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# STAMMZELL-MANIPULATION UND KARDIALE REGENERATION

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# STEM CELL MANIPULATION AND CARDIAC REGENERATION

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## CONTENTS

**ABBREVIATION LIST:**

| | |
|---|---|
| 5-bromo-2-deoxyuridine | BrdU |
| acute myocardial infarction | AMI |
| adrenomedullin | AM |
| Angiopoietin-1 | Ang-1 |
| B-cell CLL/lymphoma 2 | Bcl-2 |
| body weight | BW |
| cardiac Troponin T | cTnT |
| Cardiovascular disease | CVD |
| central canal | CC |
| central nervous system | CNS |
| cluster of differentiation | CD |
| congestive heart failure | CHF |
| coronary heart disease | CHD |
| cytomegalovirus | CMV |
| dorsal root ganglion | DRG |
| ejection fraction | EF |
| end-diastolic volume | EDV |
| endothelial progenitor cells | EPCs |
| end-systolic pressure-volume relationship | ESPVR |
| end-systolic volume | ESV |
| enzyme-linked immunosorbent assay | ELISA |
| fatty acid binding protein-4 | FABP-4 |
| heart weight | HW |
| hematopoietic stem cell | HSC |
| herpes simplex virus | HSV |
| high power field | HPF |
| human immunodeficiency virus | HIV |
| human leukocyte antigen | HLA |
| human mononuclear cord blood | hUCB |
| human nuclear antigen | HNA |
| left anterior descending coronary artery | LAD |
| left ventricle | LV |
| mesenchymal stem cells | MSCs |
| multipotent adult progenitor cells | MAPC |

| | |
|---|---|
| myocardial infarction | MI |
| nerve growth factor | NGF |
| neuron-specific nuclear protein | NeuN |
| nitrogen/phosphate ratio | N/P ratio |
| non-obese diabetic severe combined immunodeficiency | NOD/*scid* |
| Nuclear Factor-kB | NF-kB |
| phosphoinositide 3-kinase | PI3K |
| poly ethyleneimine | PEI |
| poly-L-lysine | PLL |
| propidium iodide | PI |
| protein kinases B | PKB or Akt |
| red fluorescent protein | DsRed |
| Relative light unit | RLU |
| stromal cell-derived factor-1 | SDF-1 |
| TdT-mediated dUTP Nick-End Labeling | TUNEL |
| thymocyte differentiation antigen 1 | Thy-1 |
| tyrosine kinases A | TrkA |
| vascular endothelial growth factor | VEGF |
| von Willebrand factor | vWF |
| β-Galactosidase | β-Gal |

## Structure of the thesis

To fully represent the result, the cumulative form of the Habilitation Thesis was selected. The description of the present work is organized as follows. The first chapter described the various types of stem cells, the key areas of stem cell based cardiac therapy for cardiac applications and gene enhancement of stem cell for cardiac therapy.

Chapter 2 concerns the two polyplex gene vectors for cell manipulation and describes the feasibility study of usage of the nonviral gene carrier. The ability of poly ethyleneimine (PEI) to mediate in vivo gene delivery to the Central Nervous System (CNS) by retrograde transport was explored after peripheral intramuscular injection. The development and evaluation of the cell-type specific transfecting peptide, is described also in Chapter 2.

Chapter 3 describes the latest development of stem cells and their cardiac benefit. In the earlier part of this chapter, organ-specific homing pattern of human mononuclear cord blood (hUCB) cells is investigated in a non-obese diabetic severe combined immunodeficiency (NOD/scid) mouse model. We proved that hUCB cells could specifically migrate to the heart after myocardial infarction, participate in tissue remodelling, and possibly facilitate regeneration processes after intravanous application. Furthermore, the myocardial regeneration potential of CD(cluster of differentiation) 133+ cells from bone marrow and cord blood was compared side by side in a NOD/scid mouse model of left ventricular cryoinjury. The functional and histologic consequence following direct intramyocardial delivery of human CD133+ cells has been determined.

The low cellular survival rate after stem cell transplantation into an infarcted heart within the first few days engenders only marginal functional improvement. Thus, in order to improve the efficacy of cell therapy it is necessary to reinforce stem cells against the arduous microenvironment incurred from ischemia, inflammatory response and pro-apoptotic factors. In charpter 4 we attempt to geneticly modify mesenchymal stem cells (MSCs) ex vivo with prosurvival gene bcl-2 (B-cell lymphoma 2), by nonviral vector in order to enhance their resistance to ischemic conditions from acute myocardial infarction after transplantation into the heart. The genetic modification of stem cells with Bcl-2 was found to armour stem cells settling into a deteriorative ischemic microenvironment, improve stem cell viability in the early post-transplanted period and thereby enhancing cardiac functional recovery after acute myocardial infarction.

Finally, conclusions drawn from the entire body of work are given in Chapter 5. The thesis was then followed by selected publications which contain the experimental data, the substantial results as well as the detailed discussion of the respective results.

## List of publications included as part of the thesis

(A) Wang S, **Ma N**, Gao S, Yu H, and Leong K, Transgenic expression in the brain effected by intramuscular injection of polyethylenimine/DNA complexes, Molecular Therapy, Vol. 3 (2001), 658-664.

(B) **Ma N**, Wu SS, Ma YX., Wang X, Zeng JM, Tong GP, Huang Y, and Wang S, Nerve growth factor receptor mediated gene delivery, Molecular Therapy, 2004, Vol. 9, 270-281.

(C) **Ma N**, Stamm C, Kaminski A, Li W, Kleine HD, Müller-Hilke B, Zhang L, Ladilov Y, Egger D, Steinhoff G. Human cord blood cells induce angiogenesis following myocardial infarction in NOD/scid-mice. Cardiovasc Res. 2005 Apr 1; 66(1):45-54.

(D) **Ma N**, Ladilov Y, Moebius JM, Ong L, Piechaczek C, Dávid A, Kaminski A, Choi YH, Li W, Egger D, Stamm C, Steinhoff G. Intramyocardial delivery of human CD133+ cells in a SCID mouse cryoinjury model: Bone marrow vs. cord blood-derived cells. Cardiovasc Res. 2006 Jul 1; 71(1):158-69.

(E) Li W, **Ma N**, Ong LL, Nesselmann C, Klopsch C, Ladilov Y, Furlani D, Piechaczek C, Moebius JM, Lützow K, Lendlein A, Stamm C, Li RK, Steinhoff G. Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. Stem Cells 2007 25(8):2118-27.

# Chapter 1 Introduction

## LITERATURE REVIEW: STEM CELL AND CARDIAC REGENERATION

Cardiovascular disease (CVD), which includes hypertension, coronary heart disease (CHD), stroke, and congestive heart failure (CHF), has ranked as the number one cause of death in Europe with over 2 million people dying from CVD every year. Given the aging of the population and the relatively dramatic recent increases in the prevalence of cardiovascular risk factors such as obesity and type 2 diabetes, CVD will continue to be a significant health problem well into the 21st century. However, improvements in the acute treatment of heart attacks and an increasing arsenal of drugs have facilitated survival. Still there is an unmet need for therapies to regenerate or repair damaged cardiac tissue.

Cardiac ischemia with shortage of oxygen leads to myocardial damage and cardiomyocyte loss. The cardiomyocyte (cardiac muscle cell) loss initiates a cascade of detrimental events, including immune response, formation of a non-contractile scar, ventricular wall thinning, and volume overload. The endogenous repair mechanisms, including the proliferation and stem cell recruitment from bone marrow and heart tissue are not sufficient to repair the damaged tissue. Current pharmacologic interventions for heart disease are not able to entirely restore the damaged cardiomyocytes.

It has been shown that the heart has the potential for regeneration. Clinical studies have shown that intracoronary or intracardiac transplantation of bone marrow stem cells to patients with chronic or acute ischemic heart diseases significantly improves the function and regional myocardial perfusion of the left ventricle [1-3], but the effect on heart function and the regeneration of heart muscle has been very limited [4]. Therefore, there is a great need for further improvement of stem cell property under the ischemic condition, which will brings stem cell therapies for heart disease one step closer in the daily clinic.

### *1.1 Stem cells*

Stem cells are cells that can differentiate into different kind of cells each exhibiting different characteristics such as skin, bone, liver, heart and nerve cells etc. Stem cells have two very important properties that distinguish them from other types of cells. First, they are unspecialized cells that renew themselves for long periods through cell division. The second is that under certain physiologic or experimental conditions, they could be differentiated into cells with special functions such as the cardiomyocyte or the insulin-producing cells of the

pancreas There are two kinds of stem cells: embryonic stem cell and adult stem cells. Adult stem cell could divide into hematopoietic stem cell (HSC) and MSCs. The HSC with an extensive capacity of self-renewal is responsible for the production of >95% of the body's blood cells [5]. In addition to their self-renewal and multipotent capacity, it has been demonstrated primitive HSC with a high "homing to niche" capacity has a high-marrow seeding efficiency. HSCs are marked in humans, by c-kit (CD117), thy-1(CD90), and CD34. Among these markers, CD34 has been generally used as a convenient positive selection marker for HSC. Consequently, CD133 is being newly discovered as an alternative new marker to CD34 antigen [6]. The CD133 antigen is a 120-kDa 5-transmembrane domain glycoprotein that is expressed in immature hematopoietic stem and progenitor cells [7][8]. In addition, several studies indicate that CD133 also serves as a marker for stem and progenitor cells with non-hematopoietic, pluripotent differentiation capacity, in particular the subset of CD133+ cells that is CD34– [9][10] which is highly expressed on immature stem cells but whose expression is lost during the differentiation to mature endothelial cells. CD133+ cell products may also contain mesenchymal stem cell-type cells, which have been designated as multipotent adult progenitor cells (MAPC) and might possess a particularly broad differentiation capacity [11]. The most robust line of evidence, however, indicates that CD133+ cells possess the characteristics of endothelial progenitor cells, which can readily be differentiated into endothelial cells in vitro and induce or at least participate in neoangiogenêsis processes in vivo [12-23]. For those reasons, CD133+ cells were among the first cell types to be used in clinical trials of cell therapy for myocardial regeneration, and they find increasingly wide-spread application [24-29].

MSCs can be isolated from bone marrow and many other sources [30] and can easily be expanded in an in vitro cell culture without losing their stem cell properties [31]. Because of the ease of the method of their purification and multipotent nature, they have been extensively studied in both experimental and clinical settings. In both in vivo and in vitro assays bone marrow MSCs appear able to change their fate. 5-bromo-2-deoxyuridine (BrdU) labeled marrow MSCs were reported to differentiate into cardiomyocytes after transplantation into the myocardium. Red fluorescent protein (DsRed) or GFP (green fluorescent protein) transfected human marrow MSCs was injected intraperitoneally into sheep fetuses in utero. It is showed that the human cells engrafted in the heart at a late stage of fetal development and differentiated into Purkinje fibers in the heart. Treatment of mouse MSCs with 5-azacytidine in vitro induced them to differentiate as skeletal muscle cells. Cells of two MSCs lines could differentiate as beating cells with a contractile protein profile of fetal ventricular cardiomyocytes, either spontaneously after long-term culture or after 5-azacytidine treatment [9]. Both these cell, however, expressed the cardiac-specific transcription factor Nkx2.5, which

would predispose them toward cardiomyocyte differentiation. There is no universal surface marker for the identification of mesenchymal stem cells.

They are devoid of typical HSC markers such as CD34, and CD45 and express on their surface CD44, CD90, CD105, CD106, and CD166 [32]. In addition, they express T cell receptors or their components that maintain them readiness stage to enter various pathways of differentiation upon induction [33]. It has been shown that CD34– nonhematopoietic bone marrow mesenchymal stem cells can differentiate into cardiomyocytes. The heterogeneous mononuclear fraction of the bone marrow is the most promising and extensively studied stem cell population for cellular cardiomyoplasty [34-36]. Besides, CD117 and Sca-1+ cells have also been focused to assess their cardiomyogenic potential [37][38].

Bone marrow has long been regarded as the good source of adult stem cells. Recently, stem cells have been found in specific tissue such as in the cardiac tissue of the heart, hippocampus of the brain, and olfactory bulb. It is generally believed that the embryonic stem cells have the most potential to derive into all kinds of cells. Going down the differentiation path from the embryonic stem cells are bone marrow stem cells, tissue-specific stem cells, lineage -specific precursor cells, and then terminally differentiated specific cells. Stem cells have the tendency to be maintained in culture for long period of time, with the capacity for expansion into large cell numbers for therapeutic purposes. However, the control of the differentiation path becomes more difficult the further upstream is the stem cell. Therefore, embryonic stem cells have been the most difficult to produce a pure population of cells for therapeutic applications. On the other hand, precursor cells can be induced to become the desired terminally differentiated cells in one step induction. These cells normally have less capacity for cell expansion. Also, it has been difficult to isolate such precursor cells (e.g. hippocampal neurons from the brain) from the patients. Therefore, it has been appealing to have the stem cells that can be taken from relatively abundant source such as the bone marrow, cord blood, periosteum, adipose tissues and induce them to differentiate into specific types of cells under suitable environment and conditions. Adult stem cells taken from tissue biopsy, fat tissue as the leftover of cosmetic surgery or from cadaver has been explored as the abundant source of adult stem cells as well. Some examples of the adult stem cells are hematopoietic stem cells that produce all types of blood cells, skin epithelium and epithelium of the small intestine, neural stem cells, and mesenchymal stem cells that can differentiate into cells of the musculoskeletal system.

### *1.2 Stem cell cardiac therapy*

Stem cell therapy [39] is quickly emerging as an alternative strategy in cardiovascular therapeutics. Proof-of-concept animal studies and phase I clinical trials have already shown the safety and feasibility of this approach. The preclinical and clinical studies with unmodified autologous or allogenic stem cells principally have demonstrated their potential for repair of the injured myocardium. However, there are some fundamental issues that need to be resolved to streamline research in this area. The ongoing debate on the role of stem cells cardiac therapy has pointed at controversy of underlying mechanisms, optimal cell preparation and functional integration as well as need for continuous scrutiny of the adverse effects of the therapy (immunogenicity, arrhythmogenesis, and tumorigenicity).

Cardiac benefit of stem cell transplantation has been vastly reported in vitro and in vivo[40][41] However, the exact mechanism of stem cell contributed functional improvement, its ability of adult stem cells to transdifferentiate into cardiomyocytes and integrate into the host tissue is still in the debate. The achieved cardiomyocyte phenotype was mostly demonstrated by the positive expression of various transcription factors. However, the exact function of these genes and their activation involved in cardiomyogenic differentiation remains a mystery. Many different approaches have been employed to induce cardiogenesis. Coculture of bone marrow stem cells with cardiomyocytes has been shown to preprogram these cells to adopt a cardiac phenotype. It has been shown that cell-to-cell contact during coculture of bone marrow cells with cardiomyocytes is imperative to trigger their cardiomyogenic transformation [42]. Several studies have shown that preconditioning in culture using growth factors and molecules/hormones results in enhanced therapeutic activity of target stem cells in vivo. The activity of these stimuli may enhance survival/proliferation, differentiation/stemness or engraftment ability of progenitor cells. For example, the treatment of endothelial progenitor cells (EPCs) with statins [43] led to an enhancement of proliferation/survival via phosphoinositide 3-kinase (PI3K) signalling while their pretreatment with SDF-1 (stromal cell-derived factor-1) determined an increase in their engraftment ability in ischemic tissues [44]. The use of transcriptionally active drugs such as histone deacetylase or 5-azacytidine has been further reported to enhance self renewal of the stem cells populations [45] and to promote cardiac differentiation of human bone marrow derived MSC [46]. It has been hypothesized that preconditioning of adult stem cell to adopt a cardiomyogenic differentiation before their transplantation may have better chances of their transdifferentiation into cardiomyocytes than transplantation of unmodified stem cells. The heart function of the 5-Aza-treated bone marrow cell transplanted group was better than that of other groups. The injured heart is a more efficient inducer of bone marrow cells differentiation into cardiomyocytes (64). The signals

originating from the cytokine-rich microenvironment of the injured myocardium and the direct cell-cell interaction between donor bone marrow cells and host cardiomyocytes may triggered their local protective response such as myogenic differentiation. It has been observed that the majority of endothelial cells injected into infarcted mice differentiated into myosin-positive cells with a cardiomyocytes-like morphology, whereas only a very few of them differentiated into cardiomyocytes after injection into the normal mice heart [47]. It also has been demonstrated that endothelial progenitor cells were mobilized into the peripheral blood and contributed to neovascularization in the heart during an acute infarction event in humans [48]. The angiogenic potential of bone marrow stem cells posttransplantation may be enhanced by subjecting the cells to ischemic stress before transplantation [49]. These studies suggested that the infarcted heart released into the peripheral blood, which may have trigger stem cell to differentiate into specific cell lineages. Taken together, these data suggest that the injured heart might have a greater contribution to the milieu-dependent cardiomyocyte differentiation of bone marrow cells. However, the information on the signaling molecules and responding molecular mechanisms that recruit bone marrow cells to the injured heart and transdifferentiation into cardiomyocytes is still unclear. Myocardial infarction can mobilize bone marrow cells and recruit them to injured myocardium where they get stimulated for transformation into new cardiomyocytes.

## *1.3 Gene enhancement of stem cell for cardiac therapy*

### 1.3.1 Non-viral biocompatible vector for gene modification

To deliver and express foreign genes in somatic cells *in vivo* has been a long sought-after goal of biological scientists. Gene transfer into postmitotic somatic cells would enable a direct analysis of the function and therapeutic effect of a specific protein in an intact organism. In view of this, the development of genetic intervention technology has attracted great interest not only as a means to resolve fundamental biology questions, but also as a critical step towards somatic gene therapy of genetic diseases. Indeed, the application of gene transfer is not limited to correcting genetic disorders, but can also be used to augment existing functions or provide new ones to cells for therapeutic purposes.

Successful delivery and expression of a therapeutic gene requires efficient and safe vectors that carry the gene into the cell nucleus. Vectors may be classified into two categories in terms of materials: viral and non-viral vector [50-53]. Viruses have evolved very efficient mechanisms to introduce their genetic material into host cells; therefore, they have been the obvious starting point to deveop vectors in many gene therapy protocols. However, viral vectors have significant disadvantages over nonviral vectors. Even safer, replication-defective viruses may

stimulate an immune response that can render repeat administration ineffective. Virus particles are also limited as to the sizes of the genes they can accomodate. The viral vector with higher transfection efficiency is suffered from high cytotoxicity and strong immunoresponse of virus itself. In addition, the cell transfected by virus vector may have mutagenesis. All of the remaining drawbacks limit the clinical application of viral system. In addition, nonviral vectors are typically easier and less expensive to manufacture. The plasmid DNA used in nonviral systems can be conveniently produced in bacteria such as *E. coli*, whereas packaging of genes into viral vectors in a complex procedure. The same production facilities can be used to manufacture various plasmids incorporating different genes. Nonviral gene delivery technologies include the use of naked DNA, cationic lipids formulated into liposomes and complexed with DNA (lipoplexes), cationic polymers complexed with DNA (polyplexes), a combination thereof, and DNA-carrying peptides or proteins. The non-viral vector, such as naked plasmid DNA and plasmid DNA complexed with cationized carriers, is rather safe and has no limitation in the molecular size of DNA applied. Various nonviral vectors have been designed and developed, and some of them are in clinical trials. However, nonviral vectors encounter many hurdles that result in diminished gene transfer in target cells. The main disadvantages of nonviral vectors are formulation inactivation and instability in blood, relatively low transfection efficiency, and poor targeting. Cationic vectors sometimes bind with serum proteins and blood cells when entering into blood circulation, which results in dramatical changes in their physicochemical characters. To reach target cells, nonviral vectors should pass through the capillaries, avoid recognition by mononuclear phagocytes, emerge from the blood vessels to the interstitium, and bind to the surface of the target cells. They then need to be internalized, escape from endosomes, and then find a way to the nucleus, avoiding degradation by cytoplasmic enzymes. Successful clinical applications of nonviral vectors will require a better understanding of the barriers in gene transfer and development of vectors that can overcome these barriers. In addition to these delivery barriers, an ideal nonviral vector and its carried DNA should be stable in test tubes as well as in the body, nontoxic, and nonimmunogenic.

To this end, several synthetic materials, including calcium phosphate [54-56] cationic liposomes [57-59], and cationic polymers like poly-L-lysine (PLL) [60] have been used for not only the enhancement of cell internalization and transfection but also efficient delivery of plasmid DNA to the specific area and the consequently high level expression.

In the last decade, both branched [61][62] and linear [63][64] PEI have been introduced as cationic polymers for gene delivery. Compared with other polymer, PEI exhibits highly

efficient gene transfer without the need for endosomolytic or lysosomotropic agents. PEI itself can be taken up by endocytosis and escape from endosomes [65][66]. In the presence of sufficient PEI, plasmid DNA will condense from loose, random loops to a compact structure [67]. The extent of condensation highly depends on the ratio of polymer to DNA, and PEIs [68]. The sizes of PEI/DNA complexes have been found to be within the range 20–40nm using atomic force microscopy [67]. The surface charge of PEI and PEI/DNA complexes has been examined in terms of zeta potential [69]. It was found that while a certain sample of branched PEI had a zeta potential of 37mV in solution, this value was lowered significantly to 31.5 mV when the PEI was allowed to complex with DNA (at a 7.5:1 PEI nitrogen to DNA phosphate ratio). The surface charge of the transfecting colloid is thought to be an important factor in the complexes' association with plasma membranes. The ability of PEI to transfect a wide variety of cells has been well documented. Boussif et al. found PEI-mediated transfection in 25 different cell types, including 18 human cell lines as well as pig and rat primary cells [70]. Gene deliveries have been performed in vivo using adult [71] and newborn [72] mice as well as on Sprague-Dawley rats [73]. Abdallah et al. reported no morbidity in the mice examined over their 3-month experiment course [74].

### 1.3.2 Gene manipulation of stem cell towards functional enhancement

Combining bone marrow stem cell transplantation together with the genetic modification of therapeutic genes to the injured myocardium is an alternative strategy. Among the different cell types, MSC are being expected as one of cell sources usable for cardiac reconstruction because of their differentiation potential and ability to supply growth factors. However, the therapeutic potential of MSC is often hindered by the poor viability at the transplanted site. Transplantation of mesenchymal stem cells overexpressing Akt has been shown to improve the survival of the donor cell and prevent ventricular remodeling together with improved cardiac function [75]. Non-viral transfer of Hemoxygenase 1 gene to MSCs, providing cardioprotective properties resulted in a higher number of surviving MSCs for cardiac repair and regeneration (improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector [76]. Co-overexpression of protein kinases B(Akt) and Angiopoietin-1 (Ang-1), by adenoviral transduction, was found to improve cell survival together with restoration of regional blood flow Stable therapeutic effects of mesenchymal stem cell-based multiple gene delivery for cardiac repair [77]. This multiple gene delivery study aimed to reach long term results 3 month after intramyocardial cell transplantation. Indeed extensive cell survival and myogenic differentiation were coupled with a significantly higher vessel density and smooth muscle cell covering (indicating maturation of newly formed vessels) in the study group compared to the control groups.

