A novel approach to studying the transformation of human stem cells into cardiac cells *in vivo*

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Stem cell transplantation has been proposed as a novel means of regenerating new myocardium following cardiac damage. Many laboratories have demonstrated that stem cells from different sources have the potential to transform into cardiomyocytes. Human peripheral blood CD34+ cells were transplanted into the hearts of mice with severe combined immune deficiency syndrome, and it was demonstrated that human stem cells could transform into cardiomyocytes, endothelial cells and smooth muscle cells. Using single cell preparation, cell sorting and fluorescent *in situ* hybridization, human peripheral blood CD34+ cells were transformed into cardiomyocytes mainly through cell fusion, whereas endothelial cells were derived through direct differentiation of the transplanted stem cells. This analytical method should provide a novel approach to identifying the mechanisms of stem cell transformation into cardiomyocytes *in vivo*.

Key Words: *Cardiac repair; CD34; Fusion; Stem cell*

Heart failure is a complex clinical syndrome resulting from structural changes in the myocardium that affect the ability of the ventricle to fill with or eject blood (1). It affects at least five million patients in the United States and consumed 5.4% of the health care budget in 1991 (2). Nearly one-half of a million new patients are diagnosed with heart failure each year, and the incidence of new cases is increasing each year due to aging of the population and conversion of acute cardiac problems into chronic disorders. Coronary artery disease is the cause of heart failure in approximately two-thirds of patients with left ventricular dysfunction. The remaining heart failure patients have underlying hypertension, thyroid disease, valvular disease or myocarditis. Furthermore, chemotherapy agents, such as anthracyclines and other anthraquinones, can also damage the myocardium (3). Cardiomyocytes do not normally regenerate after birth, and they respond to mitotic signals by cell hypertrophy rather than by cell hyperplasia (4). Without the ability for renewal, loss of functional cardiomyocytes due to myocardial infarction (MI) or other causes eventually leads to deterioration of pump function, resulting in heart failure. Currently, heart failure is treated medically with angiotensin-converting enzyme inhibitors, beta-blockers and diuretics. Mechanical devices and pacemakers have also been used in selective subsets of patients with heart failure. For

Une démarche novatrice pour étudier la transformation de cellules souches humaines en cellules cardiaques *in vivo*

La greffe de cellules souches a été proposée comme démarche novatrice pour régénérer le myocarde après des dommages cardiaques. De nombreux laboratoires ont démontré que les cellules souches de diverses sources peuvent se transformer en cardiomyocytes. Des cellules CD34+ du sang périphérique humain ont été greffées dans le cœur de souris atteintes d'un syndrome d'immunodéficience combinée sévère, et il a été démontré que les cellules souches pouvaient se transformer en cardiomyocytes, en cellules endothéliales et en cellules des muscles lisses. À l'aide de préparations unicellulaires, de classement cellulaire et d'hybridation *in situ* en fluorescence, les cellules CD34+ du sang périphérique humain ont été transformées en cardiomyocytes, notamment par fusion cellulaire, tandis que les cellules endothéliales étaient dérivées par différenciation directe des cellules souches greffées. Cette méthode analytique devrait procurer une démarche novatrice pour repérer les mécanismes de transformation des cellules souches en cardiomyocytes *in vivo*.

patients who have refractory heart failure, cardiac transplantation is the final resort. However, cardiac transplantation is limited by the donor source, the cost and the risk of the procedure. Recently, there has been a growing hope that stem cell transplantation may be used to regenerate the damaged cardiomyocytes (5). Stem cells are commonly defined as undifferentiated cells that can proliferate and have the capacity of both self-renewal and differentiation to one or more specialized cells.

