Genetic selection system allowing monitoring of myofibrillogenesis in living cardiomyocytes derived from mouse embryonic stem cells

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Embryonic stem (ES) cell-derived cardiomyocytes recapitulate cardiomyogenesis *in vitro* and are a potential source of cells for cardiac repair. However, this requires enrichment of mixed populations of differentiating ES cells into cardiomyocytes. Toward this goal, we have generated bicistronic vectors that express both the blasticidin S deaminase (*bsd*) gene and a fusion protein consisting of either myosin light chain (MLC)-3f or human α-actinin 2A and enhanced green fluorescent protein (EGFP) under the transcriptional control of the α-cardiac myosin heavy chain (α-MHC) promoter. Insertion of the DNase I-hypersensitive site (HS)-2 element from the β-globin locus control region, which has been shown to reduce transgene silencing in other cell systems, upstream of the transgene promoter enhanced MLC3f-EGFP gene expression levels in mouse ES cell lines. The α-MHC-α-actinin-EGFP, but not the α-MHC-MLC3f-EGFP, construct resulted in the correct incorporation of the newly synthesized fusion protein at the Z-band of the sarcomeres in ES cell-derived cardiomyocytes. Exposure of embryoid bodies to blasticidin S selected for a relatively pure population of cardiomyocytes within 3 days. Myofibrillogenesis could be monitored by fluorescence microscopy in living cells due to sarcomeric epitope tagging. Therefore, this genetic system permits the rapid selection of a relatively pure population of developing cardiomyocytes from a heterogeneous population of differentiating ES cells, simultaneously allowing monitoring of early myofibrillogenesis in the selected myocytes.

Key words: embryonic stem cells, cardiomyocytes, selection, differentiation, myofibrillogenesis, sarcomere

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Death of cardiomyocytes, mainly due to acute or chronic myocardial ischemia, may lead to progressive heart failure. While pharmacological agents may slow down the progression of the disease, they ultimately fail to prevent it. Heart transplantation is the only definitive treatment for end-stage heart failure, but it suffers from major limitations including organ donor shortage, co-morbidity of immunosuppressive treatments, and chronic graft rejection. Over the past few years, cell transplantation has emerged as a potentially useful strategy for cardiac repair. Embryonic stem (ES) cells are an appealing candidate cell type for cell therapy approaches, as they can differentiate into derivatives of all three primary germ layers (endoderm, mesoderm and ectoderm) *in vitro*, including cardiomyocytes (Kehat *et al.*, 2001; Xu *et al.*, 2002; Fijnvandraat *et al.*, 2003; Boheler *et al.*, 2005). ES cells have been established as permanent lines of multipotent undifferentiated cells derived from the inner cell mass of blastocysts (Boheler *et al.*, 2005). Cardiac differentiation in ES cell aggregates called embryoid bodies (EBs) is evidenced by the emergence of cell clusters showing spontaneous, rhythmic contractions. ES cell-derived cardiomyocytes resemble cardiomyocytes of the embryonic heart tube (Fijnvandraat *et al.*, 2003). They express α- and β-cardiac myosin heavy chain (MHC) (Sanchez *et al.*, 1991), myosin light chain (MLC) isoforms 2a and 2v (Miller-Hance *et al.*, 1993; Fijnvandraat *et al.*, 2003), cardiac troponin I (cTnI) (Fijnvandraat *et al.*, 2003), α-tropomyosin (Muthuchamy *et al.*, 1993), phospholamban (Ganim *et al.*, 1992), sarcoendoplasmic reticulum calcium ATPase (SERCA) 2a (Fijnvandraat *et al.*, 2003), atrial natriuretic factor (Miller-Hance *et al.*, 1993), and type B natriuretic factor (Boer, 1994). They exhibit electrophysiologic features of atrial-like, ventricular-like, and conduction system-like cardiomyocytes (Kolossov *et al.*, 2005). Therefore, ES cell-derived cardiomyocytes provide a unique model for studying structur-

al and functional aspects of cardiomyogenic differentiation *in vitro*.

Because pluripotent ES cells differentiate into many different cell types, enrichment of mixed populations of differentiating ES cells into cardiomyocytes is required. In fact, transplantation of pluripotent ES cells can induce tumor formation (teratoma) (Erdo *et al.*, 2003). Earlier genetic selection systems have used two different approaches to purify ES cell-derived cardiomyocytes. Klug *et al.* (1996) generated genetically modified totipotent mouse ES cells that expressed an aminoglycoside resistance gene under the transcriptional control of the α-cardiac MHC (α-MHC) promoter. Transgenic ES cell-derived cardiomyocytes enriched with G418 after *in vitro* differentiation were injected into mouse hearts where they were detectable *in vivo* 7 weeks thereafter. Several studies have used a reporter gene approach. Kolossov *et al.* generated stably transfected ES cells expressing the green fluorescent protein (GFP) gene under control of the human cardiac α-actin promoter (Kolossov *et al.*, 1998) or enhanced GFP (EGFP) under control of the α-MHC promoter (Kolossov *et al.*, 2005). EGFP expression in cardiac precursors was observed as early as 7 days of development, even before the appearance of spontaneous contractions. Müller *et al.* (2000) and Meyer *et al.* (2000) purified ES cell-derived cardiomyocytes from EBs stably transfected with the EGFP reporter gene or its cyan variant (ECFP) under control of the MLC-2v promoter. This promoter was switched on as early as day 7, as evidenced by the appearance of fluorescent cells, followed by that of spontaneous beating activity in all regions containing such cells by day 8. Moore *et al.* (2004) used a P19C16 GFP reporter line to quantify cardiomyocyte differentiation of stem cells. Hidaka *et al.* (2003) developed an *in vitro* culture system to track cell lineages positive for the cardiac transcription factor Nkx-2.5 during mouse ES cell differentiation using GFP as a reporter. Huber *et al.* (2007) generated stable transgenic human ES cell lines using lentiviral vectors that express EGFP under control of the MLC-2v promoter. ES cell-derived EGFP+ cardiomyocytes purified by fluorescence-activated cell sorting (FACS) formed stable cell grafts *in vivo* after intramyocardial cell injection.

