The Rat Myosin Light Chain Promoter-Driven DsRed Reporter System Allows Specific Monitoring of Bone Marrow Mesenchymal Stem Cell-Derived Cardiomyocytes

Seung-Cheol Choi and Do-Sun Lim†

Department of Cardiology, College of Medicine, Korea University, Seoul 136-705, Korea

ABSTRACT

Bone marrow mesenchymal stem cells (BMMSCs) have the capacity for self-renewal and differentiation into a variety of cell types. They represent an attractive source of cells for gene and cell therapy. The purpose of this study is to direct the specific expression of the DsRed reporter gene in Sca-1$^+$ BMMSCs differentiated into a cardiomyogenic lineage. We constructed the prMLC-2v-DsRed vector expressing DsRed under the control of the 309 bp fragment of the rat MLC-2v 5'-flanking region. The specific expression of the DsRed reporter gene under the transcriptional control of the 309 bp fragment of the rat MLC-2v promoter was tested in 5-azacytidine treated-Sca-1$^+$ BMMSCs over 2 weeks after the prMLC-2v-DsRed transfection. The prMLC-2v-DsRed was specifically expressed in the Sca-1$^+$ BMMSCs with cardiomyogenic lineage differentiation, and it demonstrates that the 309 bp sequences of the rat MLC-2v 5'-flanking region is sufficient to confer cardiac specific expression on a DsRed reporter gene. The cardiac-specific promoter-driven reporter vector provides an important tool for the study of stem cell differentiation and cell replacement therapy in ischemic cardiomyopathy.

(Key words : Cardiomyogenic differentiation, DsRed, Promoter, Mesenchymal stem cells, Reporter gene)

INTRODUCTION

Bone marrow mesenchymal stem cells (BMMSCs) are self-renewing, clonal precursors of non-hematopoietic tissues (Pittenger *et al.*, 1999). They can differentiate into a variety of cell types including cardiac and endothelial lineages and have been used as an important cell source for regeneration of infarcted myocardium (Pittenger *et al.*, 1999; Shake *et al.*, 2002; Toma *et al.*, 2002; Mangi *et al.*, 2003; Amado *et al.*, 2005). However, the concept of stem cell plasticity has been challenged by contradictory reports demonstrating no cardiac differentiation of transplanted cells in infarcted myocardium. Nygren *et al.* (2004) reported that bone marrow-derived hematopoietic cells generated cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation in infarcted myocardium. Furthermore, haematopoietic stem cells adopted a mature haematopoietic fate which is different from the transdifferentiation into cardiac myocytes in ischemic myocardium (Balsma *et al.*, 2004; Murry *et al.*, 2004). Therefore, it is important to determine whether transplanted cells differentiate into cardiomyocytes, and not into other cells, and to define the mechanism(s) that cause functional improvement after cell transplantation into infarcted myocardium.

A 250 bp promoter fragment of the myosin light chain 2v (MLC-2v) gene is known to confer cardiac-specific and inducible expression of a luciferase reporter gene *in vitro* and in transgenic mice (Henderson *et al.*, 1989; Lee *et al.*, 1992; O'Brien *et al.*, 1993; Lee *et al.*, 1994; Ross *et al.*, 1996). The key element for ventricular-specific gene expression is a 28 bp of HF-1 *in vitro* and *in vivo* (Zhu *et al.*, 1991; Lee *et al.*, 1994; Ross *et al.*, 1996). Furthermore, negative regulatory elements (E-box and HF-3) within the 250 bp 5'-flanking region of the rat MLC-2v gene also has been shown to mediate ventricular chamber-specific expression in transgenic mice (Lee *et al.*, 1994; Ross *et al.*, 1996). In this study, we constructed the reporter vector expressing DsRed under the transcriptional control of a 309 bp fragment of the rat MLC-2v promoter including the 250 bp 5'-flanking region of the rat MLC-2v gene to trace cardiac specific expression in Sca-1$^+$ BMMSCs.

