tension [140]. Reports showed that HIF-1α could induce both SDF-1α [77] and CXCR4 expression [97, 115], which might guide $CXCR4^+$ stem cells towards SDF-1α gradients in ischemic tissues and help to regenerate tissues. In our study, we found $CoCl_2$-induced hypoxia led to an increase in both HIF-1α expression and CXCR4 expression, which was in line with the previous reports. This indicates that HIF-1α may be a fundamental regulator of the mechanism of the SDF-1α/CXCR4 axis mediated stem cell migration following MI, which also needs to be further confirmed in hPD-MSCs.

6.3. hPD-MSCs have high cell survival and proliferation potential

The Akt protein is a key downstream target of the phosphoinositide-3 kinase (PI3K) mediated signaling pathway [141]. (Figure 14) Akt activation plays a significant role in regulating, stimulating cell proliferation, survival mechanism (by increasing nuclear factor of kappa beta (NFκB) mediated expression of pro-survival genes [142]) and neutralizing cell apoptosis. It is therefore seen as a defensive effect on post-ischemic cardiac injury [143]. Reports in experimental animals with MI showed that high Akt level in injured myocardium decreased apoptosis, reduced infarct size in the ischemic/injured myocardium and increased LV regional wall (thickening it to the pre-MI levels) [141, 144, 145]. From our data, the p-Akt was moderately increased with higher basal level of p-Akt/total-Akt in hPD-MSCs in contrary to hBM-MSCs. Since active Akt proteins can modulate numerous substrate functions related to cell survival and proliferation (even though cardiomyocytes are usually known as enormously defiant to mitotic activity and oncogenic transformation [146]), our data indicate that with a high basal level of p-Akt, hPD-MSCs naturally have high potential benefits to the regulation of cell survival, cell proliferation, and the anti-apoptotic signal transmission level than hBM-MSCs, which is a natural advance for myocardial regeneration.

The mitogen-activated protein kinases (MAPK), which include ERK, are also known to play fundamental roles in cell survival, proliferation, and apoptosis [147]. ERK is one of the keystones of the Ras/Raf/MEK/ERK pathway, a central signal transduction pathway, which functions in transmitting signals from cell surface (multiple receptors) to the nucleus (transcription factors) [148-150]. (Figure 14) The significance of ERK functions has been confirmed by many researchers, as reports showed the inhibition of ERK caused cell death in many cell lines, such as lymphocytes [151, 152] and neural cells [153]. It can be noted from our

data that p-ERK was upregulated significantly in $CoCl_2$-treated hPD-MSCs, which also suggests that hPD-MSCs have relative high tendency in cell survival and proliferation than hBM-MSCs in $CoCl_2$-induced hypoxia condition.