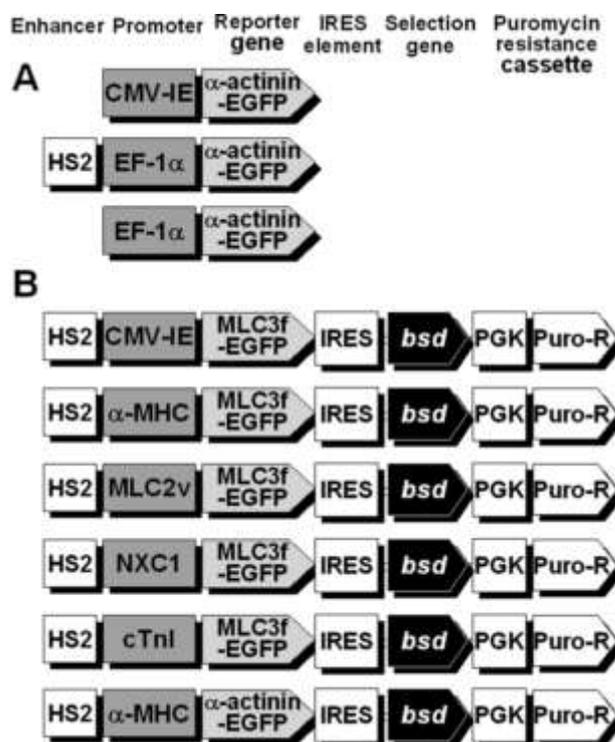The two selection modalities – drug resistance and reporter genes – have distinct advantages and limitations. While drug selection results in relatively pure populations of ES cell-derived cardiomyocytes, it does not allow direct visualization of the selected cardiomyocytes, nor of myofibrillogenesis taking place in these cells. This requires cell fixation, permeabilization, and immunostaining of myofibrillar proteins, which precludes longitudinal analysis of different stages of myofibrillogenesis in viable cells. Conversely, ES cell-derived cardiomyocytes that express a reporter gene can be readily identified and purified by FACS, but this requires enzymatic dissociation of cell aggregates to obtain a population of individual cells. Although ES cell-derived cardiomyocytes can recover after the FACS procedure and reestablish cell-cell contacts and sarcomeres in culture, this manipulation perturbs the regular unfolding of cell differentiation and myofibrillogenesis *in vitro*. Hence, combining the drug resistance and the reporter gene approach may be advantageous. Toward this goal, we generated bicistronic vectors that express both the blasticidin S deaminase (*bsd*) gene from *Aspergillus terreus* (Kimura *et al.*, 1994) and a fusion protein consisting of a sarcomeric protein – the MLC isoform MLC3f or the human α-actinin 2A isoform – and EGFP under the transcriptional control of the αMHC promoter. Additional cardiac-specific promoters were also tested. Blasticidin S treatment of EBs from transgenic ES cell lines resulted in a relatively pure population of cardiomyocytes after 3 days of selection. Correct localization of the α-actinin-EGFP fusion protein at the Z-band of the sarcomeres allowed monitoring of myofibrillogenesis in differentiating cardiomyocytes by vital fluorescence microscopy.

Materials and Methods

Gene expression vectors

The principal vectors used in the present study were derived from the bicistronic SM18.2 expression plasmid (7165 bp) that contains a MLC3f (bp 942-2159)-EGFP fusion cassette (Auerbach *et al.*, 1997) driven by the cytomegalovirus-immediate/early (CMV-IE) promoter, the Internal Ribosome Entry Site (IRES) element (starting at bp 2187; derived from pIRES, Clontech Laboratories, Palo Alto, CA, USA), the *bsd* gene (inserted 3′ of the IRES at bp 2772-3150; derived from pCMV/Bsd, Invitrogen, Basel, Switzerland), and a puromycin resistance (PuroR) cassette (bp 4126-4706) driven by the phosphoglycerate kinase (PGK) promoter

(Figure 1). The α-MHC promoter was subcloned into the SM18.2 vector as follows: the 5.5-kb α-MHC promoter fragment (Gulick *et al.*, 1991) comprising of 4.5 kb of 5' flanking sequence and 1 kb of the gene encompassing exons 1 through 3 up to, but not including, the initiation codon was amplified from the αMHC-clone26 plasmid (gift of Dr. J. Gulick, Children's Hospital, Cincinnati, OH, USA). Insertion of the *MluI/MfeI*-digested PCR-amplification fragment (primers: Table 1) from αMHC-clone26 into *MluI/EcoRI*-digested SM18.2 released the CMV-IE promoter fragment (bp 228-935). *E. coli* Top10 (Invitrogen) were transformed with the ligation product, yielding the α-MHC-MLC3f-EGFP-IRES-BSD expression plasmid. The 750-bp MLC-2v promoter fragment including 3 copies of the HF-1, HF-2 and HF-3 upstream elements that enhance promoter activity and cardiac specificity (Jin *et al.*, 1995) was amplified from the MLCp.luc plasmid (gift of Dr. Y. Jin, University of Manitoba, Winnipeg, Canada) by PCR. The *MluI/MfeI*-digested amplification fragment from MLCp.luc was subcloned into SM18.2, yielding the MLC2v-MLC3f-EGFP-IRES-BSD construct. The 2.6-kb Na$^+$/Ca2$^+$ exchanger 1 (NCX1) promoter sequence was amplified from the pGL2-Basic Vector+2.6Kb NCX1 promoter vector (Müller *et al.*, 2002) (gift of Dr. J. G. Müller, Medical University of South Carolina, Charleston, SC, USA). The *MluI/EcoRI*-digested PCR fragment was subcloned into SM18.2, yielding the NCX-MLC3f-EGFP-IRES-BSD construct. The 463-bp human cTnI promoter fragment was amplified from the -463troponinI gene construct (Bhavsar *et al.*, 2000) (gift of Dr. P. Barton, Imperial College School of Medicine, London, UK). The *MluI/EcoRI*-