MATERIALS AND METHODS

Plasmid Construction

A 309 bp fragment of the MLC-2v 5'-flanking region (GenBank #RNU26708) was amplified by PCR from the

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† Corresponding author : Phone: +82-2-920-5445, E-mail: dslmd@kumc.or.kr

Sprague-Dawley rat genomic DNA using the primers 5'-CTCGAGTCCCCTTCCTGGGTTACTTT-3' and 5'-AGAATTCAAGGAGCCTGCTG-3', which contains *Xho*I and *Eco*RI sites (underlined) at the 5'-end and the 3'-end. The PCR reaction (25 μl) contained 2.5 μl 10x buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP mix, 1 μM forward primer, 1 μM reverse primer, 50 ng of the rat genomic DNA, and 2 units of *Tag* polymerase (Genenmed Inc., Seoul, Korea). RT-PCR reactions were carried out in a thermocycler (Bio-Rad, Hercules, CA, USA) with 35 cycles of amplification according to the following program: denaturation step at 94℃ for 5 min, annealing step at 56℃ for 40 sec, polymerization step at 72℃ for 40 sec, and an extension period of 7 min at 72℃. The PCR products were cloned into the pGEM-T vector (Promega, Madison WI, USA), subcloned into the *Xho*I/*Eco*RI sites of the promoterless pDsRed vector (Clontech, Mountain View, CA, USA) and designated as the prMLC-2v-DsRed. The constructed plasmid was confirmed by DNA sequencing.

Sca-1 Expression in BMMSCs

Bone marrow cells were obtained by flushing the femurs of 5- to 6-week-female ICR mice with Dulbecco's Modified Eagle Medium (DMEM) (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (v/v) FBS (Gibco-BRL), 100 units/ml penicillin and 100 μg/ml streptomycin. The cells were cultured in DMEM with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin on 0.1% (w/v) gelatin-coated 10 cm dishes at 37℃ in a CO_2 incubator. After 3 days, the non-adherent cells were removed, and the adherent cells were expanded until 90% confluent (~6 to 7 days). Following the third passage, 3×10^4 BMMSCs were plated onto coverslips coated with 0.1% (v/v) gelatin in a 24-well plate. The cells were fixed with 4% paraformaldehyde in PBS for 10 min and washed with PBS containing 0.1% Tween 20 (PBST). The cells were blocked for non-specific binding by incubation in 5% normal goat serum (Gibco-BRL) in PBST for 30 min. Next, the cells were stained for 30 min with anti-Sca-1 antibody (1:100; BD Biosciences, Franklin Lakes, NJ, USA). The cells were then stained with a secondary Alexa Fluor-488-conjugated anti-rat IgG (1:1,000; Molecular Probes, Eugene, OR, USA) for 30 min, and washed 3 times in PBST. The nuclei were stained with 4′,6′-diamidino-2-phenylindole (DAPI), and the cells were mounted with fluorescent mounting medium (Dako, Carpinteria, CA, USA). The fluorescence images were obtained using the TE-FM Epi-Fluorescence system attached to an Olympus Inverted Microscope (Olympus, Tokyo, Japan).

Isolation of Sca-1$^+$ BMMSCs

Following passage 3, Sca-1$^+$ BMMSCs were enriched by magnetic activated cell sorting (MACS) system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, the BMMSCs were incubated with phycoerythrin (PE)-conjugated anti-Sca-1 antibody (BD Biosciences) for 15 min at 4℃, and washed with MACS buffer (PBS supplemented with 0.5% BSA, 2mM EDTA). Next, anti-PE microbeads (Miltenyi Biotec GmbH) were incubated for 15 min at 4℃, and washed once again with MACS buffer. The samples were passed through a MACS column set up in a Miltenyi magnet and the Sca-1$^+$ BMMSCs were eluted from the column by washing with MACS buffer. To increase the purity of the Sca-1$^+$ BMMSCs, magnetic sorting was performed four times.