Tissue stem cells as the basis for tissue regeneration was proposed by Pappenheim (6), based solely on morphological observations. He postulated the existence of an undifferentiated stem cell that gave rise to the plethora of blood cells via an intermediate state of progenitor cells. The hypothesis was confirmed experimentally in the 1950s with findings that hematopoietic stem cells existed in the bone marrow, which promoted hematopoietic recovery after irradiation damage (7). Subsequently, the concept of tissue regeneration from a small population of resident tissue stem cells was generally accepted and was extended to nonhematopoietic tissues (8). Since then, vigorous efforts have focused on adult tissue regeneration from stem cells. Recently, the observation of dedifferentiation and transdifferentiation of certain progenitor and mature cells has led to the re-evaluation of this definition (9,10).

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Figure 1) Adult human peripheral blood CD34⁺ cells transformed into cardiomyocytes in vivo **A** Antitroponin T; **B** Antihuman leukocyte antigen; **C** Overlay of **A** and **B**. Reprinted from reference 56 with permission

The term may be applied to a range of cell types, including differentiated cells, rather than a single entity (11).

Two sources of stem cells, from adults (somatic stem cells) and from embryos (embryonic stem [ES] cells), have been studied in different experimental and clinical settings to repair damage in a variety of organs. Although the concepts of stem cells in embryogenesis and stem cells in adult tissue regeneration were initially studied in separate approaches, they merged with the successful cloning of a mammal from the nucleus of an adult cell (12). Mouse ES cells have been studied extensively. The cell's ability to differentiate into a range of cell types, including cardiomyocytes, endothelial cells (13), hematopoietic cells (14), nerves (15), skeletal muscle (16), liver (17) and pancreatic islets (18), has been documented. Successful transplantation of ES cells into rats to regenerate cardiac muscle has been reported (19). Mouse bone marrow cells (Lin[−] c-Kit⁺) have been shown to regenerate myocytes in the infarcted region (20), and the so-called side population of bone marrow-derived cells (BMDCs) (CD34[−]/low c-Kit⁺ and Sca-1⁺) can regenerate cardiomyocytes in a mouse ischemia-reperfusion model (21). More recently, human bone marrow stem cells have been shown to differentiate into myocytes in severe combined immunodeficient (SCID) mice (22), demonstrating the potential for using adult stem cells in cardiac regeneration.

Recently, it has been shown that circulating adult peripheral blood stem cells can differentiate into mature hepatocytes and epithelial cells of the skin and gastrointestinal track (23), raising the possibility that autologous stem cells isolated from the peripheral circulation can be used to repair damaged heart tissue, obviating immunological rejection. The peripheral blood stem cells are often selected based on their surface expression of CD34. This particular population not only contains hematopoietic stem cells, but also progenitors of vascular endothelial (VE) and smooth muscle cells (24,25), which, therefore, may contribute to the regeneration of blood vessels. The sialomucin CD34 is commonly used as a marker to characterize and isolate human stem cells and progenitor cells, because surface CD34 is highly expressed by primitive cells and is downregulated as these cells differentiate into more mature cells (26).

The use of adult peripheral blood stem cells in myocardial regeneration may be superior to other forms of cell-based therapy because of the following reasons: it does not require the painful procedure of bone marrow aspiration; protocols already exist to harvest peripheral blood stem cells from patients after several days of treatment with a recombinant human granulocyte colony-stimulating factor; and autologous stem cell transplant does not require long-term immunosuppression. Thus,

myocardial regeneration using autologous, peripheral blood stem cells is a promising alternative for the treatment of heart failure. The feasibility is further supported by the recent finding that endothelial progenitors (CD34⁺) in human peripheral blood are able to differentiate into cardiomyocytes when co-cultured with cardiomyocytes of rats in vitro (24).