Thus, this genetic engineering technology is a promising strategy to enhance the therapeutic efficacy of MSC for ischemic heart disease.

### *1.4 Conclusion*

Stem cell based cardiac therapy is a promising therapeutic approach towards the functional improvement and neo-angiogenesis. Recent clinical and experimental developments demonstrate intracardiac delivery of stem and progenitor cells could significantly reduce infarct size and improve cardiac contractility. However, various problems and questions remains including the poor cell survival, the heterogeneity of the clinical results, the unclear molecular mechanism of cardiac repair, and the timing of the cell delivery. In order to further improve the extent of functional improvement, genetic manipulation with various protective genes should be further explored.

# Chapter 2 Nonviral Vector for Gene Delivery

## *2.1 Transgene expression by intramuscular injection of PEI/DNA complexes (Publication A)*

### 2.1.1 PEI Could Mediate Retrograde Axon Gene Transfer

PEI has shown high transfection efficiency both in vitro and in vivo. In particular, PEI may mediate DNA transfection in terminally differentiated nondividing neurons [78-81]. After direct brain injection, PEI/DNA complexes can provide transgene expression levels higher than those obtained with the human immunodeficiency virus (HIV)-derived vector and within the same range as that achieved with adenoviral vectors [79]. In view of the wide use of organic polymeric particles, such as dextran-coated microspheres or latex nanospheres, as retrograde tracers in neurons after endocytosis by axon termini [82], we investigated in this study whether the nonviral gene carrier PEI could be used to achieve gene transfer in the CNS after peripheral intramuscular injection.

### 2.1.2 PEI Mediated Gene Transfer in the Brain Stem Following Tongue Injection

To test the feasibility of gene delivery of PEI/DNA complexes to the CNS after intramuscular injection, we have chosen a well-characterized neuronal network, the hypoglossal system. Motor neurons of the hypoglossal nucleus in the brain stem innervate tongue muscles [83]. Marker gene (Luciferase plasmid) was complexed with PEI and injected into the tongue of the Wistar rats. We first tested PEI/pRELuc complexes at 15 mg of plasmid DNA with different charge ratios varying from 0 (i.e., naked DNA) to 25 PEI/DNA equivalents (Figure 2.1A) as well as PEI alone, produced no luciferase activity in the brain stem, indistinguishable from the tube background. At a charge ratio of 2 equivalents of PEI nitrogen per DNA phosphate where the overall charge of the PEI/DNA complexes would be negative at physiological pH, luciferase activity was $3\times10^5$ relative light unit (RLU)/brain stem. Luciferase activity was significantly higher at a charge ratio of 10 equivalents, reaching $3\times10^5$ RLU/brain stem, and reached a maximum N/P ratio of 17 equivalents, with $3\times10^6$ RLU/brain stem, and dropped to $3\times10^4$ RLU/brain stem when the charge ratio further increased to 25 equivalents. We then analyzed the effects of various amounts of DNA at a charge ratio of 17 equivalents of PEI nitrogen per DNA phosphate. A dose-dependent increase in luciferase activity was observed within the dose range of 5 to 20 mg pRELuc plasmid, with the highest value of $4\times10^6$ RLU per brain stem at 20 mg of DNA (Figure 2.1B).In terms of RLU/mg of DNA, the 10-mg dose produced $3\times10^5$ RLU/mg DNA (Figure 2.1C), roughly equivalent to 200 pg of luciferase. No further increase was observed with the 15 or 20 mg of DNA dose on a perunit basis.

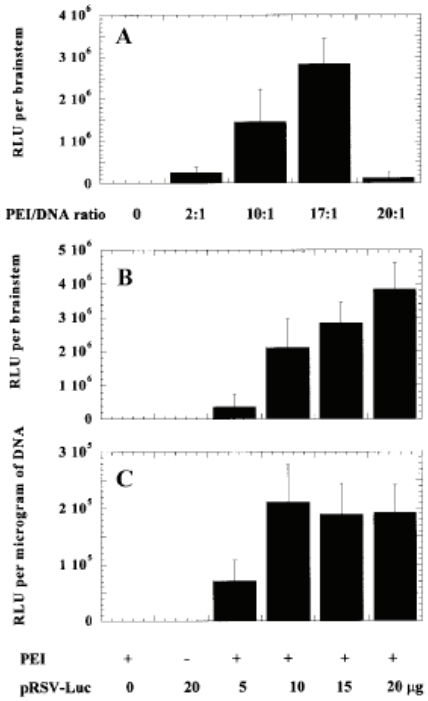


Figure 2.1 Luciferase activity in the brain stem after tongue injection of PEI/pRELuc complexes. A) Effects of PEI/DNA ratios. (B)Dose-dependent effects expressed per brain stem. (C) Dose dependent effects expressed per microgram of DNA injected.

### 2.1.3 Localization of Transfected Cells in the Brain Stem

To histologically determine the regions and cell types in the brain stem that expressed the transgene, a pcDNA3 plasmid containing the *E. coli* LacZ gene under the cytomegalovirus (CMV) promoter was complexed with PEI at N/P ratio 17/1 and injected into the tongue of the Wistar rats. The rats were sacrificed 2 days after injection. Cryostat sections of the brain stem were stained for β-Galactosidase (β-Gal) activity. Clearly labeled large neurons were evident in the hypoglossal structure (Figure2.2). Numerous motoneuron cell bodies and the proximal parts of their dendrites were densely stained. β-Gal activity was distributed throughout the whole hypoglossal nucleus in sagittal sections and β-Gal -positive cells accounted for about 90% of the total number of neurons in the hypoglossal nucleus. In contrast, no staining was detected in glial cells.Specificity of the β-Gal activity was confirmed in controls with either the human bcl-2 gene or the PEI polymer solution alone (Figure2.2).

To assess the retrograde transport of DNA constructs from the tongue into the hypoglossal nucleus, DNA was extracted from the brain stem and amplified with PCR using the primers

specific for the *E. coli* LacZ gene. The plasmid DNA was detectable in the brain stem as early as 12 h after the tongue injection and lasted for at least 14 days (Figure 2.3).

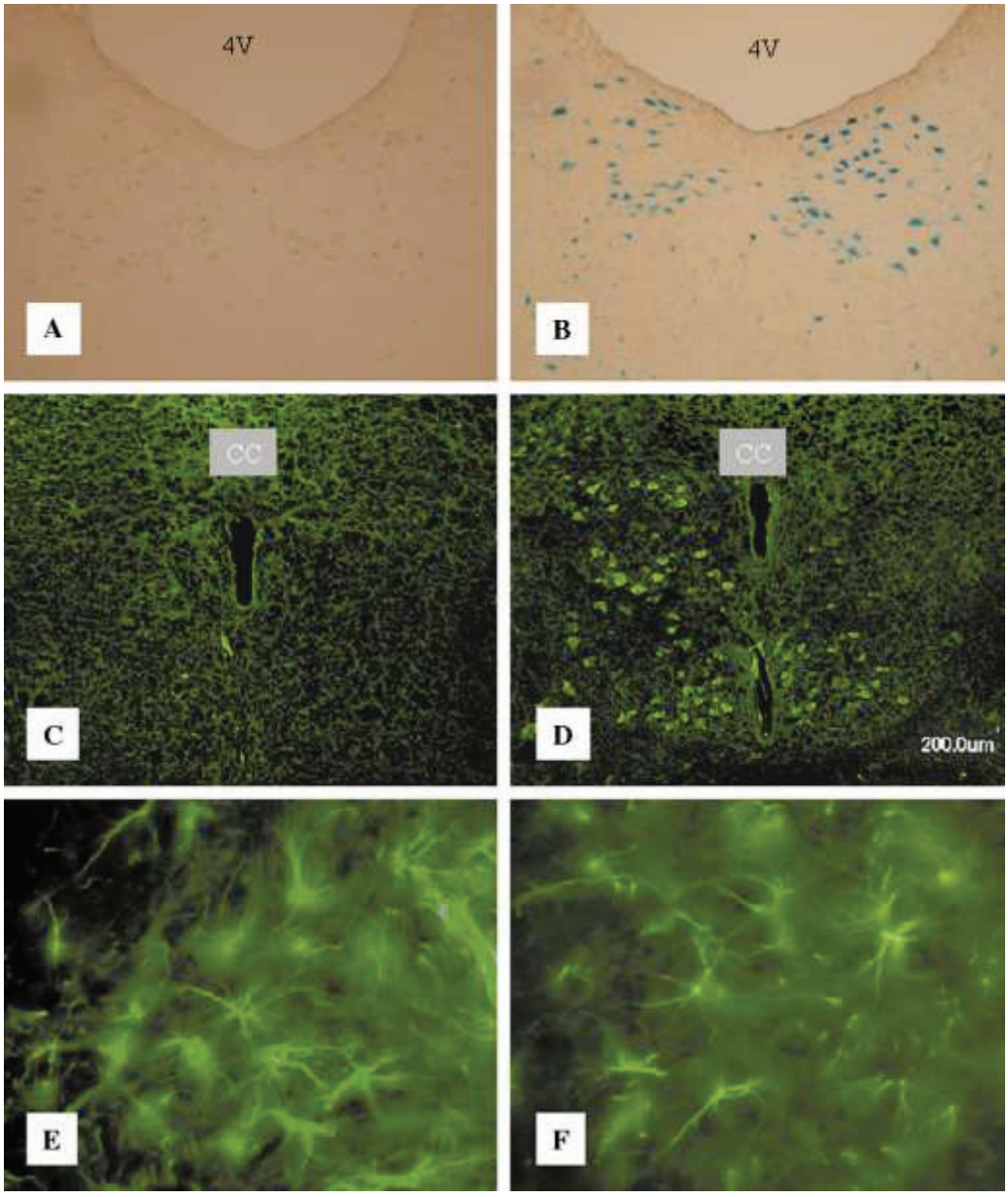


Figure 2.2 Transgene expression in the hypoglossal nuclei of the rat brain stem. (A and B) Detection of b-gal expression 2 days after tongue injection of the PEI solution (A) and the PEI/pcDNA3.1 LacZ complex (B). 4V, fourth ventricle. Original magnification: 3100. (C and D) Immunostaining of human Bcl-2 expression 2 days after injection of the PEI solution (C) and the PEI/pcDNA3 Bcl-2 complex at N/P ratio (D) into one side of the rat tongue.Fuorescence indicates the neurons overexpressing Bcl-2 mainly on one side of the hypoglossal nuclei. CC, central canal. (E and F) Immunofluorescence images of the GFAP expression in the hypoglossal nuclei. (E) Normal rat. (F) 2 days after tongue injection of the PEI/pcDNA3.1 LacZ plasmid complex at N/P ratio 30/1. Original magnification: 3400.

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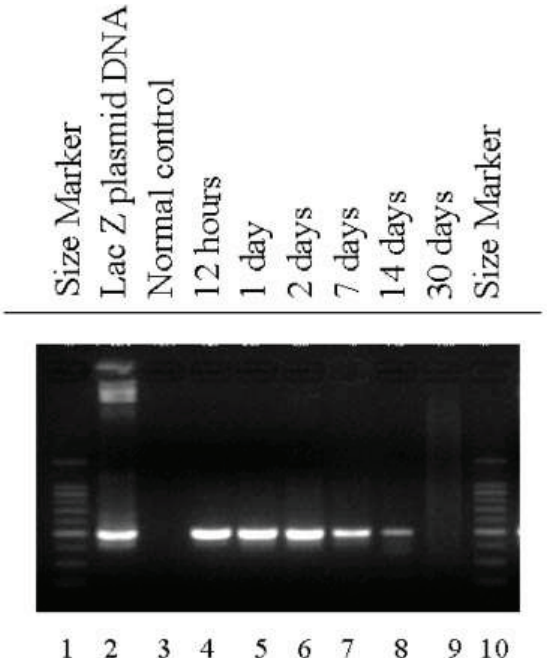



Figure 2.3 Detection of pcDNA3.1 *LacZ* with PCR analysis. PCR amplification of DNA extracted from the brain stem after tongue injection of 15 mg plasmid complexed with PEI at N/P ratio 17/1 shows a 494-bp fragment of the *E. coli LacZ* gene. Lanes 1 and 10, size marker; lane 2, positive control, 200 ng pcDNA3.1/LacZ plasmid DNA used as template; lane 3, the negative control from rats injected with the PEI solution without the plasmid construct; lanes 4–9, various time points after tongue injection of PEI/*LacZ* complexes.

### 2.1.4 Expression of the Therapeutic bcl-2 Gene in the Brain Stem

To corroborate the finding with luciferase and lacZgenes, we have also used an anti-apoptosis gene, bcl-2(B-cell CLL/lymphoma 2), for further characterization. This gene may also have a potential therapeutic value in treatment of neuronal death. Two days after injection of PEI-complexed pcDNA3 plasmid containing a human bcl-2 gene under the control of the CMV promoter into one side of the tongue, strong expression of the Bcl-2 protein was detected immunohistologically in numerous neurons of one hypoglossal nucleus (Figure 2.2). No Bcl-2-positive cells were detected in lacZ gene or PEI polymer solution controls. The expression of the human Bcl-2 could also be detected using Western blotting, after microdissection of brain-stem tissue around the hypoglossal nucleus and protein extraction. The Bcl-2 was detectable as early as 18 h after tongue injection and strongly expressed between 2 and 7 days. The expression continued for at least 14 days and became undetectable by 30 days (Figure 2.4 left).

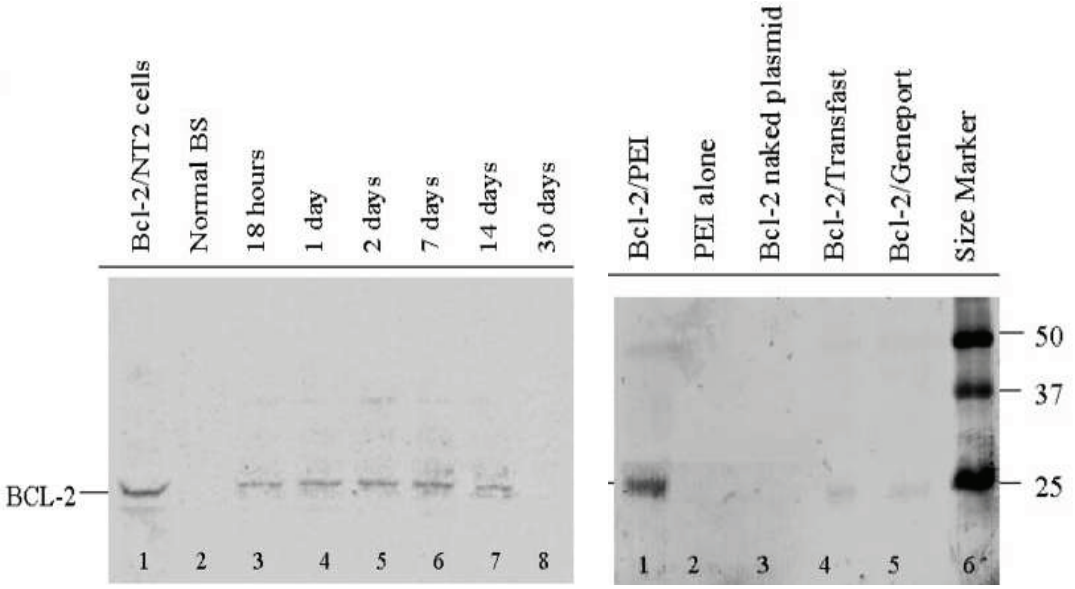



Figure 2.4 Western blot analysis of human Bcl-2 expression. (Left) The kinetic change of human Bcl-2 protein levels in the rat brain stem after tongue injection. Lane 1, positive control, Bcl-2-transfected NT2 cells; lane 2, negative control from normal rat brain stem; lanes 3–8, rat brain stem collected 18 h and 1, 2, 7, 14, and 30 days, respectively, after tongue injection of 15 mg of Bcl-2 plasmid complexed with PEI at N/P ratio 17/1. (Right) Comparison of gene transfer efficiency of different DNA carriers. Rat brain-stem samples were collected 2 days after tongue injection. Lane 1, injected with PEI/Bcl-2 complex (N/P 5 17/1); lane 2, injected with PEI solution alone; lane 3, injected with naked Bcl-2 plasmid DNA; lane 4, Transfast/Bcl-2 complex; lane 5, Gene-PORTER/Bcl-2 complex.

### 2.1.5 Discussion

We demonstrated that PEI/DNA complexes can migrate by retrograde axonal transport to neuronal cell bodies after being internalized by nerve terminals in the muscle and confirmed the feasibility of nonviral gene delivery to the CNS via peripheral injection sites. This approach bypasses the blood–brain barrier and provides a practical therapeutic strategy that is non-invasive to CNS tissues. Neurotropic adenovirus and herpes simplex virus (HSV) can deliver genes to the CNS in the same way [84-92]. These viruses invade the nervous system by directly infecting axon terminals in the periphery and then travelling to the nerve cell somata in a retrograde manner. The frequency of neuron infection by retrograde transport of the virus vectors is in the range 50–100% for adenovirus [85] and 90% for HSV vector [92]. A similar transfection frequency was observed in the present study when PEI was used to mediate neuronal gene transfer following peripheral injection. However, the PEI-mediated gene expression lasted for only 2 weeks. Gene expression may continue from 30 days for adenovirus vectors [85] to about half a year for herpes simplex virus vectors, as detected by X-gal histochemistry. In terms of safety, PEI did not invoke any inflammatory responses or alterations in GFAP immunoactivity in the brain stem following tongue injection, consistent with the report that the polymer, at concentrations for efficient DNA transfection in neurons, caused no disruptive effect on either the

cell surface membrane or the electrical properties of the cells [80]. Replication-defective adenovirus would cause inflammation in the inoculated muscles, but it caused no pathology within the CNS, where the vector expressed a recombinant gene after retrograde axonal transport [86]. HSV vectors also did not invoke any inflammatory responses either at the peripheral inoculated site or at the gene expression site in the CNS [92]. A concern of using these viral vectors is related to the clinical situation of high titers of antibodies against the vectors induced by a preexposure to the viruses in some prospective patients, which may hinder the therapeutic efficacy of the vectors [93]. As well, this preexisting immune response or an enhanced immune response to the vectors following initial inoculation is a potential risk of repeated administration of the vectors [94]. The risk of immunological complications should, in theory, be low when synthetic DNA carriers such as PEI are used for gene transfer. Furthermore, this reduced risk would allow repeated injection of nonviral DNA complexes, therefore increasing the time span of transgene expression if needed. In addition to viruses, many proteins, metallic particulates, and polymer particles can be taken up by nerve endings and retrogradely transported to neuronal cell bodies [82]. Sahenk et al. have reported a lipofectin-mediated DNA transport to spinal motor neurons after muscular or nerve injection [95], with a transfection frequency of approximately 18%. Consistent with this relatively low efficiency, two liposome systems used in the present study gave much lower levels of luciferase activities and Bcl-2 expression than PEI. Another nonviral DNA carrier that has been used to transport genes in a retrograde manner is PLL [96]. That study, although not involving any peripheral injection, has demonstrated that PLL-condensed DNA particles mediated expression of reporter genes in the retina after administration at the cut end or axon terminals of the optic nerve in the brain. However, PLL, as well as chitosan, did not mediate any gene transfer in our system.

PEI/DNA complexes are efficient at in vivo gene transfer into neurons after stereotactic injection into the brain. Under optimal transfection conditions for direct intracerebral injection, i.e., a charge ratio of 6 equivalents of PEI nitrogen per DNA phosphate, 4-3×$10^5$ RLU/mg DNA (equivalent to 0.4 ng of luciferase) was achieved [79], which was close to the values obtained using PEI to transfect neuronal cultures and newborn mouse brain ($10^6$ RLU/mg DNA) [78]. Transfection efficiencies in the brain stem observed in this study with a peripheral intramuscular injection (2-3×$10^5$ RLU/mg DNA or 0.2 ng luciferase per mg DNA injected) were close to what is achievable by direct intracerebral injection. The reason PEI gives the best result among the nonviral DNA carriers used in the present study is unclear. Size and charge of particles are probably among the factors that are critical for uptake by axonal terminals. A net positive charge may facilitate interaction with the cellular plasma membrane and endocytosis, but an excessive positive charge might cause entrapment of the DNA particles by the extracellular matrix. This is illustrated in the present study, in which the transgene expression increases with PEI/DNA ratio to the maximum, before dropping drastically at the highest ratio employed. The efficacy of PEI as

gene carrier in cell culture studies has been attributed to its proton sponge effect [78]. Comprising primary, secondary, and tertiary amino groups, PEI has been postulated to buffer the pH changes in endo/lysosomes to inactivate enzymes such as DNase 2 [97], which in turn leads to protection of the plasmid from enzymatic degradation. Since the subcellular structures that carry exogenous materials taken up by endocytosis to a neuronal cell body are endolysosomes or lysosomes, this buffering effect of PEI may be critical for DNA stability during a long distance axonal retrograde transport and, therefore, for the successful gene transfer observed in the present study. The CNS is an organ with a great diversity of cell types. This sophisticated organ is composed of many regions, each of which has unique roles. Even highly limited injury to a small area of the CNS can damage a critical function, as evidenced in Parkinson's disease, which results from the loss of dopamine neurons in a discrete anatomical region, the substantia nigra pars compacta. This feature of the CNS calls for therapies that can target specific locations or selected neuronal populations. By choosing a proper injection site, targeting some of the neuron populations deep in the CNS structure may be possible with retrograde transport-mediated gene delivery method. Jin et al. have shown that a defective HSV amplicon vector with a tyrosine hydroxylase promoter injected into the striatum may migrate by retrograde transport to the substantia nigra and mediate transgene expression in dopaminergic neurons [98]. Retrograde transport-mediated gene delivery potentially has a number of clinical applications in the peripheral nervous system as well, such as protecting degeneration of motor neurons caused by injury or genetic mutations; treating various pain states resulting from tissue injury, inflammation, or tumor invasion; and stimulating regeneration of damaged nerves.