Figure 1. Schematics of the principal expression vectors used in the present study. Boxes depict functional elements as indicated on top of figure. (A) Vectors used for analysis of the effect of the HS2 element on α-actinin-EGFP expression in ES cell lines. (B) Bicistronic HS2 element-containing vectors used in rat cardiomyocytes, COS cells, and ES cell lines (abbreviations: see text).

cut amplification fragment was subcloned into SM18.2, yielding the cTnI-MLC3f–EGFP-IRES-BSD construct. The human α-actinin 2A isoform gene was amplified by PCR from pET6-ACTN2 (Young *et al.*, 1998) (gift of Dr. M. Gautel, The Randall Centre, King's College, London, UK). The *EcoRI/NotI* MLC3f-EGFP fragment (1.2-kb) of α-MHC-MLC3f-EGFP-IRES-BSD was replaced

Table 1. PCR primers used for amplification of DNA fragments for molecular cloning: lox66-MHC.up/lox66-MHC.lo for the α-MHC promoter fragment; HumTropLox66.s/ HumTropLox66.as for the cTnI promoter fragment; MLC2vLox66.s/MLC2vLox66.as for the MLC-2v promoter fragment; NCX-upp/NCX-low for the NCX1 promoter fragment; HS2ext-s/HS2ext-as for amplification of the 523 bp fragment of the HS2 element from genomic DNA; HS2Mlu.s/HS2Mlu.as for the HS2-EF1α-EGFP fragment.

Primer	Sequence	Restriction sites
lox66-MHC.up	ccc**acgcgt**ataacttcgtatagcatacattatacgaacggtaGGATCCTGCAAGGTCACACAAGGGTC	MluI
lox66-MHC.lo	cccc**caattg**GTCGACTCAAACTCTTATG	MfeI
HumTropLox66.s	ccc**acgcgtat**aacttcgtatagcatacattatacgaacggtaGCGATTAAGTTGGGTAACGC	MluI
HumTropLox66.as	ccc**gaattc**GGGTGACCTTCAGGGTCCCA	EcoRI
MLC2vLox66.s	ccc**acgcgt**ataacttcgtatagcatacattatacgaacggtaTGTCTGGATCCCCCAG	MluI
MLC2vLox66.as	cccCAATTGCAGCTGCAGAATTCAAGGAG	MfeI
NCX-upp	ccc**acgcgt**ataacttcgtatagcatacattatacgaacggtaTAAAGTAGAAAAAGGCAAGGGAAGAG	MluI
NCX-Low	ccc**gaattc**AGGAAGGAATATTCATGAATG	EcoRI
HS2ext-s	CCCATTAATTGACCAGGTCTTCTGGCAG	AseI
HS2ext-as	CCCATTAATCAGGGTCCCCTTTTCATTG	AseI
HS2Mlu.s	GGGG**acgcgt**ATCGATTCGAATGACCAGGTCTTCTGGCAG	MluI, ClaI, BstBI
HS2Mlu.as	CCC**acgcgt**CAGGGTCCCCTTTTCATTG	MluI

with the α-actinin–EGFP fragment (Dätwyler et al., 2001), yielding the α-MHC-α-actinin-EGFP-IRES-BSD construct. The 523-bp DNase I-hypersensitive site (HS)-2 element (accession number: S73747.1) from the β-globin Locus Control Region (LCR) (Walters et al., 1996; Bulger et al., 1999; Grosveld, 1999) was amplified from mouse genomic DNA by PCR (primers: see Table 1), digested with MluI, and subcloned into the SM13.4 and FS2.2 vectors that contain an expression cassette for the α-actinin-EGFP fusion protein driven by the CMV-IE promoter and the 475-bp human elongation factor (EF)-1α promoter (Chung et al., 2002), respectively, yielding the HS2-CMV-α-actinin-EGFP and HS2-EF1-α-actinin-EGFP expression constructs. Finally, the HS2 fragment was also inserted upstream of the respective cardiac-specific promoters in bicistronic expression constructs.

Stable transfection of mouse ES cells

ES cells from the R1 cell line (Nagy et al., 1993) (gift of Dr. A. Nagy, Mount Sinai Hospital, Toronto, Canada) were used at P17 and cultured in Knock-out Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamin, nonessential amino acids, 1% sodium pyruvate, 15% Knock-out serum replacement (all from Gibco Laboratories, Grand Island, NY, USA), 1 mmol/L β-mercaptoethanol (Sigma, St. Louis, MO, USA), and leukemia inhibitory factor (LIF)-conditioned medium (0.4%), essentially as described (Smith, 1992). Electroporation, puromycin selection, and propagation of clones was performed according to standard protocols (Kolossov et al., 1998). Briefly, ES cells were trypsinized, resuspended in PBS, and diluted to a final concentration of 7×10^5 cells/mL. The cell suspension (0.8 mL) was mixed with 10 μg of vector DNA linearized with MluI and electroporated at 250 V. Cells were plated onto 100-mm plates in 13 ml ES cell medium. After 48 h, 3 μg/mL puromycin (InvivoGen; San Diego, CA, USA) was added. Stably transfected cell clones (n = 25 for each transfection vector tested) were monitored daily for appearance of green fluorescent cell clusters under a fluorescence microscope.