Cardiac Differentiation of Sca-1$^+$ BMMSCs

Sca-1$^+$ BMMSCs were seeded onto 0.1% (v/v) gelatin-coated coverslips in 24-well plates at 2×10^4 cells/well. Twenty-four hours after seeding, the cells were transfected with 1 μg of the prMLC-2v-DsRed using the Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After transfection, the cells were incubated at 37℃ for 36 hrs in a CO_2 incubator. For cardiac differentiation, the cells were incubated in DMEM with 10% (v/v) FBS, 100 units/ml penicillin and 100 μg/ml streptomycin with 1 μM 5-azacytidine (Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks. The cultures were maintained by exchanging the media every third day. To assess the cardiac differentiation of Sca-1$^+$ BMMSCs, the cells were fixed with 4% (w/v) paraformaldehyde in PBS for 10 min, washed with PBST, and permeabilized with 0.1% (v/v) Triton X-100 in PBS for 30 min. The cells were incubated in 5% (v/v) normal goat serum in PBST for 30 min, incubated overnight at 4℃ with the anti-MLC antibody (1:500; Sigma-Aldrich). After washing three times with PBST, the cells were incubated with a secondary Alexa 488-conjugated antibody (1:1,000) for 30 min. Washing three times with PBST again and the cells were stained with DAPI. The fluorescence images were collected with the TE-FM Epi-Fluorescence system attached to an Olympus Inverted Microscope.

RESULTS

Construction of the Rat MLC Promoter-Driven DsRed Vector

In order to direct specific expression of DsRed reporter gene in Sca-1$^+$ BMMSCs differentiated into a cardiomyogenic lineage, we constructed the prMLC-2v-DsRed vector expressing DsRed under control of the 309 bp fragment of the rat MLC-2v 5'-flanking region (Fig. 1). The 309 bp fragment of the MLC-2v 5'-flanking region was amplified by PCR from the rat genomic DNA, cloned into a TA cloning vector (pGEM-T) and finally subcloned

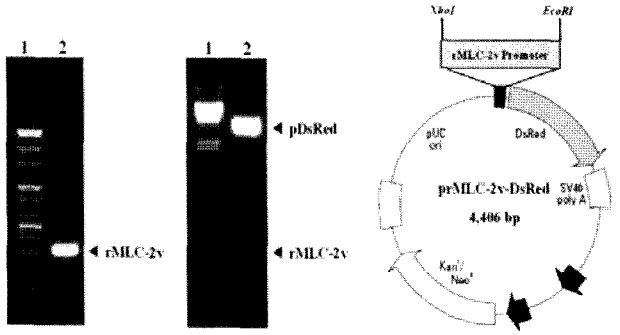

Fig. 1. Cloning of rat MLC promoter and schematic structure of prMLC-2v-DsRed. (A) A 309 bp DNA fragment of the 5' upstream regulatory sequence of the rat MLC-2v gene was amplified by PCR from the rat genomic DNA. Lane 1: 100 bp DNA Ladder. Lane 2: the 309 bp PCR product amplified from rat genomic DNA. (B) The amplified 309 bp PCR product was cloned into the promoterless pDsRed vector. Lane 1: lamda/*HindIII* size marker. Lane 2: pDsRed vector harboring the 309 bp MLC-2v promoter digested with *EcoRI*/*XhoI*. (C) Schematic structure of the prMLC-2v-DsRed.

into the promoterless pDsRed vector. By restriction mapping and sequencing, the 309 bp promoter region of the prMLC-2v-DsRed was confirmed as containing the 5'-flanking region of the rat MLC-2v gene.