## *2.2 Receptor-mediated targeted gene delivery by recombinant peptide (Publication B)*

### 2.2.1 Introduction

Targeted gene delivery to selected cell types is a crucial aspect of enhancing the specificity of transgene expression, for the purposes of gene therapy as well as functional genomics research. The transfection efficiency of a given dose of genetic material would be improved by augmenting entry into the desired cells and reducing wasteful uptake by nontarget cells. For therapeutic applications, targeted gene delivery would ensure therapeutic efficacy in the cells of interest while limiting side effects, including immune, inflammatory, and cytotoxic responses, caused by the expression of exogenous genes in nontarget cells. One approach to targeted gene delivery is to use ligand-associated delivery vectors to take advantage of the natural process of receptor-mediated endocytosis. Many ligand–receptor systems have been investigated to date for targeted gene delivery 99][100][101]. Little has been established, however, on how to use such a system to target selected subtypes of neurons.

Neurons differ in their chemical signals and receptors, among which are nerve growth factor (NGF) and its receptors. NGF is an important molecule capable of promoting the development, growth, and survival of its responsive neurons, such as basal forebrain cholinergic neurons and sympathetic sensory neurons [102]. NGF is synthesized and secreted from neuronal target cells as noncovalently bound homodimers and functions through retrograde signaling after binding to its nerve-terminal-localized receptors and being internalized in endosomes [103]. NGF interacts selectively with one of the receptor protein tyrosine kinases, tyrosine kinases A (TrkA)[104]. Internalized TrkA after NGF stimulation could be identified in endosomes and transported to neuron cell bodies to activate its signaling targets [105][106]. NGF also binds with relatively low affinity to a 75-kDa transmembrane glycoprotein, $p75^{NTR}$[107], a receptor with two main physiological functions: acting as a coreceptor to modulate Trk signaling and initiating autonomous signaling cascades that result in either the induction of apoptosis or the promotion of survival [107][108]. Since receptor-mediated endocytosis of NGF is a rapid and efficient process [103], NGF may serve as a candidate targeting ligand to direct gene vectors to TrkA- and/or $p75^{NTR}$-positive cells.

Extensive studies on the structural determinants of NGF in interacting with its receptors have identified NGF loop 1 as a key active site for $p75^{NTR}$ receptor-binding [109][110] and the N-terminal region, loop 2, and loop 4 as important structures for binding to TrkA [111][112]. Based on these studies, small bioactive peptide mimetics using sequences from the NGF loop regions have been developed [113][114][115][116]. In the current study we developed a targeted gene delivery vector by combining the NGF-receptor-binding activities of NGF loops 1 and 2 with the DNA-binding function of SPKR repeats in a single chimeric peptide. We demonstrated that the peptide enhanced gene expression mediated by DNA/polymer complexes specifically in cells.

### 2.2.2 Development of Chimeric Peptide $SPKR_4NL1$-2 and Its Biochemical and Biological Effects

$SPKR_4NL1$-2 is a chimeric peptide containing a DNA-binding domain, SPKRSPKRSPKRSPKR, and a targeting domain incorporating the NGF hairpin motif of loops 1 and 2. The two parts are linked by an α-helical linker, TYLSEDELKAAEAAFKRHNPT. The $SPKR_4$ sequence, derived from the histone H1 DNA-binding domain, and the α-helical linker were first used together by Fortunati et al. [117] in a DNA delivery vector. The linker is flanked by flexible glycine residues and serves to allow independent action of the DNA-binding and targeting domains. The targeting domain comprises a cysteine residue followed by amino acids 17–67 of human NGF, such that a disulfide bridge can form between this cysteine residue and C58 in NGF. Based on the crystal

structure of NGF [112], we judged that such a disulfide bond would help the targeting domain assume the native conformation of loops 1 and 2.

The chimeric peptide was expressed with a $His_{10}$-tag in the *Escherichia coli* strain BL21(DE3). The eluates from nickel-chelate affinity chromatography of *E. coli* lysates predominantly contained a protein similar in size to the expected molecular weight of $SPKR_4NL1$-2 at 12.9 kDa. The produced chimeric peptide was able to activate TrkA and the ERK 1 and 2 proteins in its downstream signaling pathways (Figure 2.5). We observed TrkA phosphorylation by Western blot analysis of the cell lysate of TrkA-positive PC12 cells treated with $SPKR_4NL1$-2, whereas this response was not elicited by similar treatment with a control peptide, $(SPKR)_4$, that lacked the targeting domain (Figure 2.5A). We also observed enhanced phosphorylation of ERK 1 and 2 after treatment with $SPKR_4NL1$-2 at concentrations ranging from 1 to 8 μM for 15 min, compared to that after treatment with 8μM $(SPKR)_4$ control peptide (Figure 2.5B). To demonstrate that the ERK activation occurred specifically through TrkA phosphorylation, we pretreated cells with K-252a, a TrkA tyrosine kinase inhibitor, to see if ERK phosphorylation would be reduced. Figure 2.5C shows that ERK phosphorylation induced by 8 μM $SPKR_4NL1$-2 was reduced and eventually eliminated by increasing concentrations of K-252a.

$SPKR_4NL1$-2 activates TrkA and ERK. PC12 cells preincubated in RPMI 1640 medium containing 0.5% FBS and 0.25% horse serum for 2 days were treated for 20 min with NGF or peptides diluted in serum-free RPMI 1640. The cell lysates were analyzed by immunoblotting using primary antibodies specific to either phospho-TrkA or phosphorylated ERK 1 and 2. (A) Phospho-TrkA Western blot. The cells were treated with NGF (20 ng/ml), $SPKR_4NL1$-2 (8 μM), $SPKR_4NL1$-2/DNA complexes (8 μM, N/P ratio of 5), or $(SPKR)_4$ (8 μM). (B) Phospho-ERK Western blot. The cells were treated with NGF (20 ng/ml), various concentrations of $SPKR_4NL1$-2 from 1 to 8 μM, or 8 μM $(SPKR)_4$. (C) A TrkA inhibitor blocks $SPKR_4NL1$-2-induced ERK activation. The PC12 cells were preincubated with 0, 10, 20, 50, and 100 nM K-252a (TrkA tyrosine kinase inhibitor) for 10 min before treatment with NGF (20 ng/ml) or $SPKR_4NL1$-2 (8 μM). Molecular weights of protein standards are shown on the left.

In response to NGF, PC12 cells stop proliferating and differentiate into sympathetic neuron-like cells. Within 2–3 days of NGF treatment, cell morphology changes and neurites can be seen projecting from the cells. We tested $SPKR_4NL1$-2 to see if it could also promote neurite outgrowth in PC12 cells. We treated the cells with 8 μM $(SPKR)_4$ or $SPKR_4NL1$-2 for 3 days. Compared to $(SPKR)_4$, $SPKR_4NL1$-2 displayed NGF-like bioactivity by promoting neurite outgrowth (Figure 2.6A and Figure 2.6B). We also investigated whether $SPKR_4NL1$-2 shared the ability of NGF to promote survival of PC12 cells deprived of serum for 3 days. The addition of $SPKR_4NL1$-2 (8 μM) to the serum-free medium maintained PC12 viability at 80% of the

maximal survival rate promoted by 10 ng/ml NGF (Figure 2.6C). The survival-promoting effect exhibited a dose–response trend over the range of 2–8 μM polypeptide.

$SPKR_4NL1$-2 has NGF-like bioactivity. (A and B) $SPKR_4NL1$-2 promotes neurite outgrowth. PC12 cells were treated with 8 μM (A) $(SPKR)_4$ or (B) $SPKR_4NL1$-2 for 3 days. (C) $SPKR_4NL1$-2 promotes survival of PC12 cells deprived of serum for 3 days. Different concentrations of $SPKR_4NL1$-2, from 0 to 16 μM, were added at the time of serum withdrawal and 10 ng/ml NGF was used as a positive control. Cell survival was estimated by an MTT assay and expressed as a percentage of maximal NGF-promoted cell survival.

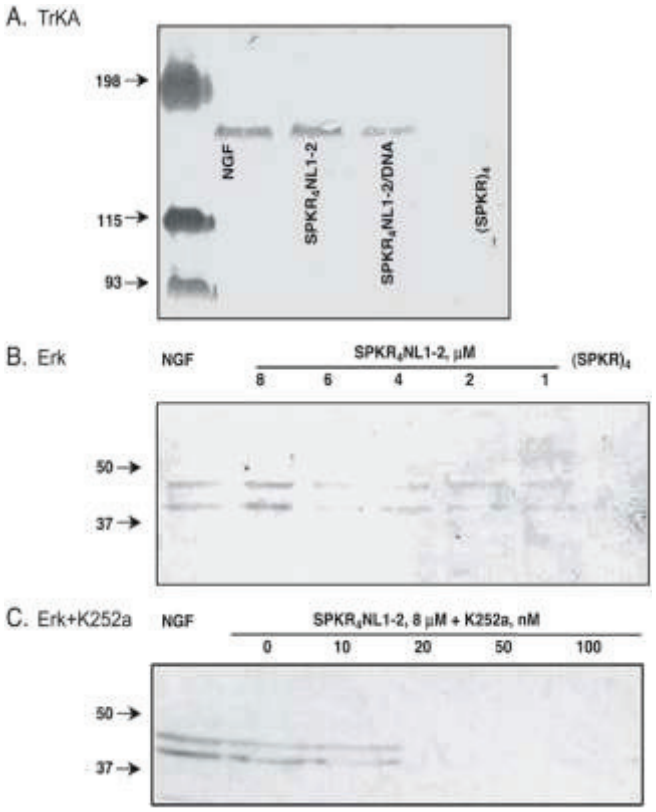



Figure 2.5 $SPKR_4NL1$-2 activates TrkA and ERK. (A) Phospho-TrkA Western blot. The cells were treated with NGF (20 ng/ml), $SPKR_4NL1$-2 (8 μM), $SPKR_4NL1$-2/DNA complexes (8 μM, N/P ratio of 5), or $(SPKR)_4$ (8 μM). (B) Phospho-ERK Western blot. The cells were treated with NGF (20 ng/ml), various concentrations of $SPKR_4NL1$-2 from 1 to 8 μM, or 8 μM $(SPKR)_4$. (C) A TrkA inhibitor blocks $SPKR_4NL1$-2-induced ERK activation.

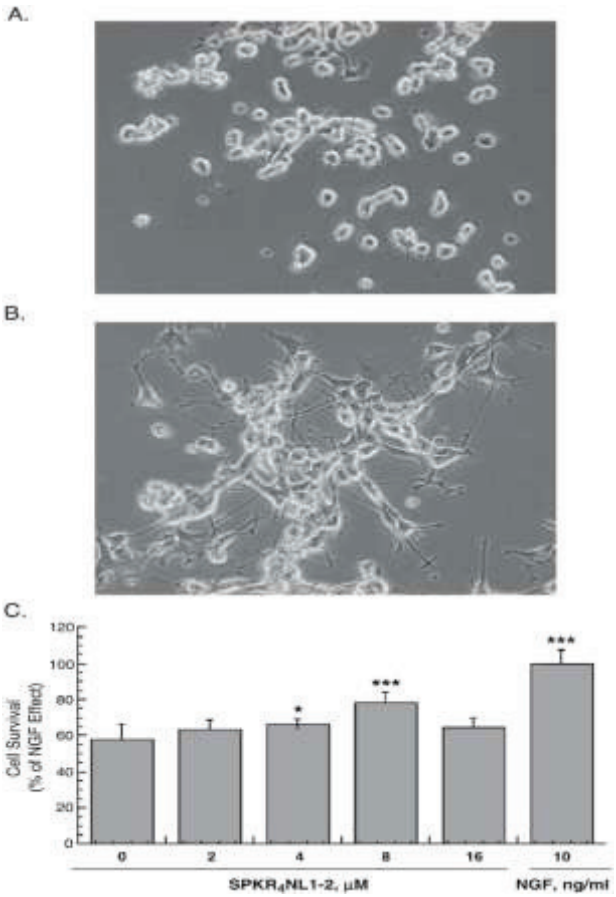



Figure 2.6 $SPKR_4NL1$-2 has NGF-like bioactivity. (A and B) $SPKR_4NL1$-2 promotes neurite outgrowth. PC12 cells were treated with 8 μM (A) $(SPKR)_4$ or (B) $SPKR_4NL1$-2 for 3 days. (C) $SPKR_4NL1$-2 promotes survival of PC12 cells deprived of serum for 3 days.

### 2.2.3 $SPKR_4NL1$-2 Binds to Plasmid DNA and Enhances Gene Delivery to PC12 Cells

We assessed the DNA-binding activity of $SPKR_4NL1$-2 first in a DNA retardation assay. When mixed with 0.1 μg of plasmid DNA, $SPKR_4NL1$-2 dose-dependently reduced the mobility of DNA through an agarose gel under electrophoresis (Figure 2.7A), indicating that the peptide had bound to the DNA and reduced its charge/mass ratio. A significant reduction in DNA migration occurred when we used 1.25 μg of peptide (Figure 2.7A). We also characterized the ability of $SPKR_4NL1$-2 to bind to and condense DNA by measuring the quenching of fluorescence when intercalated ethidium bromide was displaced from DNA by the peptide. The fluorescence dropped sharply when 8 μg of peptide was added to 0.8 μg of DNA, indicating the amount of $SPKR_4NL1$-2 needed for DNA condensation (Figure 2.7B). This result was in agreement with that from the DNA retardation assay.

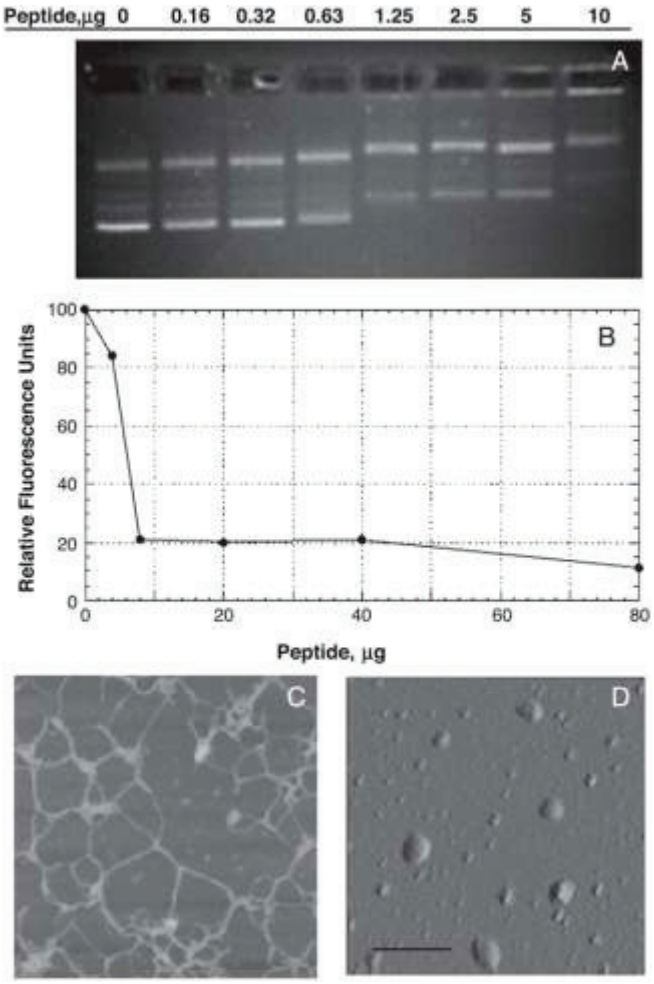



Figure 2.7 $SPKR_4NL1$-2 binds to and condenses plasmid DNA. (A) Electrophoretic mobility of plasmid DNA through a 1% agarose gel was reduced by $SPKR_4NL1$-2 binding. (B) (C and D) Atomic force microscopy images of supercoiled plasmid DNA and $SPKR_4NL1$-2/DNA/PEI600 complexes, respectively.

Atomic force microscopy revealed, however, that $SPKR_4NL1$-2 only partially condenses plasmid DNA at a nitrogen/phosphate (N/P) ratio of 2.5 (result not shown). The N/P ratio is defined as the number of protonatable nitrogen atoms per DNA phosphate group and is a frequently used measure of the charge balance in polycation or peptide/DNA complexes. For the purposes of calculating the N/P ratio for the peptides used in the study, we take the number of basic amino acid residues in the DNA-binding domain to be the number of "N" moieties per peptide molecule, that is, a total of 8 from the 4 lysine and 4 arginine residues in $(SPKR)_4$. With the addition of low-molecular-weight polyethylenimine, PEI600, at an N/P ratio of 10, the loosely looped structure of free plasmid DNA molecules was no longer seen and compact nanoparticles in the size range of 100 to 200 nm were formed (Figure 2.7C and Figure 2.7D).

Encouraged by the results from these DNA binding assays and the observation that $SPKR_4NL1$-2 could activate TrkA even after binding to DNA (Figure 2.5), we went further to test whether

$SPKR_4NL1$-2 could improve gene delivery efficiency using NGF-receptor-positive PC12 cells. Complexes made from a luciferase-encoding reporter plasmid, pCAGluc, and $SPKR_4NL1$-2 alone had relatively weak transfection efficiencies, but the addition of polycationic polymer to fully condense the DNA created an effective gene delivery system. We chose PEI600 and poly-L-lysine (PLL) for their DNA-condensing abilities and low background transfection efficiencies. In 48-well plates, we transfected PC12 cells with triple complexes containing 0.5 μg of pCAGluc, either PEI600 or PLL at an N/P ratio of 10, and various amounts of $SPKR_4NL1$-2. To prepare the complexes, we added the peptide to the DNA in OptiMEM medium and incubated them for 30 min at room temperature, after which we added the polycation and incubated the mixture for a further 30 min before use. We also added chloroquine, an endosomolytic agent, with the PLL-containing complexes. Figure 2.8A shows that $SPKR_4NL1$-2 enhanced PEI600-mediated gene transfer in a dose-dependent manner. Transgene expression using the highest amount of $SPKR_4NL1$-2 tested was 5600-fold higher than that achieved with PEI600 alone. Similarly, with PLL-mediated gene delivery, transfection efficiency with the optimal amount of $SPKR_4NL1$-2 was 1000 times higher than without $SPKR_4NL1$-2 (Figure 2.8B).

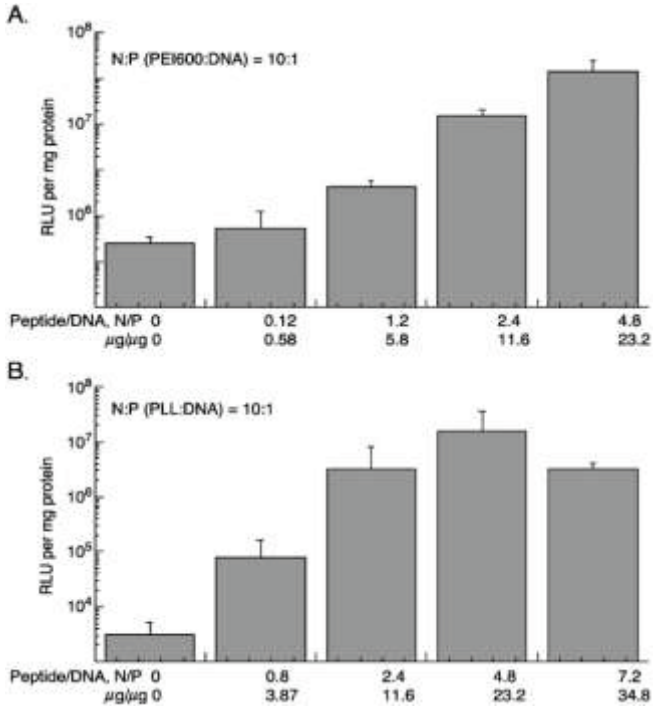



Figure 2.8 $SPKR_4NL1$-2 enhances polycation mediated gene transfection of PC12 cells. (A) PEI600 at an N/P ratio of 10. (B) Poly-L-lysine (PLL) at an N/P ratio of 10.

### 2.2.4 Specificity of $SPKR_4NL1$-2-Mediated Gene Delivery

To examine the specificity of the $SPKR_4NL1$-2 peptide in mediating gene delivery, we first compared the transfection efficiencies of the peptide and its control, $(SPKR)_4$, in PC12 cells

using a reporter gene encoding an enhanced green fluorescent protein (EGFP). When we applied $(SPKR)_4$/DNA/PEI600 triplexes, only a few EGFP-positive cells could be seen under a fluorescence microscope (result not shown). The number of EGFP-positive cells increased dramatically when we used $SPKR_4NL1$-2/DNA/PEI600 triplexes, with approximately 30% of the PC12 cells exhibiting bright green fluorescence (result not shown). The result indicates that the targeting domain designed to recognize NGF receptors was essential to the enhanced gene expression mediated by $SPKR_4NL1$-2. To demonstrate further that $SPKR_4NL1$-2-mediated gene delivery depends on the peptide binding to NGF receptors, we studied the effect of adding exogenous NGF (200 ng/ml) during transfection with $SPKR_4NL1$-2/pCAGluc/PEI600 complexes in PC12 cells. Figure 2.9 shows that the presence of NGF reduced the efficiency of intracellular accumulation of labeled plasmid DNA and the luciferase gene expression by 82 and 97%, respectively. When $SPKR_4NL1$-2 was replaced with $(SPKR)_4$, the transfection efficiency was very low and NGF treatment had no significant effects. The action of NGF as a competitive inhibitor of $SPKR_4NL1$-2/pCAGluc/PEI600 complexes indicates that $SPKR_4NL1$-2-mediated gene delivery was targeted to NGF receptors.

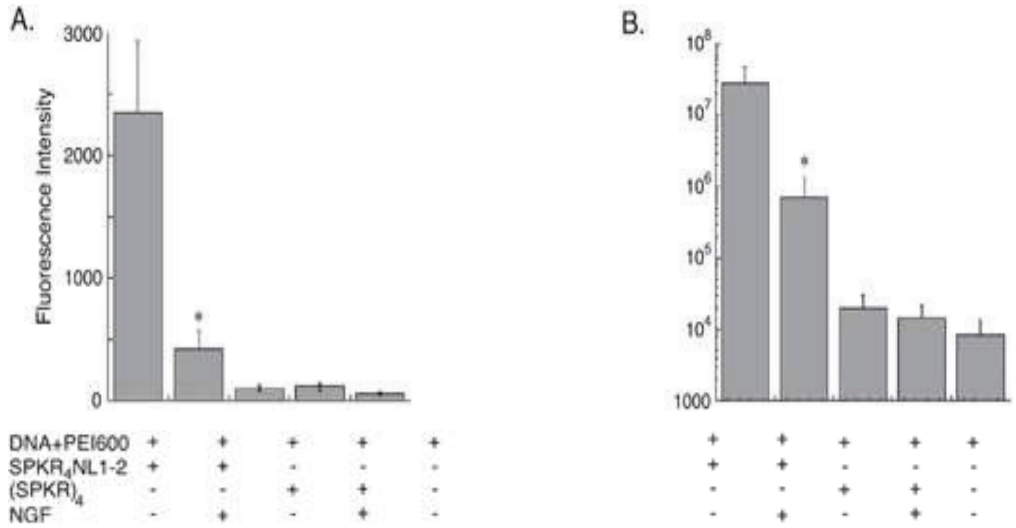



Figure 2.9 NGF inhibits (A) DNA accumulation and (B) gene expression mediated by $SPKR_4NL1$-2 in PC12 cells.