An in vivo model was developed to test both the recruitment and transformation of stem cells into different cardiac cells. First, human peripheral blood CD34⁺ cells were injected into the left ventricle of the SCID mice and the hearts were harvested at different times following injection. Figure 1 provides evidence that peripheral blood CD34⁺ cells can transform into cells of cardiomyocyte morphology with expression of cardiac troponin T. Figure 1A shows a section of myocardium that uniformly expresses cardiac troponin T. Figure 1B demonstrates that a single elongated cell that expresses human leukocyte antigen (HLA) appears to integrate into the surrounding myocardium. Integration of the HLA⁺ cell in the myocardium is demonstrated clearly when Figure 1A and Figure 1B are overlaid in Figure 1C. The single myocyte is most likely derived from a CD34⁺ cell that enters the heart through the coronary circulation rather than through direct seeding at the time of intraventricular injection. Taken together, human peripheral blood stem cells have the capacity to differentiate into troponin T-expressing cardiomyocytes in the murine heart. However, only one HLA⁺ myocyte-like cell was observed in all hearts harvested (n=7). It seems, therefore, that transformation of CD34⁺ cell into cardiomyocyte is a random, rare event when CD34⁺ cells are transplanted into normal recipients.

Because transformation of CD34⁺ cells into myocytes is a rare event in uninjured hearts, an experimental-induced MI model was used to enhance both the recruitment and possibly the transformation of recruited CD34⁺ cells into cardiac cells. Figure 2 shows the transformation of CD34⁺ cells into smooth muscle cells in the heart damaged by experimental MI. The left anterior descending coronary artery was ligated to create an MI. One million human peripheral blood CD34⁺ cells were transplanted through the tail vein of SCID mice 12 h after MI. Hearts were harvested eight weeks after cell transplantation for immunohistochemical analysis. Tissue sections were double-stained with a monoclonal antibody against antihuman HLA (Figure 2A) and smooth muscle alpha-actin (Figure 2B), which were each labelled with different secondary antibodies conjugated with fluorescent chromes of different excitation wavelengths. The antismooth muscle alpha-actin-stained blood vessels were located in the infarct zone and one of the large vessels was positively stained with anti-HLA (Figure 2A). Figure 2C (overlay of 2A and 2B) indicates that part of the

Figure 2) Transformation of adult human blood CD34+ cells into vascular smooth muscle cells in a heart injured by experimental myocardial infarction. **A** Antihuman leukocyte antigen. **B** Antismooth muscle actin. **C** Overlay of **A** and **B**. **D** Higher magnification of **C**. Reprinted from reference 56 with permission

Figure 3) Human peripheral blood CD34+ cells differentiate into vascular endothelial cells in the heart with experimental myocardial infarction. **A** Antihuman leukocyte antigen. **B** Antivascular endothelial cadherin. **C** Overlay of **A** and **B**. **D** Higher magnification of **C**. Reprinted from reference 56 with permission

vascular smooth muscle was derived from the transplanted CD34+ cells of human origin. Figure 2D shows the higher magnification of Figure 2C. The HLA+ cells were frequently observed in the hearts of all three SCID mice with experimental MI, especially in the peri-infarct area.

The injected CD34+ cells not only transformed into smooth muscle cells, but also into other cardiac cells. Figure 3B shows that one of the blood vessels stained positive for antihuman HLA. Endothelial cells of the blood vessel were identified in the sections of the heart damaged by experimental MI by a polyclonal antibody against VE-cadherin in Figure 3B. The antibody did not cross-react with cultured human smooth muscle cells when it was tested using fluorescence-activated cell sorting (FACS) analysis. Double-staining with anti-VE-cadherin and anti-HLA indicates that the transplanted CD34+ cells transformed into VE cells (Figure 3C). Figure 3D represents Figure 3C at a higher magnification. Similar to vascular

Figure 4) Adult blood CD34+ cells transformed into cells of cardiomyocyte morphology in myocardial infarction damaged hearts. **A** Antihuman leukocyte antigen. **B** Antitroponin T. **C** Overlay of **A** and **B**. **D** Higher magnification of **C**. Reprinted from reference 56 with permission

smooth muscle cells, HLA+ VE cells were frequently encountered in the infarct zone and the peri-infarct area of the hearts.