Specificity of the DsRed Expression in Sca-1$^+$ BMMSC-Derived Cardiomyocytes

We recently reported that Sca-1 molecule was expressed in BMMSCs, and Sca-1$^+$ BMMSCs have multi-potentiality differentiating into cardiac, endothelial, adipogenic and osteogenic cell lineages (Choi *et al.*, 2008). The freshly isolated BMMSCs were subcultured three times to remove the non-adherent cells. After passage 3, some BMMSCs strongly expressed Sca-1, whereas some BMMSCs weakly expressed Sca-1 (Fig. 2). To test the cardiac-specific monitoring system constructed in this study, we purified a homogeneous population of BMMSCs by using the MACS system as described previously (Choi *et al.*, 2008).

The specific expression of the DsRed reporter gene under the transcriptional control of the 309 bp fragment of the rat MLC-2v promoter was tested in 5-azacytidine treated-Sca-1$^+$ BMMSCs over 2 weeks after the prMLC-2v-DsRed transfection. Fig. 3 shows a merged image of cells expressing DsRed and MLC-2v, visualized by the primary anti-MLC and secondary Alexa-488-conjugated antibodies after 5-azacytidine-treated Sca-1$^+$ BMMSCs. This result demonstrates that the 309 bp sequence of the rat MLC-2v 5'-flanking region was sufficient to confer cardiac-specific expression on the DsRed reporter gene. In Sca-1$^+$ BMMSCs transfected with the promoterless pDsRed vector, no DsRed activity was detectable (data not shown). No red background fluorescence was detected in a negative control stained with the secondary antibody only (data not shown).

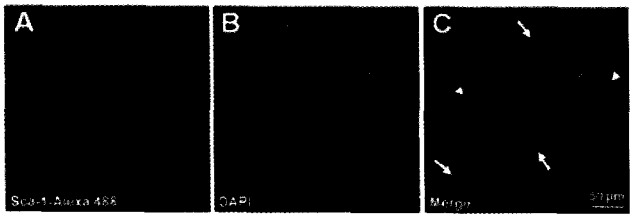

Fig. 2. Isolation of Sca-1$^+$ BMMSCs. (A) At the third passage, BMMSCs were stained with anti-Sca-1 antibodies, and visualized with Alexa Fluor-488-conjugated anti-rat antibody. (B) Nuclei were stained with DAPI. (C) Merged image with DAPI staining was shown. Some BMMSCs strongly expressed Sca-1 (arrows), whereas some BMMSCs weakly expressed Sca-1 (arrowheads). Scale bar = 50 μm.

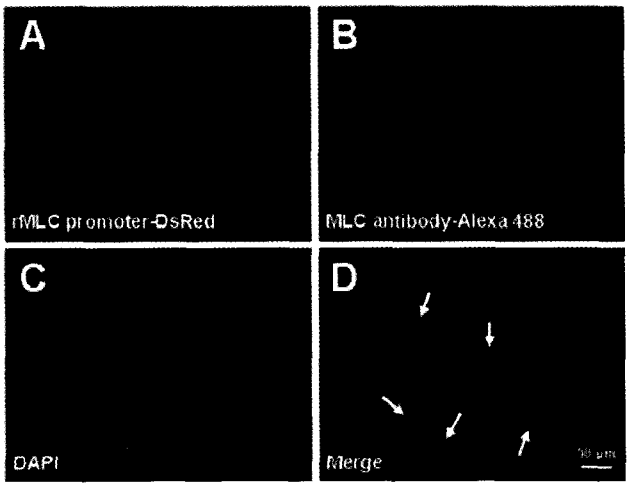

Fig. 3. Restricted expression of DsRed reporter gene in Sca-1$^+$ BMMSC-derived cardiomyocytes. Sca-1$^+$ BMMSCs were transfected with the prMLC-2v-DsRed, treated with 1 μM 5-azacytidine for 2 weeks, and their specificity was evaluated by staining with the MLC antibody. (A) DsRed-positive cells under the control of the 309 bp fragment of MLC-2v promoter. (B) Cells labeled with the MLC antibody followed by a secondary Alexa-488-conjugated antibody (C) Cells stained with DAPI (D) Merged image of cells both expressing the DsRed and the MLC proteins (arrows).