Transient transfection of neonatal rat cardiomyocytes

Newborn rat hearts were dissected, ventricles were minced, and tissue fragments were digested with collagenase (Worthington Biochemicals, Lakewood, NJ, USA) and pancreatin (Gibco), fol-lowed by purification on a Percoll density step gradient, and cultured essentially as described (Sen et al., 1988). Cells (4×10^5 cells/dish) were plated on fibronectin (10 μg/mL)-coated dishes in 67% DMEM, 17% M199, 4 mmol L-glutamine (Amimed, Basel, Switzerland), 10% horse serum (Gibco), 5% fetal calf serum (FCS; PAA Laboratories, Parker Ford, PA, USA), and 1% penicillin/streptomycin (Gibco). After 24 h, medium was replaced with maintenance medium (78% DMEM, 20% M199, 1% horse serum, 4 mmol L-glutamine, 1% penicillin/streptomycin, and 0.1 mM phenylephrine; Sigma). For transfection, 1 μg of vector DNA and 4 μL of Escort III (Sigma) were diluted in 100 μL of OptiMEM (Gibco). The transfection mix was added drop by drop to cell cultures according to the manufacturer's instructions. After cell incubation at 37°C for 4 h, transfection medium was replaced with maintenance medium.

Transfection of COS-1 cells

COS-1 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and grown at 37°C under 5% CO_2 in 89% Iscove's MEM (Amimed), 4 mmol/L glutamine, 1% penicillin/streptomycin, 10% FCS (PAA Laboratories), and 1 mmol/L β-mercaptoethanol. A mixture of 97 μL OptiMEM and 3 μL FuGENE 6 Transfection Reagent (Roche, Basel, Switzerland) was used for transfection. DNA (1 μg) was added to the transfection mixture, which was incubated for 15 min at RT, and then added to COS-1 cells according to the manufacturer's instructions. Transfected cells were incubated in the above medium under 10% CO_2 for 2 days.

Functional analysis of the HS2 element in transgenic ES cell lines

To assess the effect of the HS2 element on α-actinin-EGFP fusion protein expression, ES cells were stably transfected with either the CMV-α-actinin-EGFP or the EF1-α-actinin-EGFP vector, with or without the insertion of the HS2 element upstream of the transgene promoter. Flow cytometric analysis of EGFP expression was performed using a dual laser FACSCalibur® (Becton Dickinson, Germany). ES cells were trypsinized and resuspended to a concentration of 5×10^5 cells/mL in PBS shortly before analysis. FITC bandpass filter was used to measure EGFP fluorescence intensity excited with the 488 nm line of an argon ion

laser. Detector settings were adjusted with non-transfected ES cells of the cell line R1. Each analysis included 10^4 cells/clone (n=5 clones/vector type). Data were recorded with the CellQuest™ acquisition software (Becton Dickinson).

ES cell differentiation and selection of cardiomyocytes

To induce ES cell differentiation, ES cells (16,000/mL) were suspended in Knock-out DMEM medium supplemented with 4 mmol L-glutamine, nonessential amino acids, 1% sodium pyruvate, 15% FCS, and 1 mmol/L β-mercaptoethanol. Hanging drops were formed by plating 30 µL of cell suspension on the lids of 150-mm bacteriological dishes. After cell incubation at 37°C under 5% CO_2 for 48 h, EBs were formed. They were collected in 15 mL differentiation medium, cultured in suspension in bacteriological dishes for 2 days, and plated onto 100 and 35-mm tissue culture dishes coated with 0.1% gelatin (Fluka, Milan, Italy) at a density of 50 and 10 EBs per dish, respectively. The time point of ES cell plating was defined as day 0. EBs were monitored daily for appearance of spontaneously and rhythmically contracting myocytes under an inverted light microscope. To document beating activity, phase-contrast images were acquired at 5 sec-intervals using a Hamamatsu color chilled 3CCD camera connected to the inverted microscope, and fast cell motion was visualized by pixel intensity subtraction analysis of sequential images with Photoshop 7.0 (Adobe Systems, San Jose, CA, USA). After 10 days in differentiation medium, blasticidin S (InvivoGen) was added to cell cultures at varying concentrations (7-44 µg/mL; previous experiments showed no effect at <7 µg/mL). After 3 days of selection, 10-20 EBs were washed with PBS and then dissociated to a single cell suspension by trypsin treatment for 2-3 min. Then, 1 ml of DME + 20% FCS was added to the single cell suspension. After centrifugation (1000 rpm) for 5 min, cells were resuspended in 0.5-1.0 mL of PBS containing Ca^{2+} (1 mM) and Mg^{2+} (0.5 mM), plated on gelatin-coated dishes, cultured overnight, and immunostained with mouse monoclonal (Mm) anti-MHC (Covance, Berkeley, CA, USA) or striated muscle CH1 tropomyosin-specific antibodies (Sigma). In separate experiments, blasticidin selection was extended up to 10 days to assess toxicity.