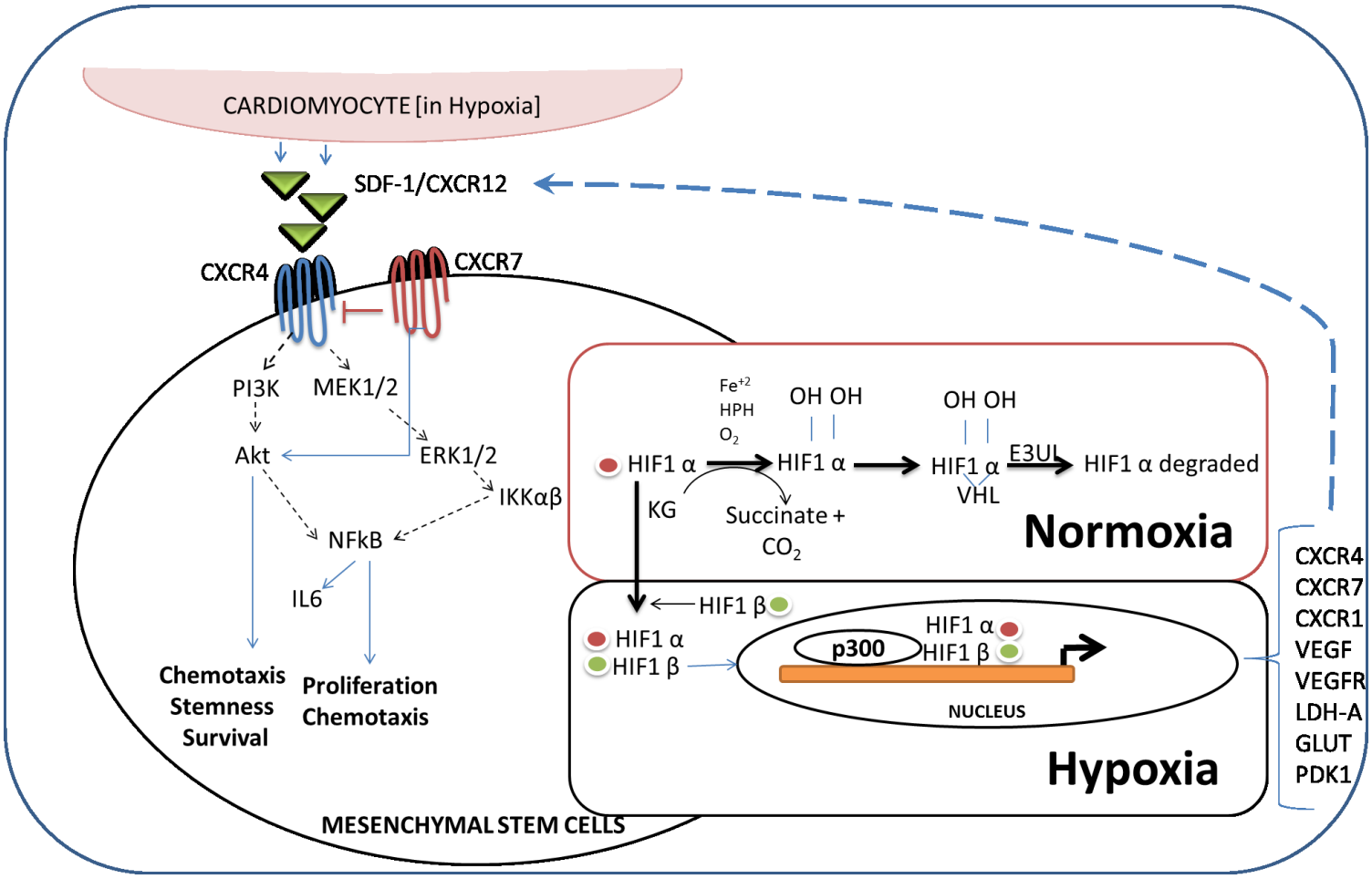



**Figure 14. Proposed pathways for improved cell migration in hPD-MSCs.** During acute myocardial infarction (AMI), cardiomyocytes start secreting soluble SDF-1α (CXCR12), which binds to surface receptor - CXCR4 of mesenchymal stem cells and triggers downstream signaling. Mainly, PI3K/Akt and MEK/ERK/IKKαβ pathways are modulated and trigger NFκB for translocation into the nucleus to activate paracrine factors including expression of CXCR4, Vascular endothelial growth factor (VEGF), and VEGF-Receptor (VEGF-R). It helps in cell migration (chemotaxis) along the gradient of SDF-1α, proliferation as well as cell survival of mesenchymal stem cells. The sensitivity of hPD-MSCs to MEK/ERK/IKKαβ pathway is relatively higher than that of hBM-MSCs, resulting in higher expression of $CXCR4^{+}$ cells in hypoxia.

### 6.4. The restricted secretion of excessive IL-6 by hPD-MSCs could reduce inflammation and help with cardiac repair

As explored and discussed in this study, besides the SDF-1α/CXCR4-axis-dependent cell homing, inflammation caused by the transplanted hPD-MSCs could also be a critical factor, which affects myocardial regeneration post-MI.

Different from previous studies, which reported the involvement of the SDF-1α/CXCR4 interaction inducing the SDF-1α-mediated IL-6 expression [89] or hypoxic stress of tumour cells resulting in high levels of IL-6 [135], our data suggested a restriction of IL-6 expression in hPD-MSCs under $CoCl_2$-induced hypoxic condition in terms of the SDF-1α/CXCR4 involved cell homing.

IL-6 is important in the dynamically orchestrated process of inflammation after MI, which is an innate reaction of human immune system. After the onset of ischemia, different immune cells are involved in the process: "neutrophils invade in the first place, and the respond of inflammation is subsequently followed by monocytes, macrophages, and fibroblasts. Meanwhile, other immune cells, such as dendritic cells and lymphocytes, also infiltrate the infarcted areas, which act as part of an attempt to maintain the immunologic homeostasis" [154]. As a result, excessive IL-6 could lead to the loss of the transplanted stem cells, as all the immune cells might eliminate them as foreign cells. Previous reports showed that the down-regulation of IL-6 can significantly reduce myocardial inflammation through reduction of cell apoptosis and necrosis of myocytes. And amelioration of extracellular matrix breakdown [155, 156] in acutely damaged hearts [157]. This is in line with the fact that IL-6 functions as a pro-inflammatory but anti-wound healing cytokine [158]. As such, inhibition of IL-6-dependent inflammation could help with cardiac repair in the infarcted region. Therefore, the restricted secretion of excessive IL-6

by hPD-MSCs observed in our study could reduce myocardial inflammation and promote the myocardial regeneration process. In addition, it will be interesting to further explore IL-6 related signaling pathways in hPD-MSCs, IL-6 manifold implications in both physiological and pathophysiological processes and its regulation of the stem cell therapy for the heart.

# CHAPTER 7. CONCLUSIONS

This study focused on comparing the cell homing, cell survival, and cell proliferation potentials of hPD-MSCs and hBM-MSCs in myocardial regeneration. The major findings of the study are as follows:

Both hBM-MSCs and hPD-MSCs endogenously express CXCR4 at a basal level. However, hPD-MSCs have a higher cell migration potential toward SDF-1α than hBM-MSCs. CXCR4 expression was more greatly increased in response to $CoCl_2$-induced hypoxic conditions in hPD-MSCs compared to hBM-MSCs. Also, the level of p-Akt was significantly higher in hBM-MSCs and hPD-MSCs when exposed to $CoCl_2$-induced hypoxic conditions rather than normoxic conditions, indicating the activation of the CXCR4/Akt pathway in both cell types. There was an increase in p-ERK levels in hPD-MSCs under $CoCl_2$-induced hypoxic conditions, however, in hBM-MSCs the p-ERK levels remained constant, which suggests the CXCR4/MEK/ERK pathway was activated dominantly in hPD-MSCs in the downstream signaling. The relatively lower expression of IL-6 by hPD-MSCs in $CoCl_2$-induced hypoxic condition indicates the dominance of IL-6 independent migration pathway, such as SDF-1α/CXCR4 axis. It might be helpful in accelerating cardiac repair by triggering less inflammatory response.

Overall, our data provides new insights into comparative molecular mechanisms that regulate mesenchymal stem cell migration derived from different tissue sources (bone and placenta).

Based on the findings mentioned above, we can conclude that hPD-MSCs could represent an effective and efficient alternative to hBM-MSCs for experimental studies and clinical trials for the treatment of myocardial regeneration post-MI. This holds great promise for the future.

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