Figure 4 shows the transformation of human blood CD34+ cells into cells of cardiomyocyte morphology in hearts injured with MI. Figure 4A shows a cluster of cells with cardiomyocyte morphology that were stained with antihuman HLA antibody. Myocardium was stained with anticardiac troponin T in Figure 4B. Overlay of Figure 4A and Figure 4B demonstrated that the CD34+ cells were integrated in the myocardium. Figure 4D represents Figure 4C at a higher magnification. Unlike the CD34+ cells found in uninjured hearts, single and clusters of CD34+ cells with cardiomyocyte morphology were frequently found in multiple sections of all three MI-damaged hearts. Therefore, injury caused by MI may play an important role in the homing and differentiation of human peripheral blood CD34+ cells in the heart.

To study the engraftment of the transplanted CD34+ cells in the heart and transformation of these cells into the cardiomyocytes, methods were developed to digest the heart with enzymes to obtain a population of single cardiac cells (27). These cells were then examined by FACS analysis using specific antibodies against HLA-ABC, a surface marker for human cells, and cardiac troponin T, a cardiomyocyte-specific marker, or Nkx2.5, a cardiac-specific transcriptional factor. HLA+ cells were detected in all four mice examined at 60 days after transplantation with human CD34+ cells. Approximately 2% ($2.0\% \pm 0.4\%$) of the total cells from the heart were human HLA+ (Figure 5A), whereas cells from control mice with induced MI, but not injected with CD34+ cells, were all HLA- (data not shown). FACS analysis of heart cells double-stained with antibodies against HLA and cardiac troponin T or Nkx2.5 demonstrated that approximately 1% ($1.1\% \pm 0.3\%$) of cells were double positive (Figure 5B), suggesting that these cardiomyocytes originated from the transplanted human cells. Furthermore, cells expressing both HLA and cardiac troponin were detected in the mouse heart 24 h (Figure 5C) and six months (Figure 5D) after transplantation. These results demonstrate the early start of the phenotypic change of the transplanted human blood CD34 cell into a cardiomyocyte and the long lasting nature of the event.

The population of double-positive cells were then collected with cell sorting and examined using fluorescent in situ hybridization (FISH) analysis in which specific probes for human and mouse X chromosomes were used simultaneously. The specificity of the probes was tested in mouse heart cells and human HeLa cells by incubating these cells with both probes, and it was confirmed that these two probes did not cross-react (data not shown). In the nuclei derived from cells that were troponin T+ or Nkx2.5+, but HLA-, only mouse X chromosomes were detected (Figure 6A). Because the recipient mice were female, two X chromosomes were observed in each nucleus. In troponin T+/Nkx2.5+ and HLA+ cells, approximately 70% of the nuclei contained both human and mouse X chromosomes (Table 1 and Figure 6B), suggesting that cell fusion had occurred. Because the human donor was male, one human X chromosome was paired with two mouse X chromosomes in each nucleus (Figure 6B). However, approximately 30% of the nuclei of troponin T+/Nkx2.5+, HLA+ cells contained only human X chromosomes (Table 1 and Figure 6C), suggesting that transdifferentiation of CD34+ cells had also taken place. FISH analysis on cells isolated from hearts harvested at 24 h and six months after transplantation also demonstrated that most of the nuclei contained both human and mouse X chromosomes, suggesting that fusion occurs as early as after 24 h and can be detected six months after transplantation (data not shown). On the other hand, FISH analysis demonstrated that almost all human CD34 cell-derived VE cells, which were sorted by co-expression of HLA and VE-cadherin, contained only human X chromosomes, indicating that transformation of these cells was due to either differentiation of the endothelial progenitor cells in the transplanted CD34+ population or transdifferentiation of the CD34+ hematopoietic stem cells (Table 2).