We subsequently tested cell-type specificity of $SPKR_4NL1$-2-enhanced PEI600-mediated gene delivery on primary cultures of NGF-receptor-expressing cortical neurons and NGF-receptor-poor glial cells isolated from 20-day-old embryonic rats. We first assayed cells for the expression of their high-affinity NGF receptor TrkA by flow cytometric analysis using an antibody against TrkA. Approximately 48% of rat primary cortical neurons were TrkA positive, while almost no primary cortical glial cells were positively stained (Figure 2.10A). We then transfected cortical neurons and glial cells in 48-well plates with $SPKR_4NL1$-2/pCAGluc/PEI600 or $SPKR_4$/pCAGluc/PEI600 complexes containing 0.25 μg/well pCAGluc, peptide at an N/P ratio of 2.5, and PEI600 at an N/P ratio of 5. Figure 2.10B shows that gene delivery by PEI600 alone was

negligible in both neuronal and glial cells. When the control peptide, $(SPKR)_4$, was added to the complexes, the additional DNA-condensing ability enhanced transgene expression in both types of cells, although the impact was higher in glia than in neurons. Triple complexes containing $SPKR_4NL1$-2 were nine times more effective in neurons than $(SPKR)_4$ triple complexes, whereas in glial cells, there was essentially no difference in performance between $SPKR_4NL1$-2 and $(SPKR)_4$.

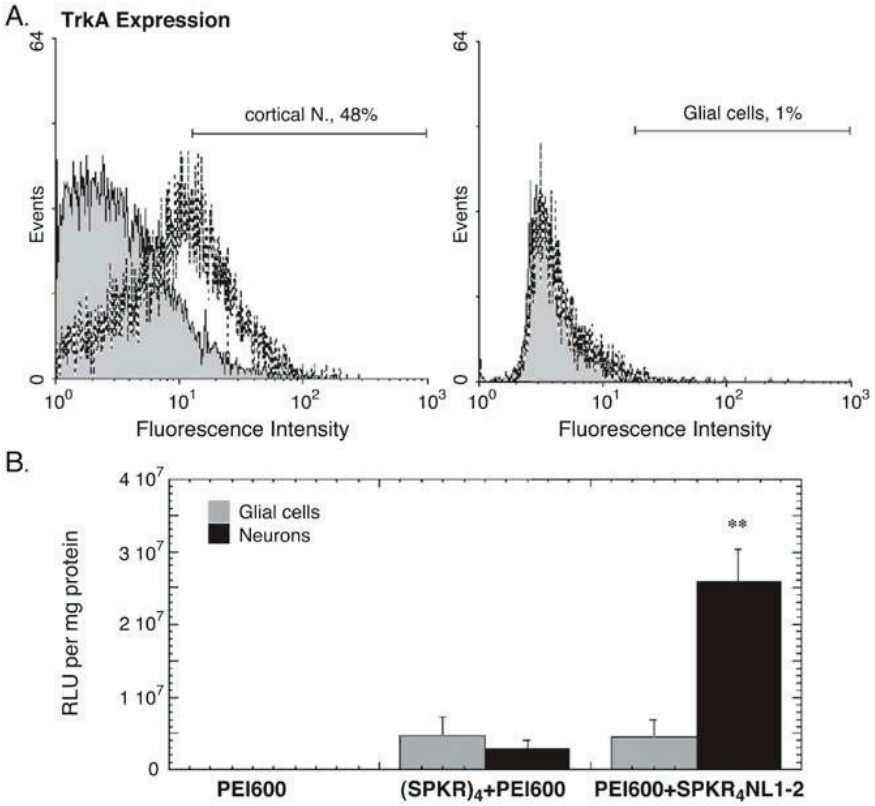



Figure 2.10 $SPKR_4NL1$-2 mediates gene delivery to primary neurons and glial cells. (A) Flow cytometric analysis of the percentage of cells expressing TrkA receptors. (B) Luciferase gene expression.

We compared $SPKR_4NL1$-2 with commonly used nonviral gene carriers PEI25kDa and Lipofectamine2000 in terms of gene delivery efficiency in primary cortical neurons and glial cells (Figure 2.11). With optimal carrier/DNA ratios, Lipofectamine2000 gave the highest transfection efficiency in both types of cells among three tested gene carriers. Although $SPKR_4NL1$-2 was less efficient in glial cells compared with PEI25kDa, it mediated the transgene expression in neurons comparable to that of PEI25kDa. Notably, $SPKR_4NL1$-2 was the only one among the three carriers giving a much higher level of gene expression in neurons compared to glial cells (Figure 2.11A). The level in neurons mediated by this peptide was 587% of that in glial cells (Figure 2.11B). The corresponding percentages produced by Lipofectamine2000 and PEI25kDa were 23 and 20%, respectively (Figure 2.11B). The difference suggested that $SPKR_4NL1$-2 preferentially functioned in neurons over glial cells.

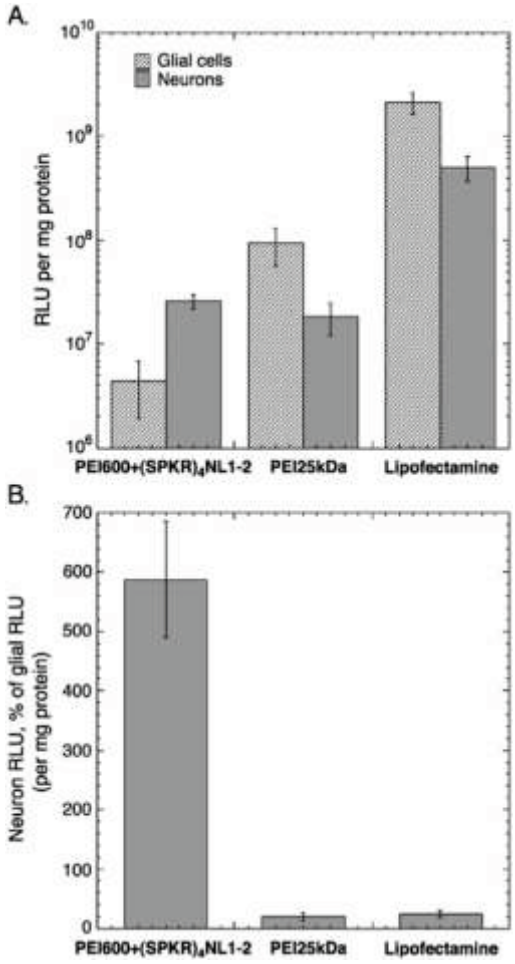



Figure 2.11 Comparison of various gene carriers for transfer efficiency in primary neurons and glial cells. Luciferase activities were measured 24 h later and are shown in RLU/mg protein in (A) and the percentage of neuronal readings from glial RLU in (B).

### 2.2.5 $SPKR_4NL1$-2-Mediated *in Vivo* Gene Delivery to Dorsal Root Ganglia (DRG)

To evaluate the efficacy of $SPKR_4NL1$-2-mediated transfection *in vivo*, we injected gene delivery complexes intrathecally into the lumbar regions of the rat spinal cord. The lumbar spinal cord is in general an NGF-receptor-poor region. A large portion of dorsal root ganglion (DRG) neurons, however, expresses TrkA receptors [118]. As DRGs lie at the distal end of the dorsal root of the spinal cord inside the apex of the dural sleeve [119], intrathecally applied peptide/DNA complexes in the cerebrospinal fluid would be able to penetrate into the DRG. We injected complexes containing 4 μg of pCAGluc per rat. We dissected the lumbar spinal cord and the lumbar DRG 3 days later and analyzed them for luciferase activity. While luciferase activity was barely detectable in the spinal cord, DRG transgene expression produced by $SPKR_4NL1$-2/pCAGluc/PEI600 complexes was 9 to 14 times that mediated by the pCAGluc/PEI600, $(SPKR)_4$/pCAGluc/PEI600, or pCAGluc/PEI25kDa controls (Figure 2.12A).

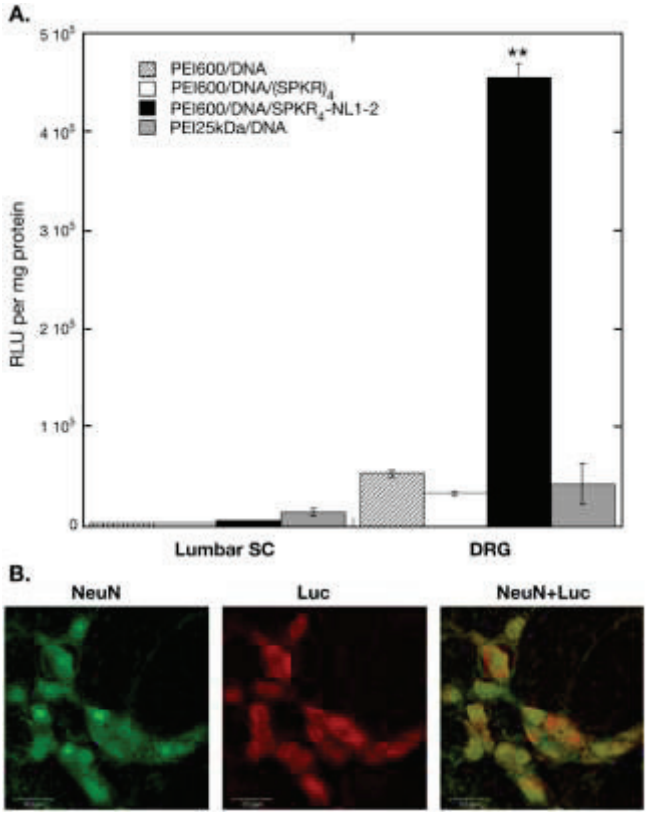



Figure 2.12 $SPKR_4NL1$-2 mediates *in vivo* gene delivery to dorsal root ganglia (DRG) 3 days after injection. (A) Luciferase gene expression. Results are expressed in RLU/mg protein. $^{**}P < 0.01$ compared to the rats treated with DNA complexed with $(SPKR)_4$/PEI600. (B) Confocal images of luciferase expression in neurons in DRG.

To evaluate *in vivo* cell-type specificity of $SPKR_4NL1$-2-mediated transfection, we carried out immunohistochemical double labeling of DRG sections using antibodies against luciferase to visualize transfected cells and antibodies against the neuron-specific nuclear protein (NeuN) to visualize neurons. The results demonstrated that almost all of the transfected cells in DRG were neurons (Figure 2.12B).

### 2.2.6 Discussion

To develop a gene delivery system capable of targeting specific subtypes of neurons in complex organs like the brain and the spinal cord, the present study has designed, developed, and tested a chimeric peptide, $SPKR_4NL1$-2, that can bind to DNA, direct DNA complexes to cells equipped with NGF receptors, and mediate intracellular gene delivery. Several lines of evidence implicate the involvement of NGF receptors in gene delivery mediated by this peptide. First, it possessed NGF-like bioactivity in NGF-receptor-positive PC12 cells, even after binding to DNA, and its activity in signal transduction was blocked by the TrkA inhibitor K252a. Second, the peptide dose-dependently mediated gene delivery to cells expressing NGF receptors, but had no enhancement effect on the cells lacking these receptors. Third, only chimeric polypeptides

containing both a receptor-targeting domain and a DNA-binding domain were functioning in mediating gene expression; the targeting domain contains NGF loops 1 and 2, two domains known to be important in interacting with TrkA and $p75^{NTR}$. Fourth, gene transfer to NGF-receptor-expressing cells was inhibited by co-incubation with an excess amount of NGF. Fifth, the gene delivery vector was able to target a region expressing NGF receptors (the DRG) even in a complex *in vivo* environment, a feature making it promising for clinical application.

Small peptide mimetics of NGF using sequences from its hairpin loop regions have been developed to activate NGF-receptor-related signal transduction and promote NGF-like neurotrophic effects [110][113][114][115][116][120]. These peptide mimetics need to be structural analogs, not just sequence analogs, of NGF loops to be biochemically and biologically active, and hence, cyclization is a common feature. Differing from the short, cyclized loops used in the previous studies, an NGF hairpin motif, a supersecondary structural element built up from two adjacent βstrands that are connected by a loop region, was utilized in the present study. The A and B strands of NGF are connected together at one end by NGF loops 1 and 2. These elements of the hairpin motif, comprising amino acids 17–67 of NGF, have been used as the targeting ligand of our chimeric peptide-based gene delivery vector. By using a hairpin motif instead of a short, artificially cyclized loop, we expect the native loop conformation to be well stabilized by hydrogen-bonding between βstrands, while the flexibility required for induced-fit ligand recognition by NGF receptors will be better conserved.

NGF, like other members of the neurotrophin family, is a noncovalently bound homodimer that stimulates dimerization of its receptors, thus triggering a signal cascade and inducing receptor-mediated endocytosis [121-123]. In designing short peptide mimetics of neurotrophins, much work has been put into emulating this effect using cyclic dimers or bicyclic compounds [110] [113-116]. In contrast, the peptide $SPKR_4NL1$-2 used in this study is monomeric and monovalent but exhibits detectable NGF-like bioactivity. During its design, we anticipated that after the polypeptide was combined with DNA, nanoparticle formation would result in many monovalent ligands being tethered close together on the particle surface, such that neighboring pairs of ligands would be able to facilitate receptor dimerization. Targeted gene delivery in the CNS is currently achieved simply through direct stereotactic injection of naked DNA or gene vectors into well-defined anatomical locations, transducing various types of cells around the injection site. Retrograde axonal transport of viral or nonviral gene vectors offers another way to target neurons in different regions by choosing an appropriate injection site either in the periphery or in more accessible regions of the CNS. This method, however, transduces all projection neurons and does not distinguish between subtypes of neurons. When used in combination with the above injection procedures, the NGF-receptor-targeting peptide reported here might be able to direct gene vectors

specifically to selected neurons in a particular region. This approach can potentially be used advantageously in gene therapy to treat disorders in which neurons expressing NGF receptors are affected. In Alzheimer's disease, the neurons with the most prominent pathological changes are basal forebrain cholinergic neurons, which express TrkA [124][125]. A gene delivery system that targets TrkA may therefore help to transfer therapeutic genes into the neurons to augment cholinergic functions.

# Chapter 3 Stem Cell for Cardiac Repair

## *3.1 Human cord blood cells induce angiogenesis following myocardial infarction (Publication C)*

### 3.1.1 Introduction

Umbilical cord blood contains circulating stem/progenitor cells with cellular characteristics that are quite distinct from those of bone marrow and adult peripheral blood [126][127]. The frequency of hematopoietic stem cells and progenitor cells equals or exceeds that of marrow and greatly surpasses that of adult peripheral blood [128]. Stem cells from cord blood expand longer in culture, produce larger hematopoietic clones in vitro, and have longer telomeres [129][130]. Cord blood also contains mesenchymal progenitor cells capable of differentiating into marrow stroma, bone, and muscle, and the immaturity of neonatal cells compared with adult cells may translate into greater cell plasticity [131]. In addition, cord blood cells are usually not infected with cytomegalovirus or Epstein–Barr virus, as is often the case with marrow cells. For all those reasons, cord blood is considered as a valuable source of cells with a potential for tissue repair in response to injury [132]. However, the homing and migration characteristics of cord blood cells as well as their capacity for transdifferentiation into various target organ cells remain largely unexamined [133]. We therefore studied the organ-specific homing pattern of human mononuclear cord blood (hUCB) cells in a NOD/*scid* (non-obese diabetic severe combined immunodeficiency) -mouse model and tested the hypothesis that hUCB cells specifically migrate to the heart after myocardial infarction(MI), participate in tissue remodelling, and possibly facilitate regeneration processes. Moreover, we investigated whether SDF-1, which has been implicated in stem cell homing processes [134][135], is involved in selective cell migration to ischemic myocardium.

### 3.1.2 Cord Blood Cell Characteristics

Thirty cord blood samples were used for mononuclear cell separation. The mean collection volume was 44.6 ± 4.1 ml, containing between 99 x $10^6$ and 294 x $10^6$ cells by manual counting after Ficoll centrifugation and washing (mean 208 x $10^6$ ± 58 x $10^6$ cells). Cell viability ranged between 42% and 98% (mean value=78 ± 18%). A representative FACS analysis is depicted in Figure 3.1, showing the forward versus sideward scatter plot (A) and the proportion of $CD14^+/CD45^+$ cells. $CD45^+$ cells are gated (C) and the percentage of $CD34^+/CD133^+$ cells among the $CD45^+$ leukocytes is shown in (D). Overall, the percentage of $CD45^+$ cells ranged between 43.8% and 91.1%. Percentages of $CD34^+$ cells (including $CD34^+/CD133^+$ and $CD34^+/CD133^{neg}$

cells) ranged between 0.11% and 1.1% (0.63 ± 0.5%). The average percentage of $CD14^+$ monocytes was 14.9 ± 0.3%.

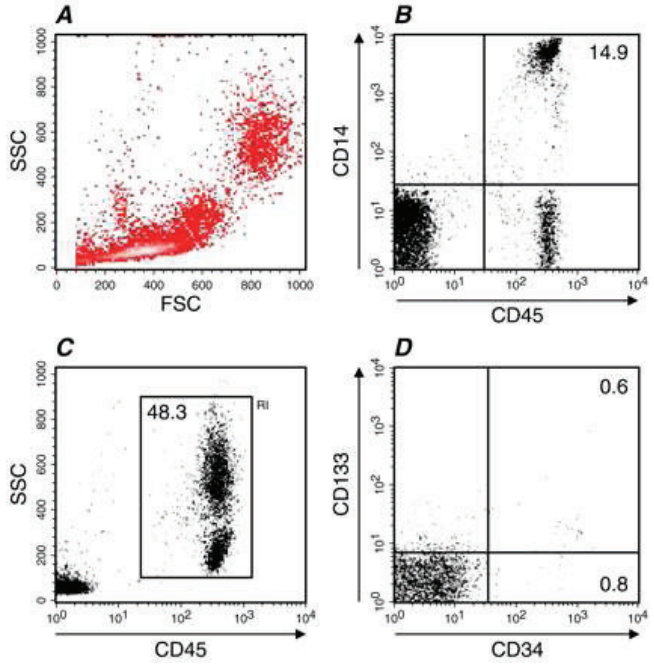



Figure 3.1 Representative FACS analysis of mononuclear cells obtained from human cord blood, showing the forward versus sideward scatter plot (A) and $CD45^+$ cell gating (C). The proportion of $CD14^+/CD45^+$ cells is shown in (B), and the percentage of $CD34^+/CD133^+$ cells among the $CD45^+$ leukocytes is depicted in (D). In this case, 48.3% of the MNCs were $CD45^+$ (C), 0.8% was $CD34^+$, and 0.6% were $CD34^+/CD133^+$ cells (D), and 14.9 of the $CD45^+$ cells were $CD14^+$ monocytes (B). Overall, the percentage of $CD34^+$ hematopoietic stem cells ranged between 0.11% and 1.1%.

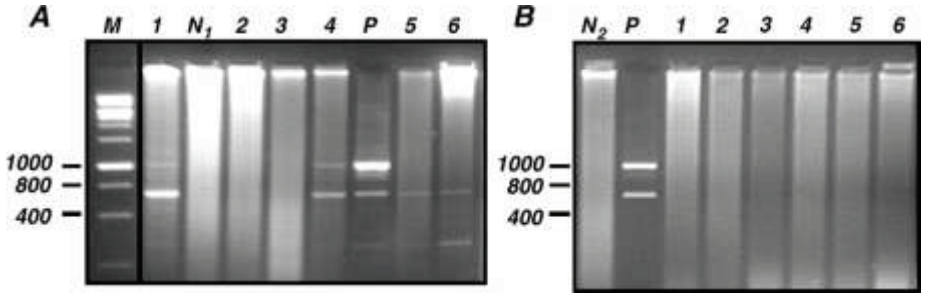



Figure 3.2 Human cord blood cell trafficking to the infarcted myocardium was detected by PCR using human-specific primers D7A and D7B of -satellite of chromosome 7 Locus D7Z. (A) Shows that 4 out of 6 MI+ mouse hearts were $hDNA^+$ 24 h after intravenous injection of human cord blood MNCs. As shown in panel (B), PCR for hDNA was negative in all hearts of MI– mice that had also undergone cell injection. Numbers (1–6) indicate different animals. M, molecular weight marker; $N_1$, negative control (animal with myocardial infarction and without cell injection); $N_2$, negative control (animal without myocardial infarction and without cell injection); P, positive control (human DNA used as template).

### 3.1.3 Detection of hDNA

Following intravenous implantation of human cord blood mononuclear cells, human DNA was detected in bone marrow, liver, and spleen of all animals (MI+ and MI–) at all time points. There was, however, a striking difference regarding hUCB cell engraftment in the heart (Figure 3.2). In 10 out of a total of 19 MI+ mice PCR confirmed the presence of hDNA, while no hDNA was detected in any of the MI– hearts ($P$=0.002). (24 h, 4 out of 6 MI+ mice; 1 week, 3 out of 6 MI+ mice; 3 weeks, 3 out of 7 MI+ mice).

### 3.1.4 Myocardial infarction

Eight animals (25%) died preoperatively prior to hUCB cell injection and were excluded from further analysis. As determined by light microscopy following H&E and Picrosirus Red staining, left anterior descending coronary artery (LAD) ligation consistently resulted in transmural myocardial infarction, exhibiting typical histological changes including thinning of left ventricular free wall and extensive collagen deposition 3 weeks after myocardial infarction. The average infarct size was 47 ± 7% in untreated MI+ mice and 39 ± 3% in the cell-treated MI+ mice, and this difference proved statistically significant ($P$=0.016;). Figure 3.3 depicts representative left ventricular sections 3 weeks following LAD ligation with (Figure 3.3A) or without (Figure 3.3B) cord blood cell injection. Within the group of cell-treated MI+ mice, infarct size was compared between hearts that were positive for hDNA by PCR, and those that were PCR-negative. Indeed, we found that infarct size was significantly smaller in MI+/cell-treated/PCR+ hearts than in MI+/cell-treated/PCR– hearts. Overall, loss or gain of ventricular tissue was also assessed by determining the ratio of heart weight (HW) to body weight (BW). The body weight did not significantly differ between groups. In MI+ mice with cord blood cell injection, the HW/BW ratio was slightly lower than in MI+ mice without cell injection, perhaps indicating less compensatory myocardial hypertrophy in cell-treated mice. As determined by computerized color analysis of the Sirius red stained sections, collagen content was lower MI+ hearts of hUCB-treated mice than in MI+ hearts of mice without cell treatment ($P$=0.02).