Immunocytochemistry

Transfected neonatal rat cardiomyocytes were washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 10 min, then washed with PBS, incubated in 0.1M glycine in PBS for 5 min, and permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 5% normal goat serum (NGS) in 1% bovine serum albumin (BSA) in PBS for 30 min. They were stained with Mm anti-rabbit skeletal α-actinin (sarcomeric; EA-53, Sigma; 1:500) antibody in PBS/1% BSA at 4°C overnight. For immunofluorescence, an appropriate Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch, Hamburg, Germany; 1:200) was used, as described (Ehler et al., 1999). EBs before and after blasticidin selection were stained with anti-sarcomeric α-actinin (1:500) and rabbit polyclonal (Rp) anti-pan-cadherin (C3678, Sigma ; 1:200) antibodies in PBS/1% BSA at 4°C overnight. For immunofluorescence, appropriate Cy3-conjugated anti-mouse (Jackson Immuno-Research; 1:500) and FITC-conjugated anti-rabbit (Cappel, Organon Teknika, Pfäffikon, Switzerland; 1:500) secondary antibodies were used, as described (Ehler et al., 1999). Controls for antibody specificity included omitting primary antibodies, replacing primary antibodies with isotype-matched control antibodies, and staining with each of the primary antibodies followed by the reciprocal secondary antibody. Actin was visualized using Alexa Fluor-633-conjugated phalloidin (Molecular Probes, Eugene, OR, USA; 1:100), as described (Ehler et al., 1999). ES cell-derived cardiomyocytes were stained with the monoclonal B4 antibody against myomesin (Grove et al., 1984), which was raised in our laboratory. Specimens were analyzed by confocal laser scanning microscopy.
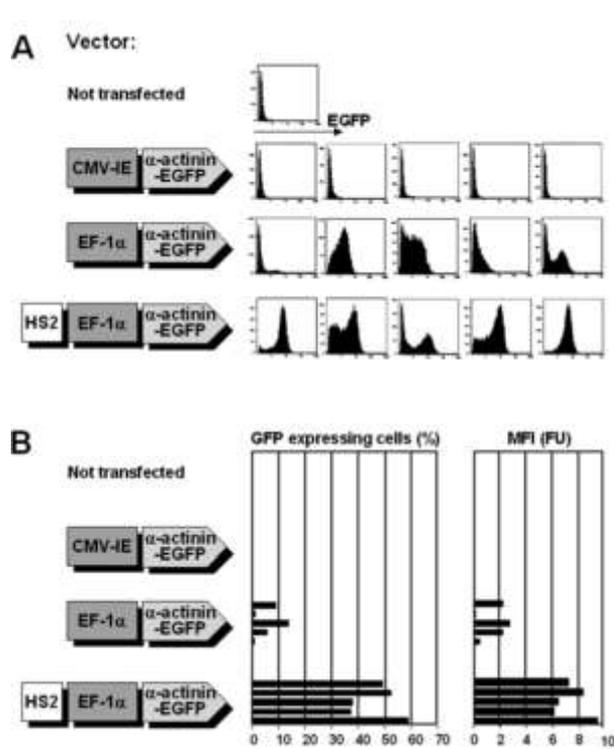
Confocal laser scanning microscopy

The imaging system consisted of a Leica inverted microscope DM IRB/E, a Leica true confocal scanner TCS SP1 (Leica Microsystems AG, Glattbruggg, Switzerland), and a Fujitsu-Siemens workstation (Fujitsu Siemens computer Bv., Regensdorf, Switzerland). The images were recorded using a Leica PL APO×63/1.4 oil immersion objective. The system was equipped with Ar and He/Ne lasers. Image processing was done using Imaris® 4.0 (Bitplane AG, Zurich, Switzerland).

Results

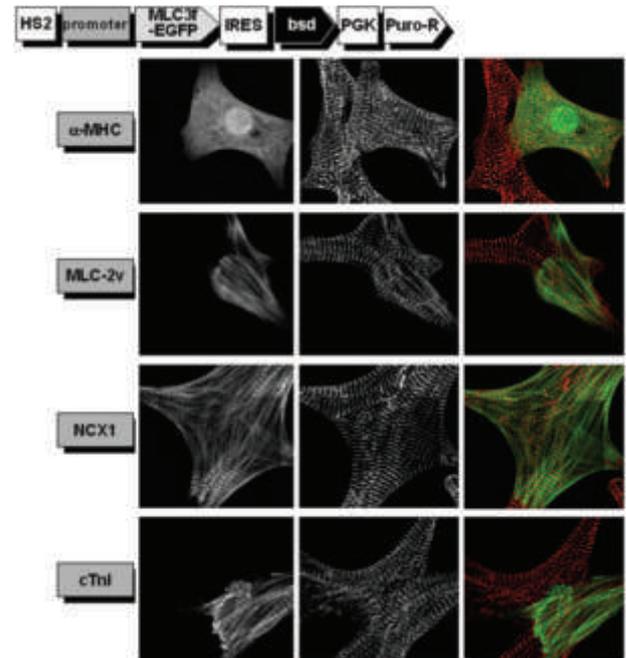### The HS2 element enhances EGFP expression in transgenic ES cell lines

The effect of the HS2 element on transgene expression in ES cell lines was assessed using an α-actinin-EGFP cassette driven by either the CMV-IE or the EF-1α promoter, with or without an upstream HS2 element. EGFP expression was detectable by FACS in ES cell lines containing the EF-1α, but not the CMV-IE, promoter-driven α-actinin-EGFP cassette. In the presence of the HS2 element, EGFP expression was detectable in 5 of 5 clones, compared with only 2 of 5 clones in its absence (Figure 2A). In EGFP$^+$ clones, presence of the HS2 element within the expression construct was associated with a approximately 10-fold increase in the number of EGFP$^+$ cells and a 5 to 10-fold increase in mean fluorescence intensity (MFI) (Figure 2B).
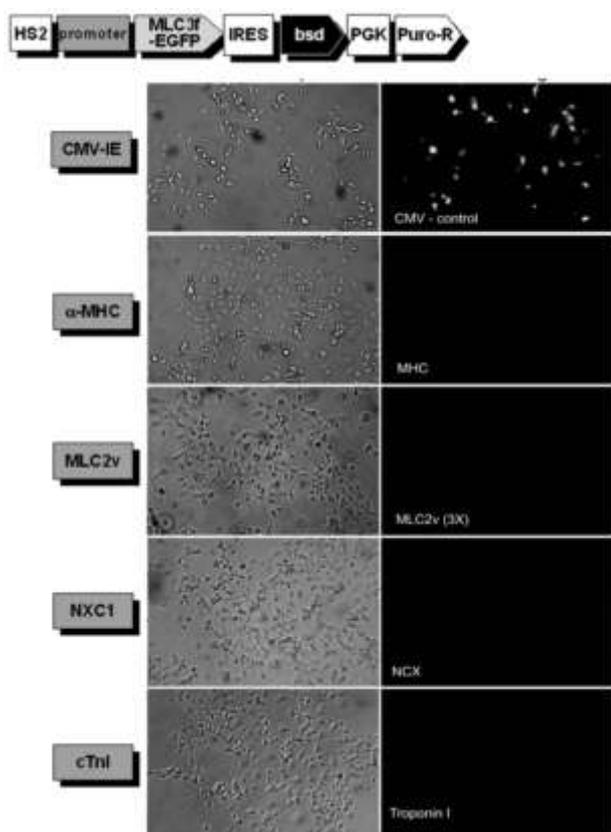
Assessment of cardiac-specific MLC3f- or α-actinin-EGFP expression in neonatal rat cardiomyocytes