During the past two years, there have been intensive debates about the biological nature of the transformation of stem cells into cells of various tissue phenotypes. Two mechanisms have been proposed. Some investigators believe that the transformation is caused by de novo generation of tissue-specific cells such as muscle cells, hepatocytes, neurons, epithelial cells and endothelial cells, which are differentiated or transdifferentiated from the transplanted stem cells in response to the local microenvironment. In fact, most of the early reports on stem cell transformation into different cell lineages failed to study the mechanism of the transformation (26,28-30). Other investigators have challenged the notion of transdifferentiation by showing that fusion between transplanted cells and host cells is responsible for the apparent transformation of stem cells. Transformation of different stem cells by cell fusion has been observed in a number of different cell types, including hepatocytes (31,32), Purkinje neurons

Figure 5 Fluorescence-activated cell sorting (FACS) of cells isolated from the whole heart (injured) from severe combined immunodeficient mice with transplanted human peripheral blood CD34+ cells. **A** Human leukocyte antigen (HLA)+ cells determined by FACS analysis. **B** HLA and troponin T double-positive cells determined by FACS analysis. **C** Double-positive cells from a heart harvested 24 h after transplantation. **D** HLA and cardiac troponin T double-positive cells from a heart examined six months after transplantation. FITC Fluorescein isothiocyanate; Ig Immunoglobulin

(33,34) and cardiomyocytes (33,35,36). It is interesting to note that these fused cells not only express the specific tissue phenotype, some of them also perform the function of the host cells (32). Some recent studies in which more reliable approaches were used to detect or exclude fusion events have demonstrated that transdifferentiation is responsible for transformation of stem cells into some host cell phenotypes (37-40), although none of the studies have completely met the proposed stringent criteria of transdifferentiation, such as clonality of injected cells, avoidance of in vitro culturing or demonstration of phenotype changes at genomic and functional levels (41).

Most of the published studies on cell fusion between the transplanted stem cells and the host tissue cells are carried out in allogeneic transplantation models. Cell fusion in these studies has been detected by using cytogenetic analysis, in which

Figure 6 Both fusion and transdifferentiation account for the transformation of CD34+ cells into cardiomyocytes in vivo. **A** Nuclei from troponin T+, human leukocyte antigen (HLA)- cells. **B** Nuclei from 70% of troponin T+, HLA+ cells. **C** Nuclei from 30% of troponin T+, HLA+ cells. Green areas: mouse X chromosome; Red areas: human X chromosome. Reprinted from reference 27 with permission

TABLE 1

Fluorescent in situ hybridization analysis of the nuclei of human leukocyte antigen-positive (HLA+) and troponin+ or natural killer (Nkx2.5+) cells (cardiomyocytes)

HLA+ and	X chromosome in nuclei			Total nuclei counted, n
	Human and mouse, n (%)	Human, n (%)	Mouse, n (%)	
Troponin+	70 (70)	28 (28)	2 (2.0)	100
Troponin+	84 (71.2)	31 (26.3)	3 (2.5)	118
Troponin+	96 (78.7)	24 (19.7)	2 (1.6)	122
Troponin+	91 (71.1)	36 (28.1)	1 (0.8)	128
Nkx2.5+	91 (78.4)	19 (16.4)	6 (5.2)	116
Nkx2.5+	47 (69.1)	17 (25)	4 (5.9)	68
Nkx2.5+	86 (74.8)	26 (22.6)	3 (2.6)	115

cell ploidy and/or appearance of donor chromosomes in host cells are used as an indication of fusion (31,32). The drawback of the approach is the potential error due to the inaccuracy of the technique. More recently, cell fusion has been detected by using Cre recombinase-transgenic mice and the Cre reporter mouse as donor and recipient animals. Cell fusion is indicated when the reporter gene is expressed after the excision of a loxP-flanked stop cassette by Cre-mediated recombination (33). However, the technique is not 100% error free. For example, the Cre recombinase may fail to express in recipient tissue or the recipient tissue may not express the inserted reporter gene, which may lead to false-negative results. We have been using a xenogeneic transplantation model in which human peripheral blood CD34+ cells are injected into SCID mice after an experimental MI is induced. Taking advantage of this model, cardiomyocytes derived from human donor cells can be readily isolated from the mouse heart by FACS based on their ubiquitous expression of the HLA-ABC antigen. The isolated cells can be used for polymerase chain reaction detection of human-specific HLA genes to determine their origin, and/or for FISH detection by using specific human and mouse chromosome probes to further confirm the cell origin and to detect cell fusion. Using this approach, it has been demonstrated that fusion between the transplanted CD34+ cells and host cardiomyocytes plays a major role in phenotypic change of the CD34+ cells (27).