### 3.1.5 Morphology of hUCB-derived cells

In hDNA-positive hearts we also traced hUCB-derived cells by immunostaining with anti-human nuclear antigen (HNA) and anti- human leukocyte antigen (HLA) class I antibodies. Human cord blood-derived cells were indentified in the heart of a MI+ mouse sacrificed 3 weeks after cell transplantation. $HNA^+$ and $HLA^+$ cells were found widely distributed in the myocardium. Typically, isolated cells and sometimes clusters of more than 10 transplanted cells were found in the subepicardial and subendocardial infarct tissue. In 100 random fields per section, the proportion of $HNA^+$ and $HLA^+$ cells was approximately 0.01–0.1% of the native mouse cells. When examined at higher magnification, $HNA^+$ and HLA-$I^+$ cells were typically localized in the immediate vicinity of myocardial blood vessels. Double-immunofluorescent staining with anti-HLA class I and anti-CD31 antibodies revealed that at least that some of the hUCB cells appeared to display the phenotype of endothelial cells. Occasionally, small blood vessels of capillary morphology presented a human–mouse micro-chimerism (Figure 3.4), consisting of both HLA-$I^+$ and HLA-$I^{neg}$ endothelial ($CD31^+$) cells.

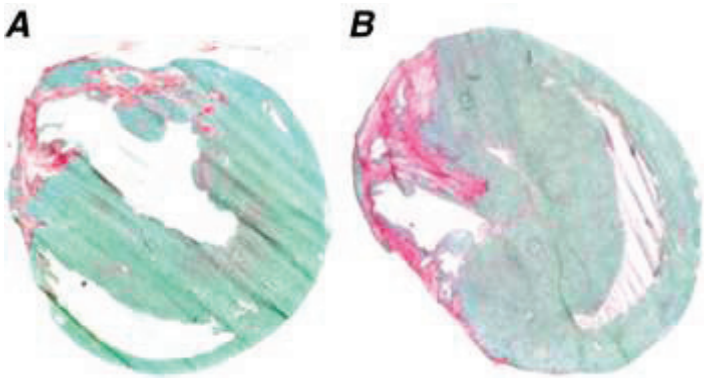



Figure 3.3 Picrosirus red staining of transverse sections through mouse hearts 3 weeks following ligation of the LAD coronary artery. Thinning of the left ventricular free wall and extensive collagen deposition in scar tissue (red) is noted. (A) Mouse heart following LAD ligation and cord blood cell injection, and (B) without cell injection.

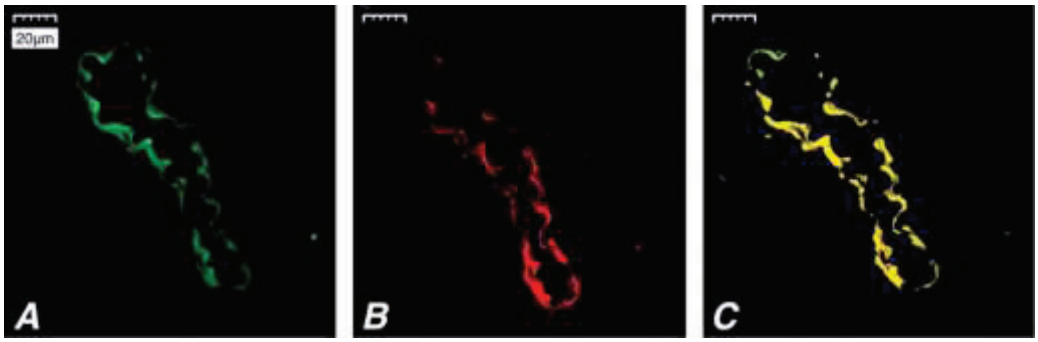



Figure 3.4 Immunfluorescent visualization of a capillary in the infarct border zone. (A) Anti-CD31 staining (green fluorescence). (B) Anti-HLA class I staining (red fluorescence). (C) Both images merged give the impression of a human (yellow) and mouse (green) vascular chimerism (original magnification 400X).

### 3.1.6 Capillary Density

Capillary density in the infarct border zone as well as in myocardium remote from the infarct was determined following immunostaining with anti-mouse Factor VIII antibody. Representative images are shown in Figure 3.5. Infarcted myocardium of mice without hUCB cell transplantation contained few capillaries, whereas capillary density in MI+ mice that had undergone hUCB cell transplantation was approximately 20% higher (Figure 3.4). In the cell transplanted group, the average vascular density within the infarct border zone was 6.19 ± 0.2 vessels per high power field (HPF; 400 ×), while in control hearts (MI+, no cell treatment) there were 5.33 ± 0.5 vessels/HPF ($p$=0.033). In myocardium remote from the infarct area, capillary density was 5.41 ± 0.49 per HPF in MI+ mice with hUCB cell transplantation, which was again significantly higher than in MI+ mice without cell injection (4.3 ± 0.57/HPF, $p$=0.013). Most of the vessels exhibiting Factor VIII-positive endothelium were capillaries with an internal diameter 20 µm. The size of the capillaries in the infarct area ranged between 5 µm and 20 µm, with a greater variation in diameter than observed in normal myocardium (10 µm to 20 µm). We also compared peri-infarct capillary density in MI+ mice with cord blood cell injection that were PCR positive with those that were negative for hDNA. Capillary density was indeed higher in PCR-pos cell-treated MI+ mice than in PCR-neg cell-treated MI+ mice (6.6 ± 0.2 vs. 5.8 ± 0.5 vessels/HPF, $P$=0.028).

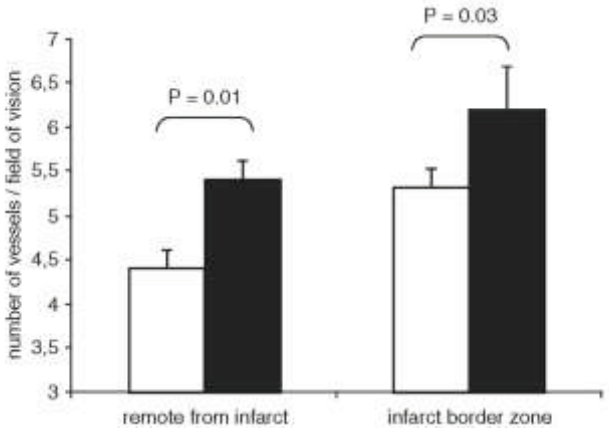



Figure 3.5 Quantitative capillary density data based on Factor VIII immunostaining. Capillaries were counted in the infarct border zone and in myocardium remote from the infarct by an investigator who was blinded with respect to the group allocation. In either situation, capillary density was significantly higher in cell-treated animals (data are mean ± S.D.).

### 3.1.7 SDF-1 Expression

To test the hypothesis that upregulation of SDF-1 occurs in infarcted myocardium and hence may contribute to hUCB-derived cell migration in ischemic hearts, we compared the expression level of SDF-1 mRNA in hearts 24 h after myocardial infarction with that in untreated control hearts. Figure 3.6 shows that the level of the SDF-1 mRNA in myocardium is approximately 7-fold higher after myocardial infarction ($p$<0.0001).

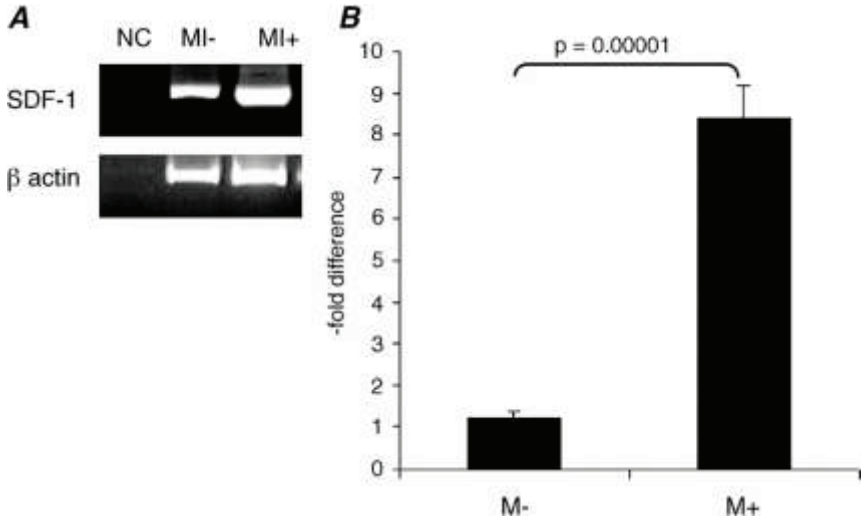



Figure 3.6 Expression of SDF-1 in murine myocardium 24 h after myocardial infarction (MI+), and in myocardium of sham-operated animals (MI–). SDF-1 mRNA expression was evaluated by real-time RT-PCR (B) and RT-PCR was performed to confirm the correct size of the amplification product and the absence of non-specific bands (A). Data represent mean ± standard deviation of 6 experiments.

### 3.1.8 Discussion

This study demonstrates that intravenously administered human umbilical cord blood cells migrate to and engraft in the heart of (NOD)/*scid* mice only following myocardial infarction. While there was no evidence of cardiomyocyte formation, hUCB cells appear to be involved in angiogenic processes. Following cell administration, capillary density in hearts with myocardial

infarction is moderately increased, but few hUCB-derived cells actually incorporate in neo-vessels, whereas most of the human cells are located in the perivascular interstitium. Cord blood cell injection also appeared to beneficially influence infarct size and collagen deposition, provided there is effective homing of hUCB cells to the ischemic heart. Since myocardial infarction also led to a marked upregulation of SDF-1 expression in the heart, we assume that SDF-1 is at least partly responsible for the selective hUCB cell trafficking to ischemic hearts, although the study design does not allow for proof of a cause–effect relationship.

Cell therapy for cardiac regeneration is currently under intensive investigation. The initial enthusiasm regarding the cardiomyogenic potential of adult stem cells [136] has faded [137][138], but substantial evidence obtained in large animal studies and even clinical pilot trials indicates that hematopoietic cells nevertheless exert beneficial effects in ischemic myocardium [139][140]. Trafficking of bone marrow-derived cells to the heart is a phenomenon that has been demonstrated in various experimental models [141] as well as in human heart transplant patients [142]. The vasculogenic potential of cord blood cells has previously been assessed in several studies. In in vitro experiments using $CD34^+/CD133^+$ human cord blood cells, Pomyje and colleagues demonstrated that those express angiopoietin-1, angiopoietin-2, and vascular endothelial growth factor as well as their receptor mRNAs, supporting a role of these cells in regulation of angiopoiesis [143]. Muohara et al. have described that transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization in the ischemic hindlimb [144], and Pesce et al. observed endothelial and also myogenic differentiation of hUCB-derived stem cells in ischemic limbs [145]. Recently, Le Ricousse-Roussanne et al. reported that ex vivo differentiated endothelial and smooth muscle cells from human cord blood progenitors home to the angiogenic tumor vasculature [146]. In our model, the inoculated hUCB cells appear to be a source of neovascularization of ischemic myocardium, since they differentiated into endothelial cells, formed chimeric capillaries, and presumably participated in post-infarct remodelling, angiogenesis, and maturation of the scar. To what extent these processes will translate into functionally relevant myocardial regeneration as has been described using human marrow-derived cells in athymic rats [139] remains to be determined. At first glance, our observation regarding the formation of a human/mouse vascular micro-chimerism appears to contradict the report by Ziegelhoeffer et al., who demonstrated that bone marrow-derived cells do not incorporate into the adult growing vasculature [147]. However, it should be noted that (i) we used cord blood cells, not bone marrow-derived cells, (ii) we utilized a myocardial infarction model, not a hindlimb ischemia or tumor model as these authors did, and (iii) we found rather few neo-vessels that stained positive for human cell markers, whereas most of the myocardial neovascularization appeared to originate from native mouse endothelial cells, with donor cells mainly located in the perivascular interstitium. Although we found evidence that hUCB-derived cells are able to incorporate in growing vessels in principle, much of the angiogenic host tissue response is most

likely do to local secretion of cytokines and growth factors [147][148]. Since 30–70% of the cord blood cells that we detected had maintained their hematopoietic phenotype, they may very well have been able to modulate the immune/inflammatory response to myocardial infarction.

The mechanisms underlying homing and engraftment of stem cells and progenitor cells in various target organs are not fully understood, but in situ chemokine and cytokine expression probably plays a role. A noted chemokine is stromal cell-derived factor-1, also known as pre-B cell growth stimulating factor. Under normal conditions, bone marrow stromal cells such as bone-forming osteoblasts produce and secrete high levels of SDF-1. SDF-1 and its receptor CXCR-4 are believed to facilitate stem cell adhesion to microvessel endothelium and trans-endothelial migration in the infarct border zone of infarcted myocardium [135]. We assume that after myocardial infarction, the local expression level of SDF-1 is upregulated, increasing the myocardial concentration of SDF-1 and attracting circulating CXCR4 positive cells [134]. However, the molecular mechanism of SDF-1 secretion by ischemic heart cells and its chemoattractant action on stem cells is still under investigation. Cord blood cell trafficking to the ischemic heart is not an all-or-nothing phenomenon, since hDNA was detectable only in 10 out of 19 MI+ hearts. In intact biological systems subjected to complex pathophysiologic stimuli, the molecular and cellular response is usually gradual, and chemokine expression may need to reach a certain threshold to attract relevant numbers of progenitor cell. The variables involved are manifold: Initial infarct size, magnitude of cytokine expression per unit ischemic tissue, or the responsiveness of injected hUCB cells may vary considerably. The necessity of relevant hUCB cell trafficking to the ischemic heart for a protective or regenerative effect on the myocardium is underscored by our finding that infarct size is significantly smaller and capillary density is higher in hearts of cell-treated MI+ mice that were PCR+ for hDNA than in those that were PCR–.

Naturally, our study possesses several methodological limitations. We did not determine the functional relevance of myocardial hUCB cell migration in terms of left ventricular contractility or relaxation properties. Furthermore, the co-localization studies that showed the incorporation of hUCB-derived cells into murine neo-vessels were performed using fluorescence microscopy, which has been implicated in producing false-positive results [138] [147]. Another possible pitfall is the complete absence of hUCB-derived cells in normal control hearts and the presence of hUCB-derived cells in approximately 50% of the ischemic hearts. Would these data rely on microscopy-based methods such as immunohistology or in situ hybridisation only, one could argue about an inherent lack of sensitivity. Therefore, we chose to perform PCR for detection of human DNA in mouse hearts, and used human chromosome specific α-satellite primers to track cells. In preliminary experiments, we amplified human alpha satellite DNA from in vitro somatic cell hybrids containing only a handful of human cells. PCR analysis with centromere-specific primer sets can be performed to characterize somatic cell hybrids more effectively and perhaps

more accurately than by cytogenetic means. To our knowledge, this is the first time that a chromosome specific α-satellite DNA primer has been used to distinguish different genetic backgrounds, and we are confident that PCR-negative murine myocardium does in fact not contain a relevant number of hUCB-derived cells.

## *3.2 Comparation of human CD133+ cells derived from bone marrow and from cord blood in a SCID-mouse model (Publication D)*

The concept of cardiac cell transplantation to improve myocardial function is tremendously attractive, and it has repeatedly been suggested that cells derived from skeletal muscle, bone marrow, blood, or other tissues might have a regenerative capacity in the mammalian heart. Among those, CD34+ or CD133+ progenitor cells are of particular interest, because the corresponding antibodies can be used in clinical enrichment protocols, applying methods for primary cell isolation that comply with standard regulations for clinical use. Nevertheless, few experimental studies have systematically assessed the myocardial regeneration potential of CD133+ cells in vivo. We therefore sought to determine the functional and histologic consequences following direct intramyocardial delivery of human CD133+ cells derived from bone marrow and from cord blood in a SCID-mouse model of left ventricular cryoinjury.

### 3.2.1 Characteristics of CD133+ Cells from Cord Blood and Bone Marrow

The average purity of the CD133-enriched cell products was 91.2±4% (CD133+ cells in percent of total mononuclear cells), with no difference between UCB and BM-derived cells. To test whether the CD133-enriched cell products from UCB and BM had a different composition of subpopulations, cells were counted by flow cytometry following labelling of CD34, CD133, CD45, and CD117. Representative results are shown in Figure 3.7. Cell viability was quantified by propidium iodide staining, and was consistently higher than 90%. There were no differences in the expression of CD45 and CD34. $BM^{CD133}$ expressed the CD133 antigen not as strong as $UCB^{CD133}$ cells. On average, 70% of the $BM^{CD133}$ cells were also positive for CD117, whereas only up to 35% of the $UCB^{CD133}$ cells were CD117+. The frequency of $BM^{CD133+/CD117+}$ cells in every examined $BM^{CD133}$ cell product was markedly higher than that of $UCB^{CD133+/CD117+}$ cells among the $UCB^{CD133}$ cell products (Figure 3.7A and B).

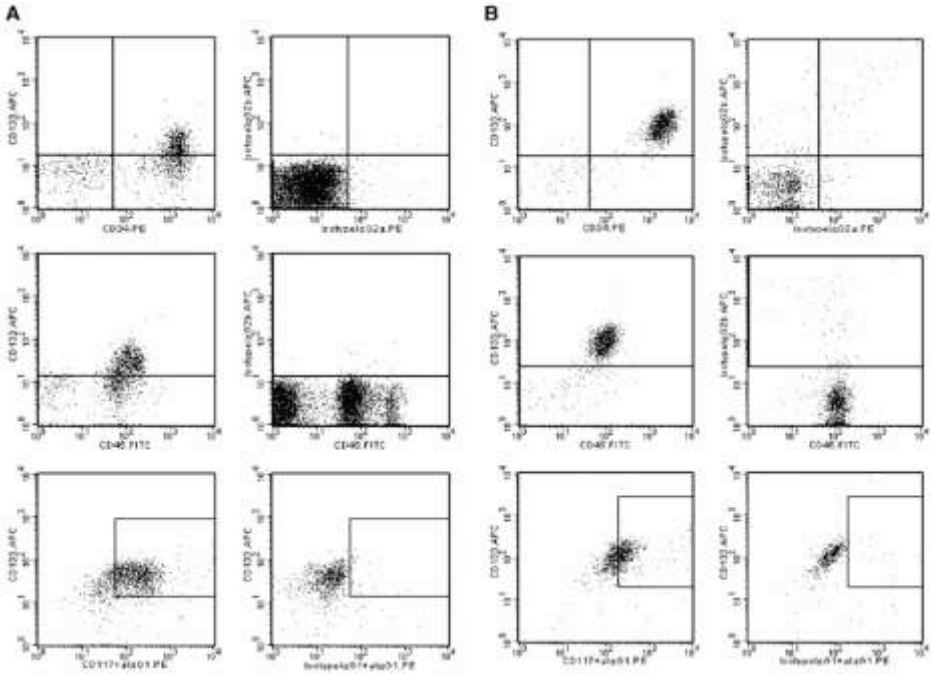



Figure 3.7 (A) Multicolor flow cytometric analysis of CD133+ cells derived from bone marrow. First row, left: CD133-selected cells were found to have a dim expression of CD133 and are all CD34+; right: isotype control. Second row, left: $BM^{CD133}$ cells are also CD45 dim; right: isotype control. Third row, left: 68.9% of all $BM^{CD133}$ cells were found to be positive for CD117; right: isotype control. (B) Multicolor flow cytometric analysis of CD133+ cells derived from cord blood. First row, left: All $UCB^{CD133}$ positive cells were found to be positive for CD34, right: isotype control; Second row, left: All $UCB^{CD133}$ cells also express CD45; right: isotype control. Third row, left: 34,1% of all $UCB^{CD133}$ cells were found to be positive for CD117; right: isotype control.

### 3.2.2 In Vitro Plasticicty Studies

When cultivated on matrigel coated culture dishes and exposed to VEGF, both $BM^{CD133}$ and $UCB^{CD133}$ cells formed capillary network-like structures after 14 days of culture (Figure 3.8A and B). Approximately 75% of the thus cultivated cells expressed endothelial cell-specific von Willebrandt factor (vWF)(Figure 3.8C and D). Moreover, most of those cells were also positive for uptake of Dil-*ac-LDL* (Figure 3.8E-H), further evidence of an endothelial cell-like differentiation commitment. In this respect, no apparent difference in behavior was found between $BM^{CD133}$ and $UCB^{CD133}$ cells. On the other hand, after up to 21 days exposure to myogenic differentiation conditions, neither with or without 5-Azacytidine, no expression of β-myosin heavy chain and no spontaneously beating cell was detected in any of the samples, indicating that differentiation in myocyte-like cells, at least under the given in vitro conditions, did not occur.

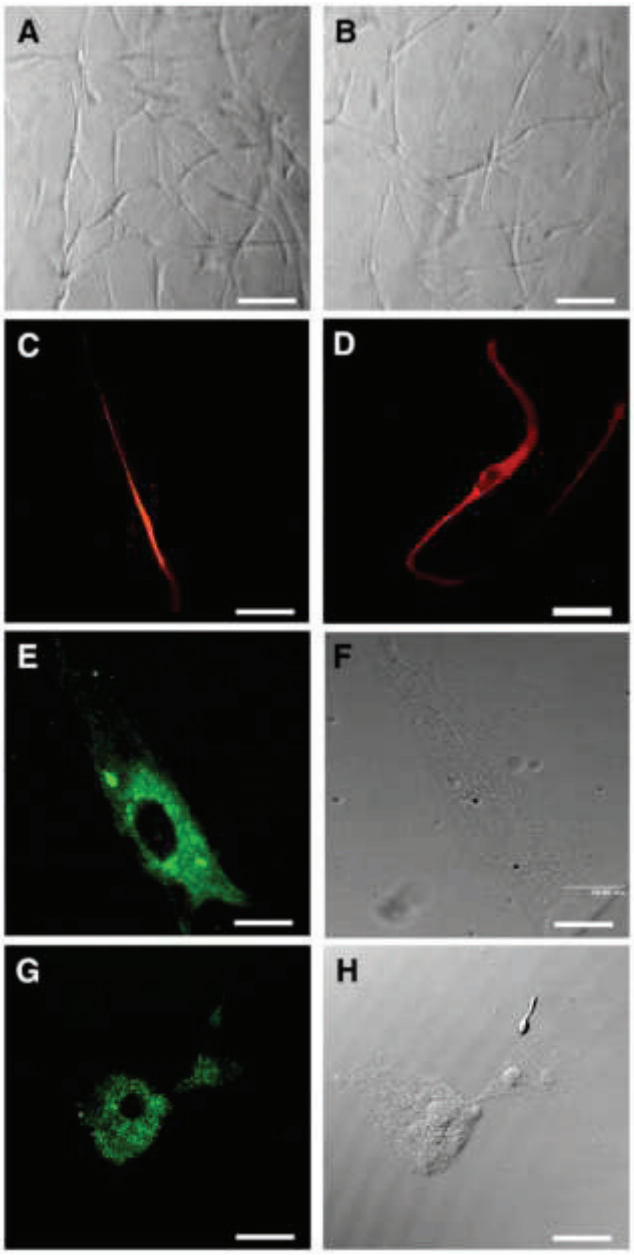



Figure 3.8 In vitro differentiation of CD133 cells towards an endothelial cell phenotype. (A) After cultivation on Matrigel for 2 weeks, $UCB^{CD133}$ cells form capillary-like tubules (scale bars=100 μm). (B) $BM^{CD133}$ cells display the same behaviour. (C, D) Immunostaining of $UCB^{CD133}$ (C) and $BM^{CD133}$ (D) derived endothelial progenitor cells for vWF (red fluorescence, scale bars=50 μm). (E, F) Cellular uptake of acLDL (green fluorescence) by endocytosis in $BM^{CD133}$ derived endothelial cells (F, differential interference contrast image). (G, H) The same observation in $UCB^{CD133}$ derived cells (scale bars=20 μm).