We generated bicistronic vectors containing the HS2 element, a cardiac-specific promoter (the α-MHC, MLC-2v, NCX1 or the cTnI promoter), either the MLC3f-EGFP or α-actinin-EGFP fusion gene, the IRES element, and the bsd cassette (Figure 1). These vectors were first tested in transiently transfected neonatal rat cardiomyocytes and COS-1 cells (the latter as a control for tissue specificity). MLC3f-EGFP gene expression driven by each of the four cardiac-specific promoters was detectable by fluorescence microscopy in transfected neonatal rat cardiomyocytes (Figure 3), but not in COS-1 cells (Figure 4). While vectors containing the MLC-2v, NCX1 or the cTnI promoter resulted in the correct localization of the MLC3f-EGFP fusion protein to the Z-band of the sarcomeres, the HS2-α-MHC-MLC3f-EGFP-

Figure 2. Effects of the HS2 enhancer element on α-actinin-EGFP expression in stably transfected ES cells. (A) Flow cytometry histograms showing EGFP expression in 5 clones for each vector tested. (B) Numbers of EGFP$^+$ cells (%) and mean fluorescence intensity (MFI; FU, arbitrary fluorescence units). Horizontal bars indicate individual clones.

Figure 3. Confocal laser scanning micrographs of neonatal rat cardiomyocytes transiently transfected with bicistronic vectors expressing the bsd gene and a MLC3f-EGFP cassette driven by the indicated cardiac-specific promoters (see Figure 1). Columns: left, green fluorescence microscopy; middle, sarcomeric α-actinin immunostaining; right: color merge images (EGFP, green; α-actinin, red). Correct incorporation of MLC3f-EGFP at the Z-band of the sarcomeres was observed with the MLC-2v, NCX1 and cTnI promoters, but not the αMHC promoter (diffuse intracellular distribution of EGFP).
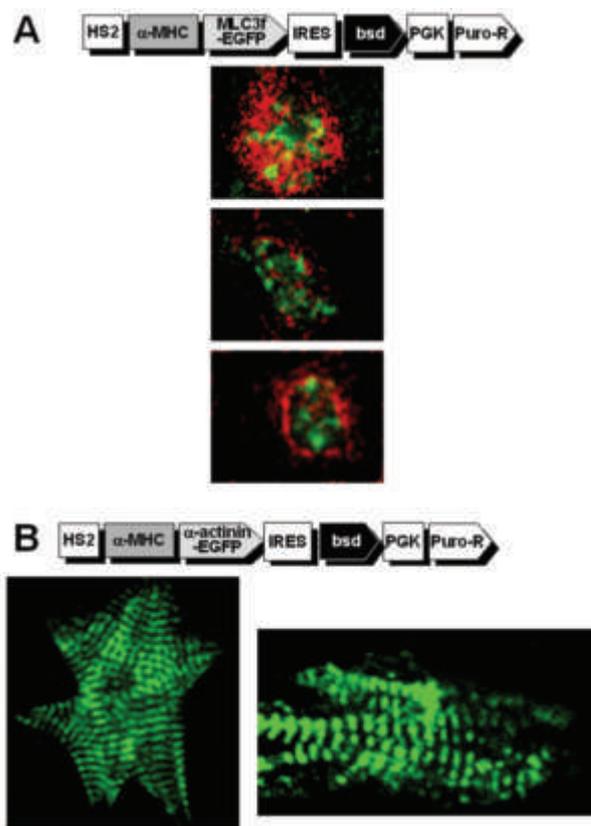
Figure 4. Confocal laser scanning micrographs of COS-1 cells transiently transfected with bicistronic vectors expressing the *bsd* gene and a MLC3f-EGFP cassette driven by the indicated cardiac-specific promoters (see Figure 1). A CMV-IE promoter-containing vector was used as a control. Columns: left, phase-contrast microscopy; right, fluorescence microscopy. No EGFP expression was observed using cardiac-specific promoters.

Figure 5. Vital fluorescence microscopy of EBs. (A) Three different HS2-α-MHC-MLC3f-EGFP-IRES-BSD ES cell lines showing green fluorescent regions. Pixel intensity subtraction analysis depicts spontaneously contracting regions in red. All green spots were co-localized with red areas. (B) High-magnification, vital fluorescence micrographs of HS2-α-MHC-α-actinin-EGFP-IRES-BSD ES cell-derived cardiomyocytes showing sarcomeric tagging.

IRES-BSD vector resulted in a diffuse intracellular distribution of the fusion protein (Figure 3). This problem was not observed using the α-actinin-EGFP cassette driven by the α-MHC promoter, which resulted in the correct epitope tagging in ES cell lines.