DISCUSSION

Although the MLC-2v promoter is 3 kb long, previous studies have identified the first 250 bp sequence of the promoter to be critical for the mediation of cardiac-specific gene expression *in vitro* (Henderson *et al.*, 1989; Zhu *et al.*, 1991). A deletion assay of the 5'-flanking region of the rat MLC-2v gene demonstrated that a 250 bp of the MLC-2v 5'-flanking region was sufficient to confer cardiac specific expression on a luciferase reporter gene, inducing at least a 70-fold increase in the level of luciferase activity in the cardiac cells versus non-cardiac cells

(Henderson *et al.*, 1989). The 250 bp of the 5'-flanking region of the rat MLC-2v gene containing CArG motifs and additional *cis* elements was conserved in the sequence and relative position in the chick cardiac MLC-2v gene, suggesting its importance in the tissue specific and regulated expression of the cardiac MLC-2v gene (Henderson *et al.*, 1989). A conserved 28-bp element (HF-1) in the rat cardiac MLC-2v gene was shown to be critical both for cardiac-specific and inducible expression in transient assays (Zhu *et al.*, 1991).

In addition, the 250 bp of the MLC-2v 5'-flanking region conferred cardiac ventricular specificity when used to target gene expression in the ventricle of transgenic mice (Lee *et al.*, 1992; O'Brien *et al.*, 1993; Lee *et al.*, 1994; Ross *et al.*, 1996). Point mutations within the conserved regulatory sites HF-1a and HF-1b significantly obstructed ventricular muscle specificity in transgenic mice (Lee *et al.*, 1992). Transgenic mice harboring a 250 bp MLC-2v promoter fragment fused to a luciferase demonstrated reporter gene activity in the primitive murine heart tube (O'Brien *et al.*, 1993). Lee *et al.* (1994) reported that positive elements (HF-1a and HF-1b) and negative regulatory elements (E-box and HF-3) mediated ventricular chamber-specific expression of the 250 bp of MLC-2v promoter-luciferase fusion genes in transgenic mice. Mutations in the E-box regulatory domain within 250 bps of the rat MLC-2v promoter element abolished transgene expression in multiple transgenic lines (Ross *et al.*, 1996). Therefore, we constructed the prMLC-2v-DsRed vector including the first 250 bp 5'-flanking region of the rat MLC-2V gene. Consistent with previous reports, we showed that the 309 bps of the rat MLC-2v promoter also conferred specific expression in the Sca-1$^+$ BMMSC-derived cardiomyocytes.

Cardiac-specific promoter systems driving reporter genes have been primarily used to isolate cardiomyocytes from embryonic stem (ES) cells or embryonal carcinoma cells. Meyer *et al.* (2000) demonstrated that the MLC-2v promoter conferred ventricular specificity to the enhanced cyan fluorescent protein (ECFP) expression within the embryoid body as revealed by MLC-2v costaining of ECFP fluorescent cells with a CGR8 ES cell clone harboring MLC-2v promoter driving ECFP. Ventricular-like cardiomyocytes were selected from ES cells stably transfected with the enhanced green fluorescent protein (EGFP) under control of the MLC-2v promoter and 0.5 kb enhancer element of the cytomegalovirus (Müller *et al.*, 2000). Hattan *et al.* (2005) demonstrated that cardiomyocytes purified from BMMSCs transfected with a 2.7 kb of the MLC-2v promoter-driven EGFP vector could produce stable intracardiac grafts in mice. Similar to previous reports, we also demonstrated that prMLC-2v-DsRed allowed specific monitoring of the Sca-1$^+$ BMMSCs differentiating into cardiomyocytes under fluorescent microscopy. Gruber *et al.* (2004) showed that direct injection of a 3 kb MLC-2v promoter-driven luciferase into mouse myocardium allowed *in vivo* detection in the hearts of live mice over 3 weeks. These results suggest that the cardiac tissue-driven reporter system could be used as an important tool for cell replacement therapy for ischemic cardiomyopathy.

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