### 3.2.3 Survival

Overall, left ventricular cryoinjury produced an early mortality of approximately 30% within the first 4 h after completion of the surgical procedure, and there was no difference in peri-procedural mortality between control and cell-treated groups (Figure 3.9). In sham operated animals no mortality was observed. During the subsequent phase of recovery from the operation, another third of the mice in the cryoinjury control group died (late mortality=32%). This delayed mortality was abolished by both $BM^{CD133}$ and $UCB^{CD133}$ cell transplantation ($p<0.05$ vs. Control). In the time-related Kaplan–Meier analysis, survival of mice in the cryolesion control group was 43% (70% confidence interval 33–53%) at 28 days, 75% (64–86%) in $UCB^{CD133}$ cell treated mice, and 68% (60–74%) in $BM^{CD133}$ cell treated mice. Mean survival time was computed as 13 days

(70% confidence interval 11–16 days) for control mice, 19 (16–22) days for $BM^{CD133}$ cell treated mice, and 21 (18–24) days for $UCB^{CD133}$ cell treated mice. Post-mortem histological analysis of mice that died during the observation period revealed the typical transmural cryolesion of the left ventricle.

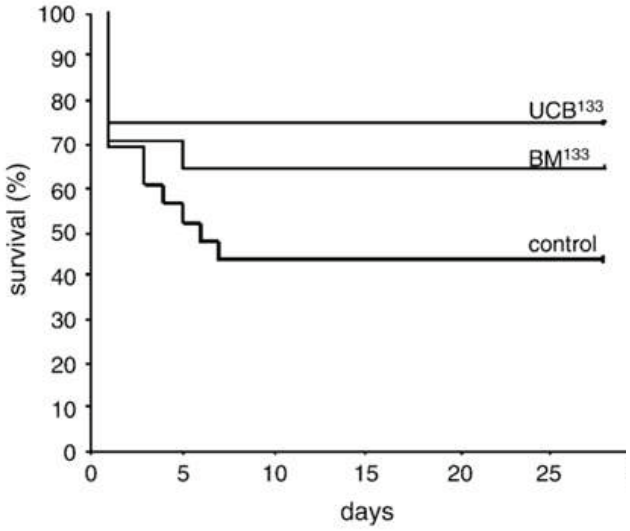



Figure 3.9 Kaplan–Meier estimated survival of mice with 28 days after cryoinjury of the left ventricle. Surgical mortality was similar in all cryoinury groups. Other than that, no mice died late after simultaneous injection of $UCB^{CD133}$ cells, and only one mouse died after delivery of $BM^{CD133}$ cells. In contrast, mice without cell treatment (control) had a marked mortality during the first postoperative week. In the sham operated group (thoracotomy without cryolesion) no animals died peri- or postoperatively (data not shown).

### 3.2.4 Heart function

Left ventricular contractility was measured by transthoracic echocardiography before and 4 weeks after cryoinjury or sham operation. No differences in shortening fraction were found between groups before surgery. At the end of the observation period, LV contractility in the control group was significantly reduced in comparison with sham operated animals (41.3±0.8% vs. 46.2±1%, $p$=0.01) (Figure 3.10). Injection of human $UCB^{CD133}$ cells in the cryolesion had no beneficial effect on shortening fraction (SF=40.8±0.7%, $p$=0.7 vs. control). In contrast, treatment with human $BM^{CD133}$ cells almost completely prevented the decrease of contractility (SF=45.4±0.9%, $p$=0.02 vs. control). In order to evaluate heart function under stress conditions, anaesthetized mice were treated with dobutamine (200 µg/kg, s.c.). Dobutamine produced a similar increase in heart rate in all groups (from 405±8 to 543±8 beats per minute). Contractility also markedly increased in all groups, however, the relative increase was more pronounced in sham operated mice (18.0±1.5%) than in the cryoinjury groups (10.5±1.6%, $p$=0.02 vs. sham). Under dobutamine stress, shortening fraction in $BM^{CD133}$ cell treated mice remained significantly higher than in control mice or $UCB^{CD133}$ treated hearts.

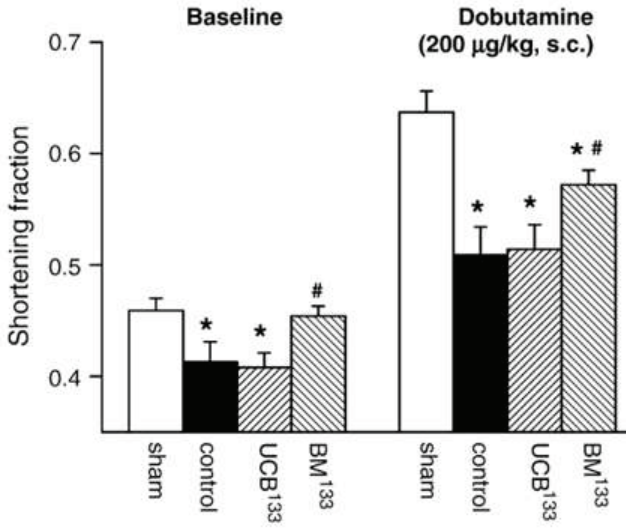



Figure 3.10 Left ventricular shortening fraction as a measure of myocardial contractility measured 28 days postoperatively by transthoracic echocardiography, both at rest ("baseline") and under catecholamine stress provoked by injection of dobutamine ("dobutamine"). Compared with sham-operated animals, cryoinjury led to a significant reduction in shortening fraction ("control"). Injection of $UCB^{CD133}$ cells had no measurable effect on contractility, but $BM^{CD133}$ cells completely prevented or reversed to loss of contractile function at rest, and partially under dobutamine stress. *$p$<0.05 vs. sham group, #$p$<0.05 vs. control group.

### 3.2.5 Heart morphology

Histological analysis demonstrated that cryoinjury consistently produced transmural myocardial necrosis (Figure 3.11). In the injured region profound scar formation and wall thinning was observed after 28 days. The size of scar area did not significantly differ between $BM^{CD133}$ cell-treated, $UCB^{CD133}$ cell-treated, and control animals (Figure 3.12A). There was also a significant increase in heart weight-to-body weight ratio in all cryoinjured mice, again without relevant differences between the treatment groups (Figure 3.12B). The development of LV dilatation and partial compensatory hypertrophy was also mirrored by increased left ventricular end-diastolic dimensions on echocardiography (data not shown). Again, there was no measurable effect of $BM^{CD133}$ or $UCB^{CD133}$ cell transplantation.

### 3.2.6 Identification of transplanted cells

Forty-eight hours after transplantation, human cells could readily be detected by immunostaining using human specific antibodies (hNuc or HLA-1) in both $BM^{CD133}$ and $UCB^{CD133}$ transplanted hearts (Figure 3.13A). There was no evidence of transplanted human $BM^{CD133}$ or $UCB^{CD133}$ cells that co-expressed myosin heavy chain. One month after cell transplantation, neither $BM^{CD133}$ nor $UCB^{CD133}$ derived hNuc+ or HLA-1+ human cells could be visualized in any of the sections studied by immunofluorescence. However, human DNA was clearly present in all cell-treated hearts and was identified by PCR using a human specific primer (Figure 3.13B and C).

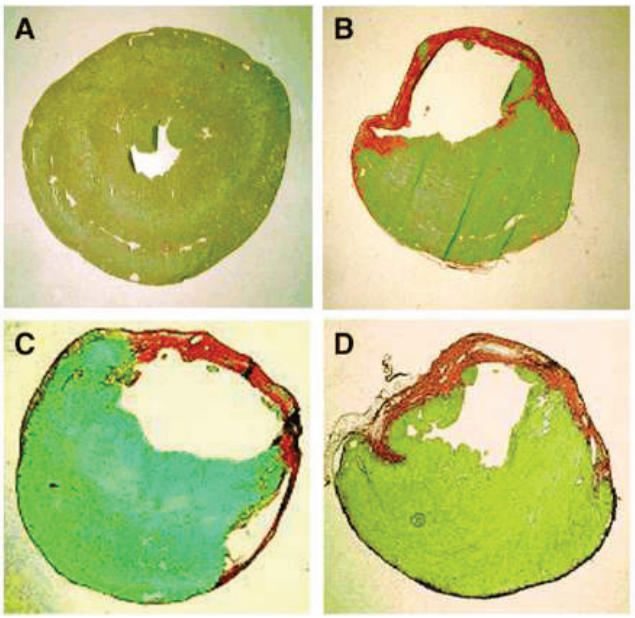



Figure 3.11 (A) Gross morphology following Sirius red staining of a normal left ventricle (transverse section) in the sham operation group, and (B) a heart 4 weeks after cryoinjury (control group) without cell injection. (C) 4 weeks after cryoinjury and injection of $UCB^{CD133}$ cells, and (D) after injection of $BM^{CD133}$ cells.

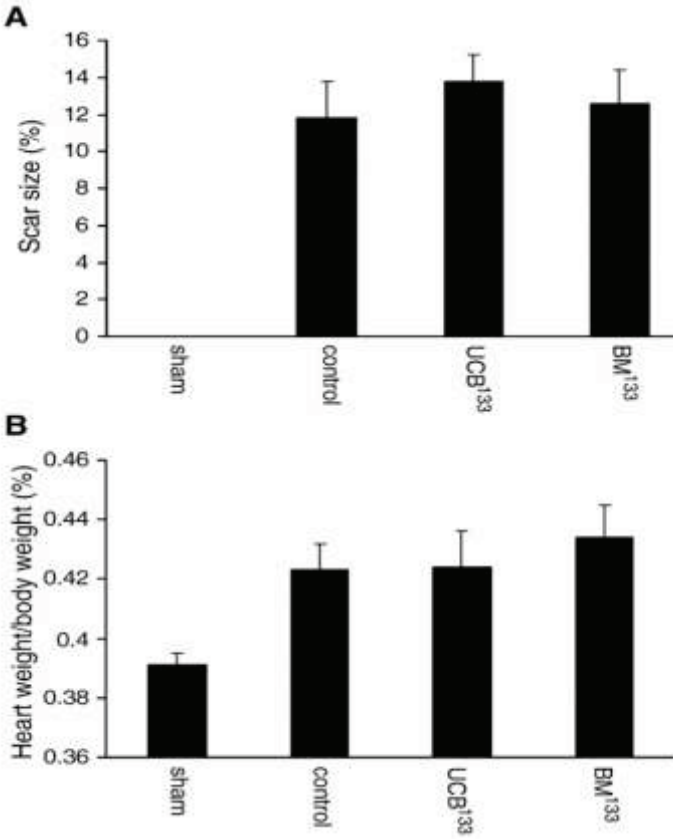



Figure 3.12 (A) Scar size after 4 weeks in hearts of sham operated animals ("sham") and mice that underwent cryoinjury alone ("control") or with injection of $BM^{CD133}$ or $UCB^{CD133}$ cells. Scar size was measured by computerized planimetry on Sirius Red stained sections from mid-level of the lesion. There was no difference between control and cell-treated hearts. (B) Heart weight-to-body weight (HW/BW) ratio in %. Mice with cryoinjury had a higher HW/BW ratio, indicating some degree of compensatory hypertrophy of the remaining viable myocardium. Cell injection had no effect on this parameter.

### 3.2.7 Capillary density

Capillary density in the border zone of the cryolesion was determined based on CD31 immunostainingOverall, capillary density in cell-treated hearts was approximately 25% higher than in control hearts with a cryolesion. (Figure 3.14), whereas there was no significant difference in capillary density between $BM^{CD133}$ and $UCB^{CD133}$ cell-treated hearts (control, 10.7±0.7

vessels/HPF; $BM^{CD133}$, 13.3±2.3 vessels/HPF, $p$=0.04 vs. control; $UCB^{CD133}$, 14.3±2.6 vessels/HPF, $p$=0.005 vs. control, $p$=0.2 vs. $BM^{CD133}$).

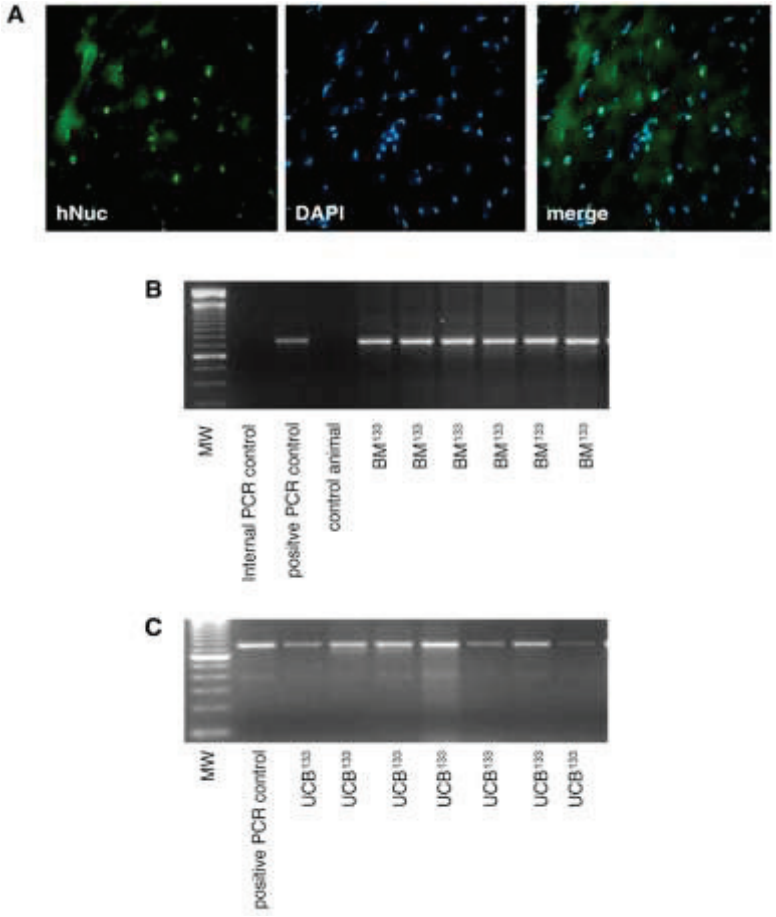



Figure 3.13 (A) Detection of human cells 48 h after cryoinjury and $BM^{CD133}$ cell injection by immunostaining for human nuclear antigen (hNuc) and counterstaining of all nuclei with DAPI (original magnification 200 x). Both $BM^{CD133}$ and $UCB^{CD133}$ cells could readily be detected 48 h after injection. After 4 weeks, however, hNuc staining was negative in all examined sections. (B, C) Detection of human DNA 4 weeks after cryoinury and cell injection by PCR using the human-specific primers D7A and D7B of α-satellite of chromosome 7 Locus D7Z1. All examined hearts that had been treated with $BM^{CD133}$ (B) or $UCB^{CD133}$ (C) cell injection were PCR-positive. MW=molecular weight marker

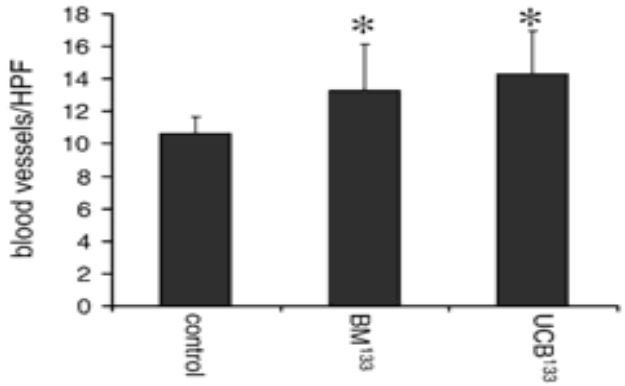



Figure 3.14 Blood vessel density in the border zone of the cryolesion 4 weeks postoperatively.Average blood vessel count per high power field counted in 10 randomly chosen high-power fields (HPFs) in 2 sections per heart and 6 hearts per group. In cell-treated hearts, blood vessel density was approximately 25% higher than in control hearts. *$p$<0.05 vs. control.

### 3.2.8 Tunel assay

Numerous Tunel-positive nuclei were identified in the area of the cryolesion 24 h postoperatively (Figure 3.15). In general, they also showed condensed nuclei as another typical feature of apoptosis. In control animals, the apoptotic nuclei count was approximately 5-fold higher in the center of the cryolesion than in remote normal myocardium, and 12-fold higher in the necrosis border zone. Compared with $BM^{CD133}$ or $UCB^{CD133}$ cell-treated hearts, there was no difference in TUNEL-positive nuclei count in the center of the cryolesion (Figure 3.15B). However, injection of $BM^{CD133}$ cells resulted in a significant reduction of cardiomyocyte apoptosis in the border zone (3.9±1.6% vs. 7.5±1.3% in control, $p$=0.02). In the border zone of $UCB^{CD133}$ cell-treated hearts, apoptosis seemed to be reduced to a similar degree, but the difference did not reach statistical significance (4.3±0.7%, $p$=0.14 vs. control). In the remote normal myocardium as well as in the center of the cryolesion, the percentage of TUNEL-positive cardiomyocytes was not significantly different between the groups.

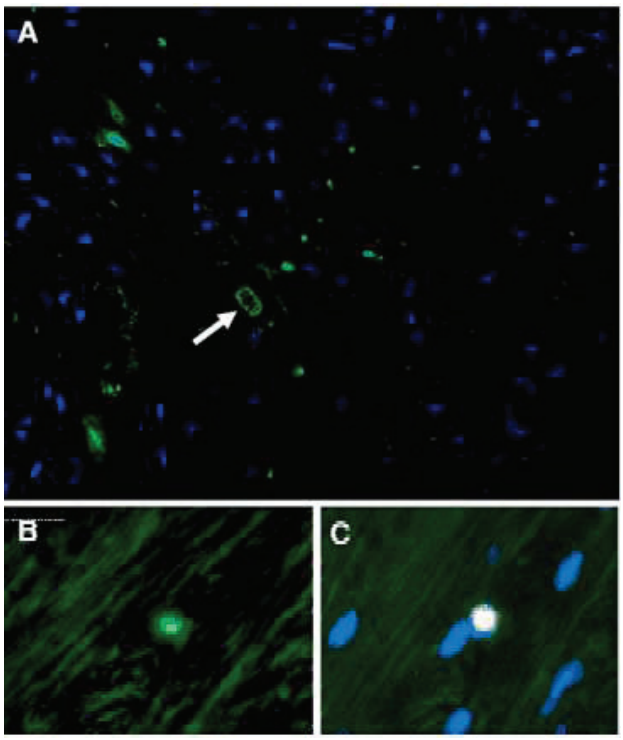



Figure 3.15 Apoptosis in the myocardium following cryoinjury with or without cell injection. (A) TUNEL staining with DAPI counterstaining of all nuclei, depicting several TUNEL+ nuclei and one TUNEL+ capillary (arrow). (B) Detail magnification of a TUNEL+ nucleus embedded between or within myofibrils. (C) The same image merged with DAPI stain. The TUNEL+ nucleus appears to be part of a multinucleated myofiber, however, it can not be clearly distinguished from an interstitial cell.

### 3.2.9 Discussion

Although the notion of cardiomyocyte-differentiation of unmodified adult stem cells appears to be unrealistic at present [149], a variety of experimental studies in small and large animals have indicated that haematopoietic stem cells and/or endothelial progenitor cells can improve heart function upon delivery to diseased myocardium. While basic researchers have identified very promising adult stem cell types utilizing complex ex vivo cell isolation and manipulation strategies [150][151][152], clinicians are currently limited to using clinically available cell products. In this context, the CD133 antigen has attracted interest because it is expressed on the

surface of endothelial progenitor cells and presumably also on cells with a particularly high degree of stemness (i.e. CD133+/CD34 – cells), and because protocols for large-scale isolation by immunomagnetic selection of native cells are readily available. Following the advent of clincial trials using CD133+ bone marrow or blood-derived cells, Agbulut et al. first demonstrated the functional efficacy of human CD133+ marrow cells in an immunodeficient rat model of ischemic myocardial infarction [153]. Similar to their observation, we were not able to detect transplanted $BM^{CD133}$ cells by immunofluorescence several weeks after implantation, although the persistence of their progeny in the heart was evidenced by PCR. The reasons for the disappearance of histologically detectable cells are not clear. The immunodeficient NOD/SCID mouse model has been extensively used in xenogenic cell transplantation studies [154], but some residual immunologic host activity against the xenogenic cell graft cannot be completely excluded. Alternatively, pharmacologic immunosuppression might be used, but this could also interfere with cellular proliferation and differentiation. It is not clear, either, why hDNA can be detected by PCR in the absence of positive immunostating for human cells. Possible, albeit largely speculative, explanations are fusion of transplanted human cells with host cells [155][156], persistence of hDNA following phagocytosis or lysis of graft cells, or simply the different levels of sensitivity of both detection methods.

Although the echocardiographic assessment of LV contractility in mice may be less accurate than intracardiac recording of pressure–volume loops in rats (as was done by Agbulut et al. [153]), our data confirm the functional benefit derived from $BM^{CD133}$ cells. The possible reasons for this increase in contractile function are manifold, but, admittedly, the results of our experiments are not sufficiently conclusive to point out one specific mechanism-of-action. Along with many other groups, we did not find convincing evidence of cardiomyocyte-like differentiation of $BM^{CD133}$ cells, but our in vitro experimental approach was certainly limited. It should not be ruled out that some myocyte differentiation potential of human hematopoietic/endothelial progenitor cells does exist, provided there is direct contact with host cardiomyocytes, as has been indicated in several in vitro co-culture or in vivo studies [157]. Nevertheless, we did detect a significant impact on vascularization of the injured myocardium [158], and found that the presence of $BM^{CD133}$ cells appears to reduce the number of apoptotic cells in the infarct border zone, although this phenomenon did not result in a measurable difference in infarct size. In fact, we did not expect to see a relevant impact on the size of the necrosis area, since the cryolesion instantly produces an irreversible transmural necrosis that could only be expected to decrease in size when substantial neo-myogenesis would occur. The most solid albeit least specific line of evidence, however, is the beneficial impact on post-injury mortality. Whatever the mechanism may be, some benefit of CD133+ cells in the heart does exist.