Establishment and specificity of α-MHC promoter-driven MLC3f- or α-actinin-EGFP expression in ES cell lines

Transgenic ES cell lines wexre generated by stable transfection with the HS2-α-MHC-MLC3f-EGFP-IRES-BSD or HS2-α-MHC-α-actinin-EGFP-IRES-BSD vectors. Cell clusters in EBs exhibiting bright green fluorescence were detectable around days 7-8 by fluorescence microscopy in 3 of 25 clones tested for each of the two vectors. The appearance of fluorescent cell clusters preceded the onset of spontaneous con-
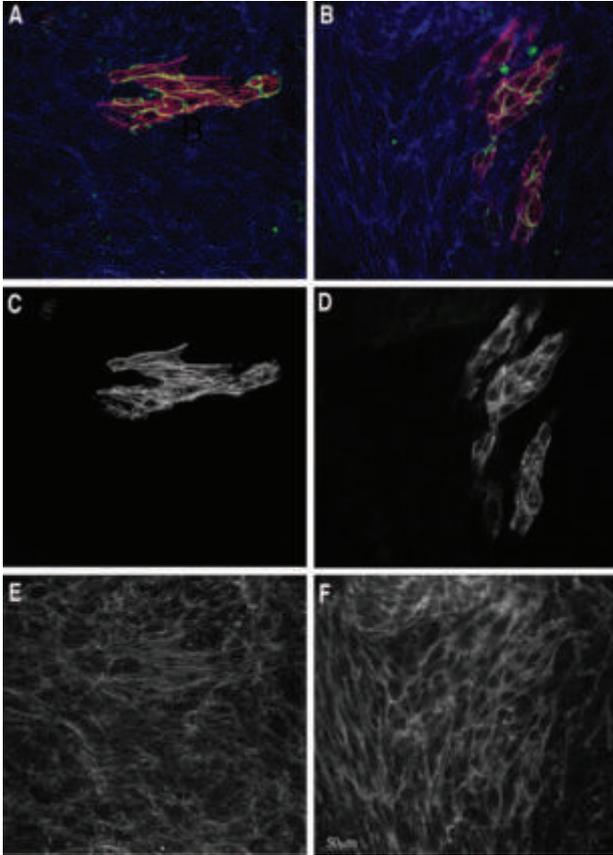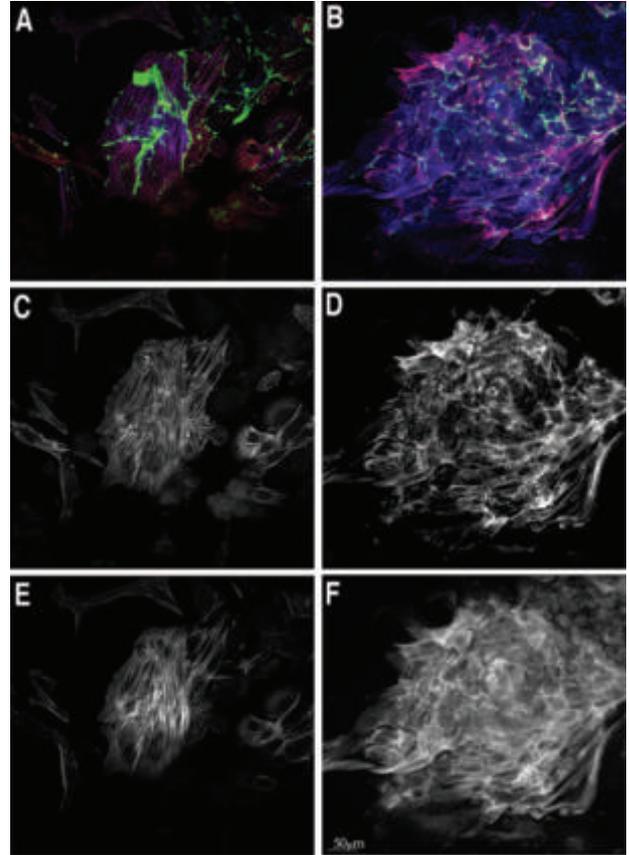
tractions in the same regions on days 8-9 (Figure 5A). No green fluorescent area was located outside contracting regions. Again, the α-actinin-EGFP, unlike the MLC3f-EGFP, fusion protein was correctly localized to the Z-line of sarcomeres (Figure 5B). No green fluorescence was detectable in ES cell clones generated with MLC-2v, NCX1 or cTnI promoter-containing vectors (n=25 clones/vector).

Blasticidin S selection and characterization of ES cell-derived cardiomyocytes

ES cell-derived cardiomyocytes in EBs stained positive for sarcomeric α-actinin and pan-cadherin (Figure 6). Pan-cadherin delineated the whole perimeter of cell membranes rather than being concentrated in intercalated disks, which is characteristic for embryonic cardiomyocytes. In the parent R1 ES cell line, 12 μg/mL blasticidin selection started on day 10 caused massive cell death by 48
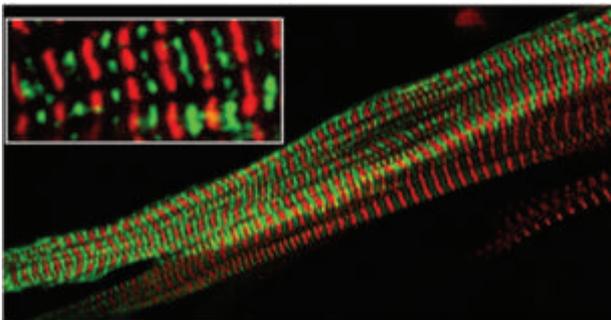
Figure 6. Confocal laser scanning micrographs images of EBs before blasticidin selection. Left and right columns show two examples of ES cell-derived cardiomyocyte clusters. Upper row: color merge images (immunostaining for pan-cadherin, green; sarcomeric α-actinin, red; phalloidin, blue); middle row: α-actinin; lower row: phalloidin.