Findings that cell fusion accounts for the transformation of the transplanted stem cells into different cell types has raised a fundamental question about the value of stem cell transplantation in tissue regeneration. It is known that fusion between transplanted bone marrow-derived stem cells and host hepatocytes leads to liver regeneration and cure of the genetic liver disease fumarylacetoacetate hydrolase deficiency (32). It has been demonstrated that even macrophages can fuse with the host hepatocyte, resulting in a complete rescue of mice with the genetic liver disease (42). However, the mechanism for the rescue is unclear. Genes of donor hematopoietic cells may be reprogrammed after fusion with hepatocytes, and as a result, the fused cells express the hepatocyte phenotype and rescue the damaged liver. Gene reprogramming has also been reported in bone marrow-derived Purkinje neurons (43), in which fusion between bone marrow cells with Purkinje neurons resulted in stable heterokaryons that are dominant over the BMDCs, as the morphology is typical of functional Purkinje neurons, with complex dendritic trees and axons. Moreover,

TABLE 2

Fluorescent in situ hybridization analysis of the nuclei of human leukocyte antigen-positive (HLA+) and vascular endothelial-cadherin-positive (VEC+) cells

HLA+ and VEC+	X chromosome in nuclei			Total nuclei counted, n
	Human and mouse, n (%)	Human, n (%)	Mouse, n (%)	
Mouse 1	1 (1)	101 (97.1)	2 (1.9)	104
Mouse 2	0 (0)	127 (97.7)	3 (2.3)	130
Mouse 3	1 (0.8)	119 (98.4)	1 (0.8)	121
Mouse 4	0 (0)	136 (99.3)	1 (0.7)	137
Mouse 5	5 (3.6)	131 (95)	2 (1.4)	138
Mouse 6	3 (2)	141 (96)	3 (2)	147

cytoplasmic factors within the Purkinje neurons reprogrammed the fused BMDC nuclei, resulting in activation of a Purkinje neuronspecific transgene, L7-GFP (43).

Many investigators have reported that stem cell transplantation leads to improvement of cardiac function after MI in both animal experiments (44-49) and human studies (50-54) in which a variety of donor cells and different transplantation routes have been employed, and a general beneficial effect has been confirmed. However, it is difficult to explain the apparent improvement of the cardiac function by simple fusion between the donor cells and host cardiomyocytes. One possibility is that cell fusion also leads to reprogramming of the donor gene expression, which could be initiated by an altered intracellular milieu provided by the host tissues. As a result, transplanted stem cells may express the cardiac phenotype and re-enter the cell cycle, which may contribute to myocardial regeneration. This has been proved in an in vitro study in which cardiomyocytes could fuse with endothelial cells, cardiac fibroblasts and bone marrow cells, and the fused cells could re-enter the cell cycle (55). On the other hand, genes of the recipient cell may also be reprogrammed and their expression may be activated or inactivated. The fate of the recipient cardiomyocytes may be changed. For example, apoptosis of cardiomyocytes after MI may be stopped or reversed. Therefore, gene reprogramming may rescue the host cardiomyocyte that is designated to die from apoptosis after MI to reserve the myocardium, or bestow the remaining cardiomyocyte ability of proliferation that may facilitate myocardial regeneration. The approach that we described here could be used to study the mechanism and the long-term consequence of cell fusion in myocardial regeneration.

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