In the typical clinical target population, older patients with advanced ischemic heart disease, the regenerative capacity of autologous adult stem cells is probably reduced [159], and cord blood-derived cells have been suggested as an attractive alternative with enhanced angiogenic and hematopoietic potential [160-164]. In a previous study, we have shown that human cord blood mononuclear cells injected intravenously migrate to the ischemic mouse heart and facilitate angiogenesis processes [165]. Similarly, Leor et al. described beneficial functional effects of CD133+ cord blood cells after intravenous infusion in athymic rats with myocardial infarction [166], and Yang et al. reported on the angiogenic capacity of expanded $UCB^{CD133}$ in ischemic hind limbs [167]. We therefore decided to directly compare the efficacy of $BM^{CD133}$ and $UCB^{CD133}$ cells in the present study, and found a similar effect on post-injury survival and blood vessel density. In vitro, both $BM^{CD133}$ and $UCB^{CD133}$ cells were able to form capillary-like structures and to differentiate into endothelial cells and/or their immediate progenitors. Besides primary endothelial differentiation of CD133+ cells, the increase in blood vessel density in vivo might also be attributed to the generation of angiogenic growth factors. In this context, Pomyje et al. demonstrated the expression of angiopoietin-1, angiopoietin-2 and vascular growth factor as well as their receptor mRNAs in CD34+/CD133+ human UCB cells in vitro, supporting the notion of a paracrine action of these cells in the regulation of angiogenesis [168]. The increase in blood vessel density most likely increases local blood flow in the necrosis border zone, thus delaying or preventing the onset of apoptosis. Alternatively, direct anti-apoptotic actions due to secreted cytokines or other graft-host cell interactions may also play a role.

On the other hand, there was no measurable impact of $UCB^{CD133}$ cells on LV contractility, and the reduction of apoptosis did not reach statistical significance. Taken together, $UCB^{CD133}$ cells appear to less potent than $BM^{CD133}$ cells in preventing or reversing myocardial injury, at least in the model used here, although in vitro they appear to be similarly potent endothelial progenitor cells. In an attempt to find an explanation for this counterintuitive finding, we compared the expression profile of a number of "stemness"-related surface markers. The only difference we found is an approximately 50% lower percentage of CD117+ cells among $UCB^{CD133}$ cells. The proto-oncogene c-KIT/stem cell factor receptor/CD117 is thought to modulate cellular differentiation and proliferation processes but also to inhibit apoptosis, and one might assume that less CD117+ cells in a given cell product indicate an overall lower functional capacity [169]. However, our study is only observational and does not provide data to prove this notion. There is a multitude of other confounding factors that might also help explain the differences between $BM^{CD133}$ and $UCB^{CD133}$ cells, including the UCB preservation protocol and possibly a higher content of mesenchymal-type stem cells in BM-derived cell products.

In summary, we found that CD133+ cells from human bone marrow clearly have beneficial effects on the hearts subjected to an extensive cryolesion, improving survival, contractility and

vascularization, and inhibiting myocardial apoptosis. The experimental support for the ongoing clinical pilot trials using similar cell products therefore seems to grow. The corresponding cell product derived from cord blood appears to be less potent, and further studies are required to elucidate the reasons for this discrepancy.

# Chapter 4 Genetic Manipulation towards Better Stem Cell Survival (Publication E)

## *4.1 Introduction*

Considerable experimental and clinical evidences have demonstrated that using different cell types to replace the necrotic tissue in the myocardium is safe and can contribute to the improvement of angiogenesis and heart functions [170-175]. Among the various cell types investigated, bone marrow MSCs are self-renewing clonal precursors of non-hematopoietic stromal tissues [176]. They can be isolated from the bone marrow or adipose tissue and expanded in culture based on their ability to adhere to culture dishes and proliferate *in vitro*. MSCs are multilineage potential cells [177] and can give rise to osteoblasts, chondrocytes [178], neurons [179], skeletal muscle [180] and cardiac muscle [181][182] under appropriate conditions. Furthermore, studies on human, baboon and murine MSCs showed that MSCs are immunosuppressive [183][184]. Therefore, MSCs appear to be an appealing cell source for cardiac transplantation [171][185] because of the ease of harvest and expansion *ex vivo*. However, the low cellular survival rate after transplantation into an infarcted heart within the first few days engenders only marginal functional improvement [182][186]. Thus, it is necessary to reinforce MSCs against the arduous microenvironment incurred from ischemia, inflammatory response and pro-apoptotic factors in order to improve the efficacy of cell therapy.

The 26-kDa Bcl-2 anti-apoptotic protein belongs to the Bcl-2 family of proteins, which was originally found to be overexpressed in B-cell lymphoma. It serves as a critical regulator of pathways involved in apoptosis, acting to inhibit cell death. Bcl-2 gene was found to be up-regulated in failing hearts [187][188] as well as aging hearts [189]. It acts to prevent programmed cell death of ventricular myocytes [190].The protective effects of Bcl-2 for conditions of the diseased heart have been approved by previous studies [190][191]. Interestingly, overexpressing Bcl-2 in transgenic mice improved the heart function and inhibited cardiomyocyte apoptosis.

In this study, adult rat bone marrow-derived MSCs were genetically modified to overexpress Bcl-2, which aimed at vivifying the stem cells and enhancing their resistance to ischemic conditions from acute myocardial infarction after transplantation into the heart. We proposed that genetic modification of stem cells with Bcl-2 would armour stem cells settling into a deteriorative ischemic microenvironment, improve stem cell viability in the early post-transplanted period and thereby enhancing cardiac functional recovery after acute myocardial infarction.

### *4.2 MSCs express markers distinct from hematopoietic stem cells*

Cell isolation, expansion, characterization and differentiation of rat MSCs have been established according to previous reports [177]. The morphology of rat MSCs from bone marrow displayed a homogenous spindle-shaped population and maintained a similar morphology during the subsequent passages. FACS analysis was employed to identify the surface marker expression. The MSCs culture was shown to be devoid of CD34 and CD45 (Figure 4.1D and E), which are the markers for hematopoietic cells. In contrast, a high expression of CD29, CD44, CD90 markers were observed (Figure 4.1A, B and C).

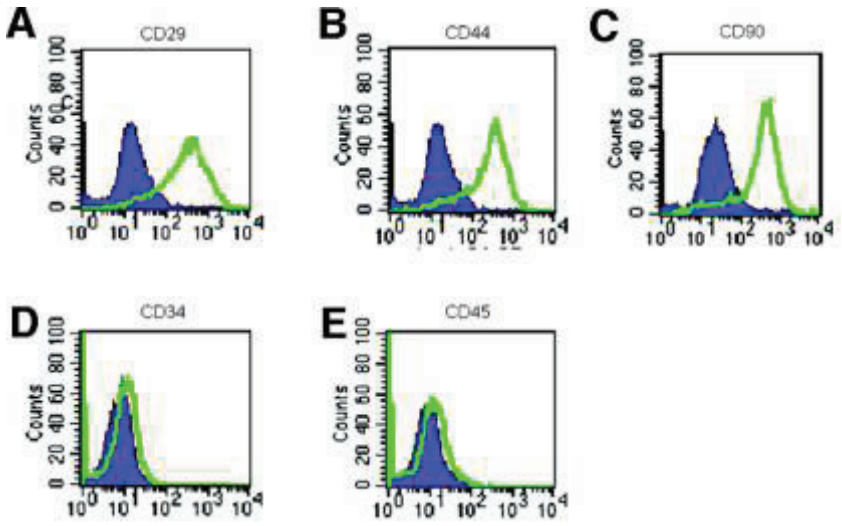



Figure 4.1 Immunophenotypic profile of MSCs. Flow cytometry histograms after three passages show the expression (unshaded) of selected surface molecules; **(A):** CD29; **(B):** CD44; **(C):** CD90; **(D):** CD34; and **(E):** CD45. Control cells labeled without primary antibodies (shaded). The rat MSCs were positive for CD29, CD44, and CD90 but negative for CD34 and CD45 surface markers, which are commonly found on hematopoietic cells.

### *4.3 Overexpression of Bcl-2 in genetically modified MSCs*

The transfection efficiency of Polyethylenimine for genetic modified MSCs was evaluated by pcDNA3.1/GFP as an internal control after 24 h gene delivery. More than 50% of MSCs were transfected based on FACS analysis (Figure 4.2B). To evaluate the expression of Bcl-2 protein in the genetically modified MSCs *in vitro*, Western blot analysis was performed on cell samples at various time points (24 h, 72 h, 7 days and 21 days) post-transfection. A significantly high expression of Bcl-2 was observed in Bcl-2-MSCs as early as 24 h, which remained detectable on day 7 (Figure 4.2C). In contrast, a weak expression of Bcl-2 was detected in vector-MSCs 24 h after transfection, indicating the low level of endogenous Bcl-2 proteins in MSCs. The expression of internal housekeeping β-actin gene was at the same level. Our observation showed that the MSCs were successfully modified with Bcl-2. The up-regulation of Bcl-2 was transient and this

may minimize the risk of malignant transformation or late cell failure. No apparent change of MSCs phenotype after transfection was observed (data not shown).

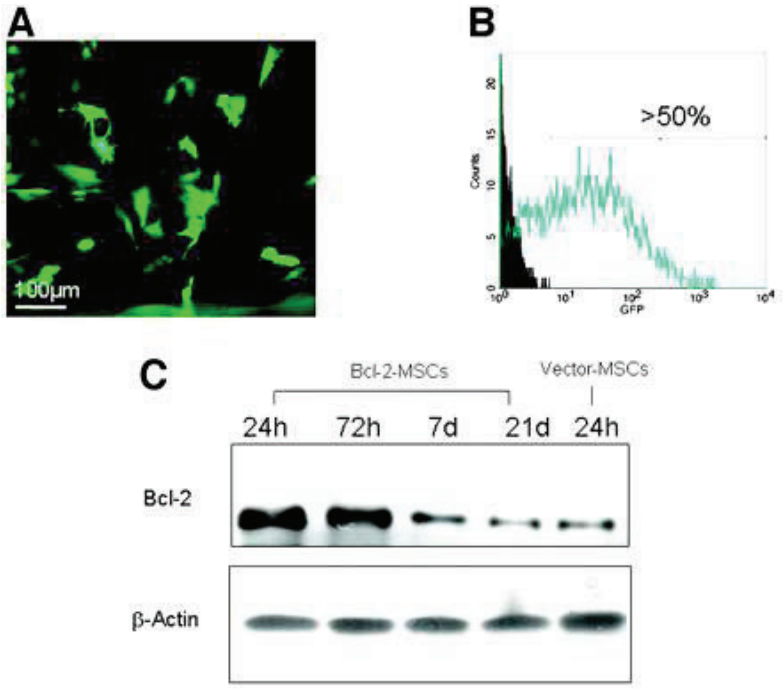



Figure 4.2 Transfection of MSCs with nonviral vector. **(A):** Representative photomicrograph of MSCs transfected with pcDNA3.1/GFP. **(B):** Fluorescence-activated cell sorter analysis showing the transfection efficiency of GFP transfected MSCs (unshaded) compared with untransfected MSCs (shaded). **(C):** Representative Western blots showing that Bcl-2 was overexpressed in Bcl-2 modified MSCs and lasted for 7 days after transfection. Housekeeping protein ß-actin served as loading control. Abbreviations: d, days; GFP, green fluorescent protein; h, hours.

### *4.4 Bcl-2 modified MSCs maintain their multi-differentiation capacity*

MSCs can differentiate into multi-lineages (such as bone, cartilage, adipose tissue and cardiac muscle) and this ability is taken as a functional criterion defining MSCs precursor cells. To verify if the Bcl-2 modification affected the differentiation capacity, the genetically modified MSCs underwent myogenic, adipogenic and chondrogenic differentiation using the methods previously described [181][192] at 48 h post-transfection. After 21 days of induction towards an adipogenic lineage, a characteristic morphological change with accumulation of lipid vacuoles was observed (Figure 4.3A). Immunostaining revealed the presence of fatty acid binding protein-4 (FABP-4), which is a marker protein for adipocytes. Chondrogenesis was assessed by immunostaining for aggrecan after 4 weeks of culture under chondrogenic conditions. The chondrocyte-like cells showed positive staining for aggrecan protein (Figure 4.3B, C and D). For cardiomyocyte differentiation, Bcl-2-MSCs were exposed to 5-azacytidine for 24 h. After two weeks, cardiomyocyte-like cells and positive immunostaining for Nkx2.5 were noted (Figure 4.3E, F, and G). Taken together, this evidence indicated that Bcl-2-MSCs retain their multi-differentiation potential into adipogenic, chondrogenic and cardiomyocyte lineages.

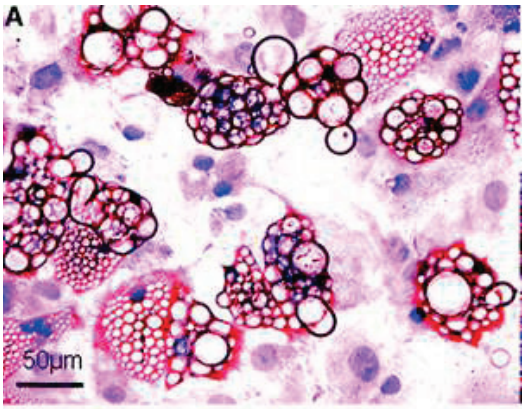
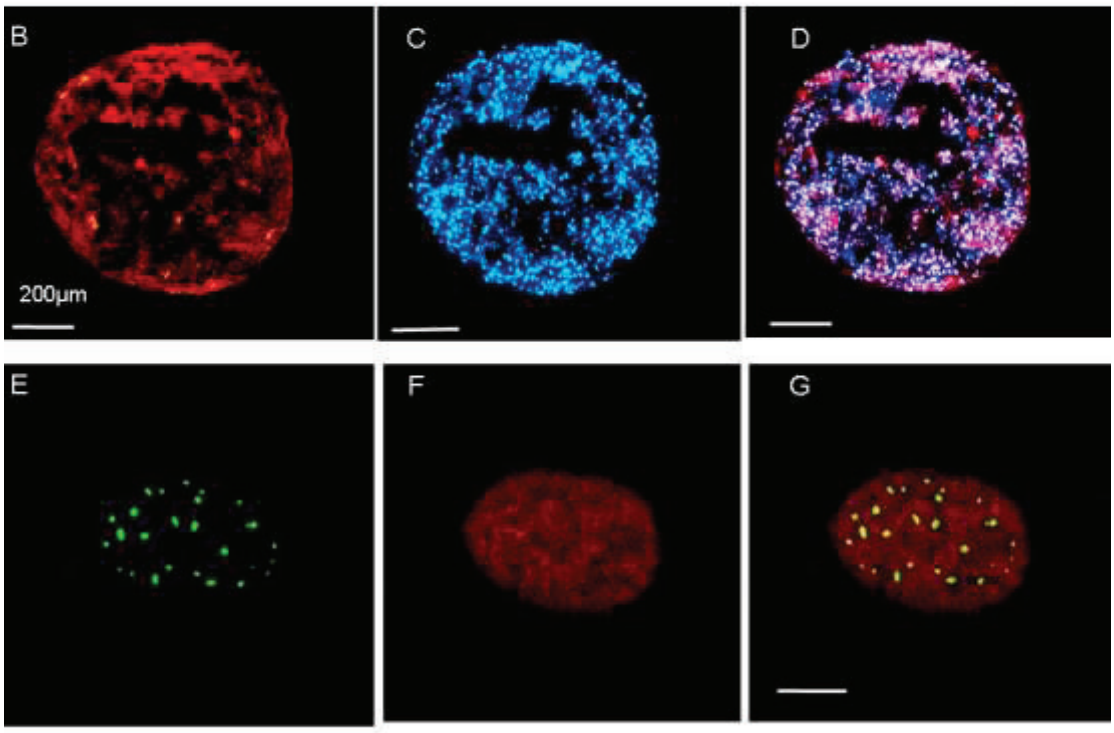



Figure 4.3 Differentiation capacity of Bcl-2-MSCs. MSCs transfected with Bcl-2 were cultured in adipogenic, chondrogenic, and myogenic medium for up to 2 months. **(A):** Adipogenic differentiation. Immunostaining with fatty acid binding protein-4 (brown). **(B–D):** Chondrogenic differentiation. Immunostaining for aggrecan (**[B, D]**, red). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (**[C, D]**, blue). **(E–G):** Cardiomyocyte-like differentiation. Immunostaining with anti-Nkx2.5 antibody (**[E, G]**, green). Nuclei were counterstained with propidium iodide (**[F, G]**, red).




### *4.5 Bcl-2 modified MSCs protect against apoptosis in vitro*

To test the capability of Bcl-2-MSCs to protect against apoptosis *in vitro*, the modified MSCs were treated under hypoxic conditions for 24 h. The evaluation of apoptosis was carried out using TUNEL assay (Figure 4.4A) and Hoescht 33342 staining (Figure 4.4B). Under hypoxia, the rates of cell apoptosis in MSCs and vector-MSCs exceeded those of Bcl-2-MSCs by 1.5 fold. The apoptotic cell number was significantly reduced by the Bcl-2 genetic modification. Quantitative assay showed that the number of TUNEL-positive cells was decreased from 53±3.5% of MSCs

and 51±4.7% of vector-MSCs to 36%±1.8% (n=6, $p$<0.001) of Bcl-2-MSCs. However, there was no significant difference in apoptotic cell numbers between MSCs, vector-MSCs and Bcl-2-MSCs when evaluated under normoxia. A further confirmatory assay was based on nuclear chromatin morphology after staining with Hoescht 33342. Apoptosis was evidenced by cell shrinkage; nuclear condensation and DNA fragmentation that occurred in cells treated with hypoxia (Figure 4.4B). The quantitative assay based on Hoescht staining showed that the number of apoptotic cells was decreased from 46±3.1% of MSCs and 45±4.2% of vector-MSCs to 30±2.6% of Bcl-2-MSCs (n=6, $p$<0.001). These findings demonstrated that the Bcl-2 modified MSCs could protect against apoptosis under hypoxic conditions. It is anticipated that Bcl-2 modified MSCs may retain their anti-apoptotic properties in the ischemic myocardium.

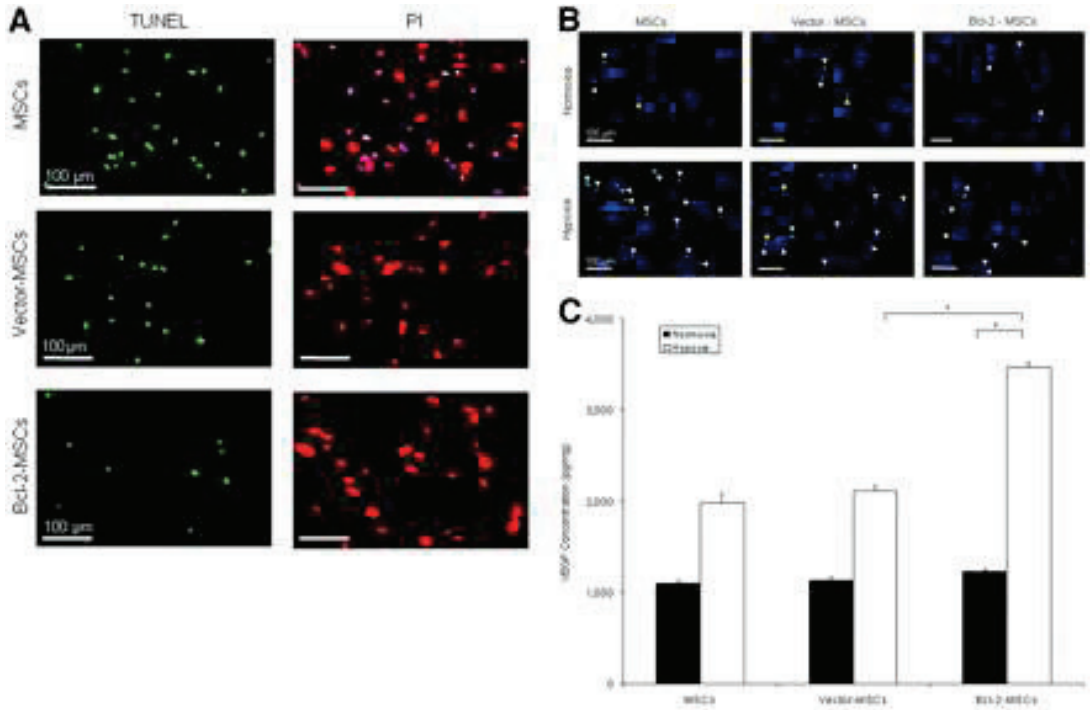



Figure 4.4 Antiapoptotic effect and upregulation of VEGF secretion of Bcl-2-MSCs under hypoxic conditions. **(A):** Representative photomicrographs of TUNEL-positive (left panels) and total (right panels) cells after 24 hours of hypoxic treatment. **(B):** Representative photomicrographs of apoptotic cells (arrows) with chromatin condensation by Hoechst 33342 staining after 24 hours of hypoxic treatment. **(C):** VEGF secretion by Bcl-2-MSCs under hypoxic conditions. After 24 hours of incubation, conditioned medium from normoxia (solid bar) and hypoxia (empty bar) treated cells ($n$ = 6) was subjected to VEGF enzyme-linked immunosorbent assay (ELISA) assay. VEGF concentration values are mean ± SD (* $p$ < .001). ELISA data are representative of three independent experiments. Abbreviations: PI, propidium iodide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP end labeling; VEGF, vascular endothelial growth factor.

### *4.6 Bcl-2 modified MSCs upregulated angiogenic cytokine VEGF under hypoxia*

To identify potential paracrine mechanisms responsible for the therapeutic effect of Bcl-2-MSCs, we examined the secretion of VEGF. MSCs, vector-MSCs and Bcl-2-MSCs were cultured under either normoxic or hypoxic conditions for up to 24 hours and VEGF secretion was quantitated by

Quantikine rat VEGF immunoassay. A more than 60% increase in secretion of VEGF was detected in Bcl-2-MSCs compared to MSCs alone and vector-MSCs when the cells were cultured under hypoxic conditions (Figure 4.4C). However, there was no significant difference among the groups when the cells were cultured under normoxic conditions. The angiogenic cytokine VEGF was significantly up-regulated in Bcl-2-MSCs in response to hypoxic conditions. The high level expression of VEGF from Bcl-2-MSCs may provide cardioprotective and pro-angiogenic effects.