Figure 7. Confocal laser scanning micrographs images of EBs after 3 days of blasticidin selection. Left and right columns show two examples of ES cell-derived cardiomyocyte clusters. Upper row: color merge images (pan-cadherin, green; sarcomeric α-actinin, red; phalloidin, blue); middle row: α-actinin; lower row: phalloidin. Compared to Figure 6, phalloidin-positive but α-actinin-negative regions surrounding α-actinin-positive areas have disappeared.

h. Virtually all cells were dead and detached from the plates by 72 h. Treatment with 34 μg/mL blasticidin caused detachment of virtually all of the

Figure 8. Confocal laser scanning micrograph of selected, cultured HS2-α-MHC-α-actinin-EGFP-IRES-BSD cardiomyocytes. EGFP, green; myomesin immunostaining, red. Insert: high-magnification; the α-actinin-EGFP fusion protein is located at the Z-line of sarcomeres.

cells by 48 h. In EBs containing green fluorescent beating regions, virtually all cells outside those regions detached after treatment with 34 μg/mL blasticidin for 48 h, followed by 12 μg/mL for 24 h. Surviving cell clusters stained positive for sarcomeric α-actinin and pan-cadherin (Figure 7). They survived treatment with 44 μg/mL blasticidin during 10 days with no apparent toxicity. FACS analysis of cells dissociated from EBs before selection showed 5.1±3.4% and 4.5±2.2% of cells staining positive for α-MHC and tropomyosin, respectively, as compared with >98% after selection. Selected cardiomyocytes grew normally in culture and exhibited correct incorporation of the newly synthesized α-actinin-EGFP fusion protein at the Z-band of the sarcomeres (Figure 8).

Discussion

ES cells-derived cardiomyocytes are both a useful model to study cardiomyogenesis *in vitro* and a potential source of cells for repairing failing hearts. However, enrichment of heterogeneous ES cell-derived populations into cardiomyocytes remains a major challenge. Here we describe a genetic selection system based on bicistronic expression vectors that contain both the *bsd* resistance gene and an EGFP reporter gene expressed from the α-MHC promoter. Selection with blasticidin S resulted in a relatively pure population of ES cell-derived cardiomyocytes. Insertion of the HS2 element from the β-globin LCR upstream of the transgene promoter resulted in enhanced EGFP expression in ES cell lines. Previous data showed that transcriptional enhancers may act *in cis* to suppress position-effect variegation (Walters *et al.*, 1996). Using a stably integrated reporter gene, recombinase-mediated excision of the HS2 element caused a dramatic increase in the number of K562 erythroleukemia cells showing transgene silencing. This observation suggests that the HS2 element may inhibit transgene silencing. In the present study, insertion of the HS2 element in the EF1-MLC3f-EGFP expression vector significantly enhanced EGFP expression, as assessed by flow cytometry. EGFP was not detectable using a CMV promoter, consistent with previous data showing transcriptional activity of the EF-1α, but not the CMV, promoter in naïve ES cells and EBs (Chung *et al.*, 2002). A peculiar feature of our selection system is the use of a fusion protein consisting of sarcomeric α-actinin protein and EGFP for epitope tagging. The newly synthesized EGFP fusion protein was correctly incorporated at the Z-band of the sarcomeres, suggesting that the exogenous protein was assembled into myofibrils in ES cell-derived cardiomyocytes using the same molecular interactions that govern assembly of the corresponding endogenous protein. Therefore, this system may be applicable to transgenic mouse models where it may allow characterization of mutant proteins involved in cardiomyopathies. However, sarcomeric localization was lost using the MLC3f-EGFP fusion protein expressed from the α-MHC promoter, despite being maintained using the MLC-2v, NCX1 and cTnI promoters. The reason for this promoter-specific effect is unclear. An additional aspect of novelty of the present selection system is the use of the *bsd* gene from *Aspergillus terreus* (Kimura *et al.*, 1994). This results in faster selection (3 days), as compared to other systems including neomycin or puromycin resistance genes (6-8 days) (Klug *et al.*, 1996). No toxic effects of blasticidin S on ES cell-derived cardiomyocytes was apparent even after 10 days of treatment. Another practical advantage of the *bsd* gene is that, unlike neomycin and puromycin resistance genes, it is not part of a large number of expression vectors used for molecular cloning. In our model, blasticidin S selected for a relatively pure population of ES cell-derived cardiomyocytes that showed normal morphology, myofibrillar architecture, cell-cell contacts, and growth in culture. A limitation of the present study is that ES cell lines expressing EGFP fusion proteins were generated using the α-MHC, but not the MLC-2v, the NCX1 or the cTnI promoter, as assessed by microscopical screening of 25 clones for each gene construct. Although low-level EGFP expression in some of these clones remains a possibility, bright green fluorescent cell clusters were not observed under the fluorescence microscope. This contrasts with our observations in transiently transfected neonatal rat cardiomyocytes, in which the MLC2v, NCX1 and cTnI promoter directed MLC3f-EGFP expression detectable at the microscopical level. Differences in gene expression between stable and transient transfection conditions have been observed in a number of cell systems (Makar *et al.*, 1998), reflecting the importance of nuclear matrix or chromatin interactions, or of additional transcriptional regulatory elements outside the promoter region for appropriate gene expression. Earlier studies reported that the endogenous MLC-2v, NCX1 and cTnI promoters are active on day 7 of ES cell differentiation (Boheler *et al.*, 2005). Moreover, the MLC-2v promoter (Franz *et al.*, 1993; Meyer *et al.*, 2000; Müller *et al.*, 2000), the NCX1 H1 promoter (Müller *et al.*, 2002), and the cTnI promoter (Opherk *et al.*, 2007) have been shown to drive cardiac-specific transgene expression in mouse embryos. Of note, earlier patchclamp studies showed that EGFP expression driven by the α-MHC promoter is restricted to pacemaker and atrial, but does not include ventricular, ES cell-derived cardiomyocytes (Kolossov *et al.*, 2005). In contrast, the MLC-2v promoter allows selection of cardiomyocytes with ventricular specification (Meyer *et al.*, 2000; Müller *et al.*, 2000). In conclusion, we have described a genetic system that allows rapid and effective selection of ES cell-derived cardiomyocytes and simultaneous monitoring of myofibrillogenesis in differentiating myocytes by vital fluorescence microscopy.

Acknowledgments

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