### *4.7 Cell engraftment with Bcl-2 modified MSCs in the infarcted heart*

To assess the efficacy of Bcl-2-MSCs transplantation, $6\times10^6$ Bcl-2-MSCs or vector-MSCs from passage 3 were transplanted into the viable (Left Ventricle)LV myocardium bordering infarction. Medium was injected into control animals. The assessment of cell engraftment was carried out 4 days, 3 weeks and 6 weeks after cell transplantation. BrdU positive nuclei were identified by immunostaining (Figure 4.5A-E). Figure 4.5A and B depicted representative images 4 days following cell transplantation. Figure 4.5C-E showed representative images of Bcl-2-MSCs cells in the heart of a MI+ rat sacrificed 3 weeks after cell injection. The number of surviving cells in Bcl-2-MSCs group was greater than in vector-MSCs following cell transplantation and the differences proved to be statistically significant, with 2.2-fold enhancement of cell survival on day 4 (n=10, $p<0.01$), 1.9-fold enhancement on day 21 (n=9, $p<0.05$) and 1.2-fold enhancement after 6 weeks (n=6, $p<0.05$). Manipulation MSCs with Bcl-2 gene enhanced cellular survival after myocardial infarction.

Furthermore, double immunofluorescence staining with BrdU and anti-vWF antibody revealed that at least some of the MSCs cells (3.1±1.3%) appeared to display endothelial cell-like phenotype. Occasionally, blood vessels consisted of BrdU positive cells (Figure 4.6A and B). 3 weeks after cell transplantation, we observed a very few number of MSCs colocalized with cardiac Troponin T (cTnT) (Figure 4.6C-E). The colocalization was further confirmed by the 3-D reconstruction of the tissue acquired by a Leica TCP2 confocal microscopy (Figure 4.6F -H). The frequency of cTnT –BrdU double positive cells from the engrafted stem cells was extremely low (Bcl-2-MSCs group 0.05±0.02% cTnT). There was no significant difference between Bcl-2-MSCs and MSCs group. It was not clear whether the transplanted cells had fused or differentiated into cardiomyocytes. There is no evidence for tumor formation and MSCs differentiation into bone, adipose and cartilage after 3 and 6 weeks cell transplantation (data not shown).

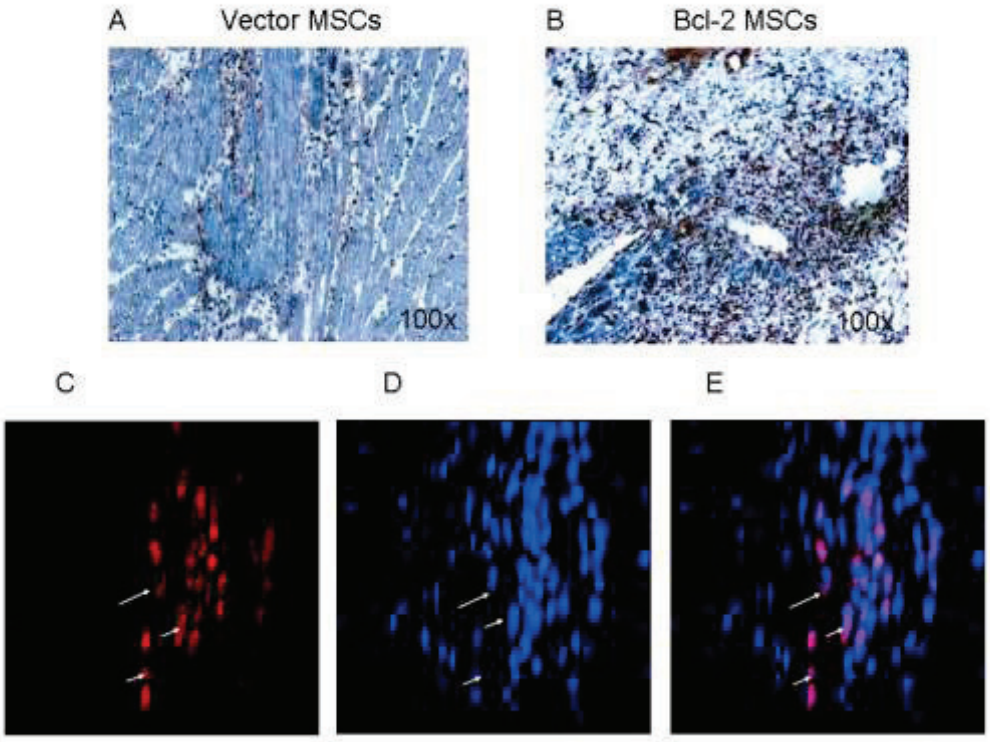



Figure 4.5 Engraftment of Bcl-2-MSCs in ischemic myocardium. **(A, B):** Representative images from immunostaining of grafted MSCs in the infarcted rat myocardium 4 days following MSC injection. BrdU-labeled MSCs (brown) were clearly identified. **(C–E):** Immunofluorescence staining of BrdU-labeled cells in the Bcl-2-MSC group 3 weeks after cell treatment with high magnification. **(C):** BrdU (red). **(D):** DAPI (blue). **(E):** Merged image (×1,000 in all panels). Arrowheads indicate the cell engraftments.

### *4.8 Capillary density*

Capillary density at the border zone of the acute myocardial infarction (AMI) was determined based on vWF immunostaining 3 weeks after cell transplantation. There was a significant increase in capillary density in vector-MSCs (20.5±1.9 vessels/HPF) and Bcl-2-MSCs (23.6±1.3 vessels/HPF) groups when compared with medium-treated hearts with AMI (14.4±2.1 vessels/HPF) (n=8, $p<0.001$) and no obvious angiogenesis was detected in the medium group. In addition, the capillary density was 15% higher in Bcl-2-MSCs treated hearts than in vector-MSCs treated hearts with significant difference (n=8, $p=0.002$).

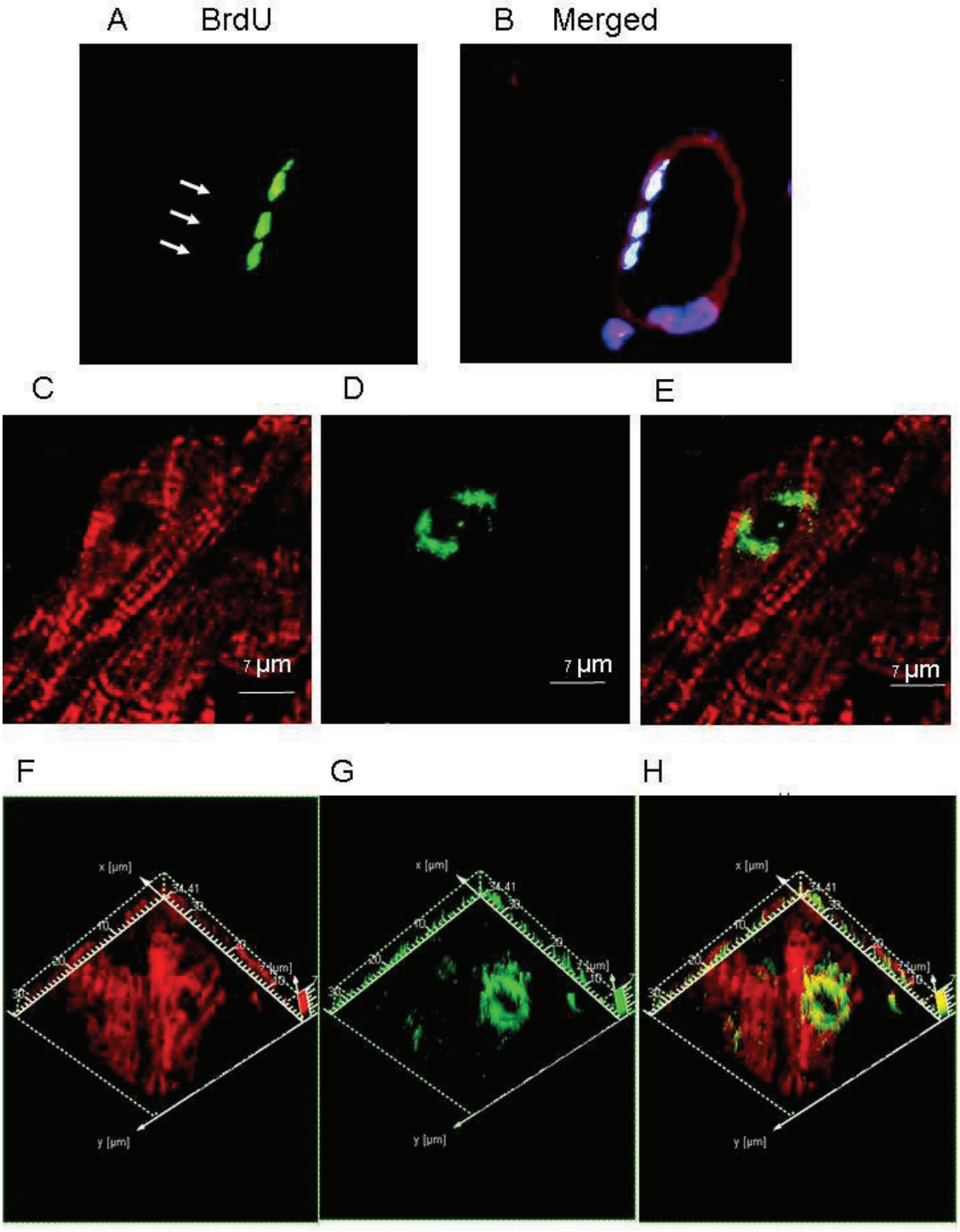



Figure 4.6 Bcl-2-MSCs in ischemic myocardium. **(A, B):** Double immunofluorescence staining revealed BrdU-labeled Bcl-2-MSCs (green) incorporated into the endothelial lining of blood vessels (red) near the infarct border zone 3 weeks after transplantation. **(C–E):** Three weeks after transplantation, sections near the infarct zone were double-stained for BrdU (green) and cardiac marker Troponin T (red). BrdU-positive cell colocalized with cardiac Troponin T. **(F–H):** Three-dimensional reconstruction of the tissue section using confocal microscopy illustrates that BrdU-positive nucleus (green) is within a cell body that expresses cardiac Troponin T. Cardiac-like cells were stained for Troponin T; BrdU-positive cells were identified (primary antibody: monoclonal mouse anti-BrdU antibody; secondary antibody: Alexa 488)..

### *4.9 Infarct size and cardiac function*

LAD ligation consistently resulted in transmural myocardial infarction, exhibiting typical histologic changes including thinning of the left ventricular free wall and extensive collagen deposition 3 weeks after myocardial infarction. Both vector-MSCs treated and Bcl-2-MSCs treated MI+ animals showed smaller infarction size (34.2±6.0% in vector-MSCs group; 28.3± 5.8% in Bcl-2-MSCs group) compared with the medium treated animals (47.8±6.3%) and the difference was statistically significant (n=8, $p<0.001$ vector-MSCs group vs. medium group; n=8, $p<0.001$ Bcl-2-MSCs vs. medium group). Further, the collagen content was 17% lower in Bcl-2-MSCs treated hearts than in vector-MSCs treated hearts (34.2±6.0% of vector-MSCs group vs. 28.3± 5.8% of Bcl-2-MSCs group) (n=8, $p=0.029$).

Hemodynamic parameters of left ventricular function demonstrated that max dP/dt and min dP/dt were improved significantly in Bcl-2-MSCs group than in vector-MSCs group (n=6, $p<0.001$). Compared with medium group, both Bcl-2-MSCs and vector-MSCs group had significant improvements on hemodynamic parameters such as end-systolic volume (ESV), ejection fraction (EF) , Maxium dP/dt and Minimum dP/dt. Furthermore, there were significant improvements in Bcl-2-MSCs group on end-diastolic volume (EDV), end-systolic pressure-volume relationship (ESPVR) and Emax compared to medium group (Bcl-2-MSCs group: 239.2±35.9 µl of EDV, 0.58±0.14 mmHg/µl of slope ESPVR, 1.50±0.34 mmHg/µl of Emax vs. medium group: 292.1±39.0 µl of EDV, 0.36±0.16 mmHg/µl of slope ESPVR, 0.93±0.39 mmHg/µl of Emax) (n=6, $p<0.05$). However, there was only a positive trend toward improvement on EDV, ESPVR and Emax between vector-MSCs group and medium group, and the difference did not reach statistical significance. No significant difference was observed on the values of $V_0$ between Bcl-2-MSCs group (83.4±14.1 µl) and vector-MSCs group (79.4±13.0 µl), or between Bcl-2-MSCs group and the medium group (87.2±15.8 µl).

### *4.10 Discussion*

MSCs derived from adult bone marrow have been proposed as a promising cell source for the regeneration and restoration of infarcted heart function. However, poor survival of implanted cells hampered the therapeutic efficacy. In this study, the key roles of Bcl-2 played in protecting grafted MSCs survival under hypoxia-ischemia were identified *in vitro* and *in vivo*. We confirmed that Bcl-2 genetically modified MSCs retained their differentiation capacity and could give rise to different lineages *in vitro*. Overexpression of Bcl-2 reduces MSCs death and apoptosis under hypoxic conditions. *In vivo* transplantation of Bcl-2-MSCs enhances the survival rate of MSCs in the LAD ligation model. It could attenuate post infarction left ventricular (LV) remodelling and restore LV function, which may have attributed to the enhanced anti-apoptotic properties of the modified MSCs. These observations suggest that genetic modification of MSCs

with Bcl-2 could be of significant value in improving the efficacy of stem cell therapy following a broad range of cardiac diseases. Our results agree with the earlier findings that transgenic mice over-expressing Bcl-2 in the heart reduced infarct size and improved recovery of cardiac function after ischemia/reperfusion injury [191][193][194].

There are several mechanisms contributing to the high level of stem cell death within 4 days after implantation into ischemic hearts [182][186]. These include host inflammatory response, loss of survival signal from matrix attachments or cell-cell contact, delivery of oxygen and substrates via diffusion [182][186], various pro-apoptotic or cytotoxic factors in the ischemic [195] and ischemic/reperfusion damage to the implanted stem cells incurred from repeated bouts of ischemia [196]. Furthermore, MSCs are extremely sensitive to the hypoxic and inflammatory environment in ischemic hearts [182]. Our present studies revealed that genetic modification of MSCs with Bcl-2 effectively protects transplanted MSCs against ischemia and increases cell survival after implantation. Our investigation, consistent with previous findings from Mangi et al. [176] and Tang et al. [196], showed that the therapeutic efficacy of MSCs was closely related to the *in situ* survival of cells implanted in the hostile environment of hypoxia, inflammation and scarring from myocardial infarction. Hence, the survival essential factors for the MSCs, such as cytokines, chemokines, integrins and other adhesion molecules need to be further addressed quantitatively.

It was recently reported that persistent overexpression of VEGF in nonischemic myocardium may cause the formation of vascular tumors [197]. The present study for the first time, demonstrated the high level expression of VEGF from Bcl-2-MSCs in response to the hypoxic conditions. Therefore, transplantation of Bcl-2-MSCs could provide adequate magnitude and duration of VEGF expression in the ischemic myocardium without formation of vascular tumors. The high level expression of VEGF from Bcl-2-MSCs transplanted to the ischemia-damaged myocardium would provide cardioprotective effects and induce functional collateral vessels which contribute to the salvaging of ischemic myocardium and decrease the infarct area.

However, it is still not clear whether intramyocardially transplanted MSCs function as newly differentiated cardiomyocytes and endothelial cells or serve as the paracrine cells by secretion of cardioprotective proteins. Our study and those of other groups [196][198][199] confirmed that grafted implanted MSCs can secrete important survival factors, including VEGF, insulin-like growth factor, hepatocyte growth factor, SDF-1 and basic fibroblast growth factor. Considering the low frequency of new cardiomyocytes and endothelial cells [196][200], this paracrine effect could be even more important for the functional recovery of the infarcted heart than the transdifferentiation potential of stem cells. Therefore, a quantitative assay of the paracrine

cytoprotective factors from stem cells cocultured with cardiomyocytes subjected to hypoxia deserves significant attention. The cocktail of paracrine cytoprotective factors may bring fresh therapeutic options to a number of diseases, including heart failure.

Our current work, together with many other studies demonstrated that bone marrow MSCs implantation could induce therapeutic angiogenesis in infarcted heart [201-204] or ischemic tissue [205]. Our data suggested that the viability of transplanted cells is one of the important factors for preservation of myocardial function and the therapeutic angiogenesis is not linearly correlated with the survival rate of the transplanted cells. The underlying mechanism of angiogenesis is very complicated. For instance, MSCs are able to differentiate into vascular endothelial cells in the ischemic myocardium and generate capillary like structure [201][202][204]. Meanwhile, MSCs enhance angiogenesis partly by increasing endogenous levels of vascular endothelial growth factor and vascular endothelial growth factor type 2 receptor [205]. Our evidence support the theory that the paracrine effect of MSCs might be one of the major reasons for therapeutic angiogenesis. Using genetic modification approach may further enhance the cytoprotective effect and restore MSCs angiogenesis effect.

In the present study, a significant increase in nucleosomal DNA fragmentation and DNA condensation by TUNEL and Hoechst 33342 nuclear staining was observed in MSCs subjected to hypoxic conditions compared with the normoxic control cells, a finding concordant with previous data verifying that hypoxia provokes apoptosis of MSCs [176]. It is known that hypoxia can induce Bcl-2 down regulation through Nuclear Factor-kB (NF-kB) in endothelial cells [206] and myocytes [207]. We provide the first direct evidence that Bcl-2 upregulation from exogenous delivery activates a survival pathway that is sufficient to suppress hypoxia-induced apoptosis of MSCs. The underlying mechanism by which Bcl-2 protects MSCs under hypoxia is still unknown. We speculate that NF-kB might play a pivotal role in the cytoprotective effect of Bcl-2 on MSCs under hypoxic conditions. Further studies need to be conducted in order to support the hypothesis.

Our hemodynamic data are in consistent with previous reports which demonstrated cardiac functional improvement after MSCs transplantation [208][209]. We found that Bcl-2-MSCs transplantation further reduced EDV and significantly enhanced the systolic functional recovery determined by slope ESPVR and Emax. These beneficial effects of Bcl-2-MSCs may, in part, attribute to VEGF secretion in response to the hypoxic environment, angiogenesis and the improved cellular survival after pre-treatment with the anti-apoptotic Bcl-2 gene.

In this investigation, MSCs were genetically modified with Bcl-2 by a cationic polymer based vector with several unique advantages such as easy surface modification with well-defined structure and chemical properties [210], relatively low toxicity, high capability for carrying large therapeutic genes and lack of immune responses. More importantly, transient overexpression of Bcl-2 modified MSCs benefited from the non-viral gene delivery vector may minimize the potential risk of tumorigenesis while providing safe and sufficient protection for transplanted MSCs from short-term ischemic damage, which plays a critical role in transplanted stem cell death [182]. Further works on the long term persistence, function and phenotype of Bcl-2 modified MSCs treated with non-viral vectors need to be determined to safely realize the full potential of MSCs for myocardial regeneration.

### *4.11 Conclusion*

In summary, we have confirmed that genetic modification with anti-apoptotic Bcl-2 gene resulted in high-level VEGF expression in response to the hypoxic conditions. It enhanced the survival of engrafted MSCs in the heart after acute myocardial infarction. The transplantation of Bcl-2 modified MSCs ameliorated LV remodeling and improved LV function. Genetically engineering cells by Bcl-2 using a non-viral vector could be an effective strategy for increasing cell survival after cell transplantation while minimizing the potential risk of tumorigenesis. Transplantation of gene-engineered MSCs may provide a novel and effective approach in the treatment of acute myocardial infarction.

## Chapter 5 Summary

Stem cell therapy holds great promise in the future treatment of cardiac disease such as acute myocardial infarction, congestive heart failure, and chronic ischemic heart disease. Accumulating clinical and experimental evidence suggested there is the possibility of regenerating damaged myocardium using adult stem cells, which have generated enthusiasm for therapeutic developments towards cardiac regeneration.

Several different stem cells have been demonstrated to increase cardiac function when injected into infarcted hearts in experimental models. However, the underlying mechanism by which such structural and functional recoveries are achieved is still unclear. Although it has been confirmed that the endothelial progenitor cells have the capacity to form new vessels which may contribute to cardiac remodelling and functional improvement, it is still under debate that adult bone-marrow-derived mesenchymal stem cells (MSCs) may differentiate into cardiac myocyte-like cell for the damaged myocardium.

A major limitation to the efficacy of cell therapy is the poor viability of the transplanted cells. Indeed, the functional improvement from stem cell therapy has been quite modest. Mechanisms contributing to the high level of stem cell death after implantation into ischemic hearts include host inflammatory response, loss of survival signal from matrix attachments or cell-cell contact, delivery of oxygen and substrates via diffusion, various pro-apoptotic or cytotoxic factors in the ischemic and ischemic/reperfusion damage to the implanted stem cells incurred from repeated bouts of ischemia. Furthermore, stem cells are extremely sensitive to the hypoxic and inflammatory environment in ischemic hearts. Genetic modification of stem or progenitor cells may represent an important advancement in regenerative medicine. By combining gene with cell therapy, one may be able to enhance stem cell function and viability. Indeed, genetic modification can improve survival, metabolic characteristics, contractility, proliferative capacity, or differentiation of the stem cells. Furthermore, the cells may become a vehicle for gene therapy whose secreted gene products can exert paracrine or endocrine actions that may result in further therapeutic benefits. Our present studies revealed that genetic modification of MSCs with Bcl-2 effectively protects transplanted MSCs against ischemia and increases cell survival after implantation. Our investigation, consistent with previous findings, showed that the therapeutic efficacy of MSCs was closely related to the in situ survival of cells implanted in the hostile environment of hypoxia, inflammation and scarring from myocardial infarction. Hence, the survival essential factors for the MSCs, such as cytokines, chemokines, integrins and other adhesion molecules need to be further addressed quantitatively. All these studies proved that an

ideal combination of cell and gene therapy might represent a future strategy for myocardial repair and regeneration.

The field of stem cell research is still at its very preliminary stage. A clear understanding of the ideal approach in terms of choice of cell type, time, and method of delivery may take years of investigation. Continued development and application of genetic manipulation approaches in stem cells will undoubtedly lead to more efficient guidance and control of stem cell fate and improved stem cell function by promoting survival, migration, proliferation, differentiation or reprogramming of stem cells, which eventually leads to the success of cardiac regeneration.

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Nan Ma

**Appendix**

**Copy of the original articles**