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ORIGINAL ARTICLE

A lentiviral vector with a short troponin-I promoter for tracking cardiomyocyte differentiation of human embryonic stem cells

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Human embryonic stem cells (hESCs) may become important for cardiac repair due to their potentially unlimited ability to generate cardiomyocytes (CMCs). Moreover, genetic manipulation of hESC-derived CMCs would be a very promising technique for curing myocardial disorders. At the present time, however, inducing the differentiation of hESCs into CMCs is extremely difficult and, therefore, an easy and standardizable technique is needed to evaluate differentiation strategies. Vectors driving cardiac-specific expression may represent an important tool not only for monitoring new cardiac-differentiation strategies, but also for the manipulation of cardiac differentiation of ESCs. To this aim, we generated cardiac-specific lentiviral vectors (LVVs) in which expression is driven by a short fragment of the cardiac troponin-I proximal promoter (TNNI3) with a human cardiac α-actin enhancer, and tested its suitability in inducing tissue-specific gene expression and ability to track the CMC lineage during differentiation of ESCs. We determined that (1) TNNI3-LVVs efficiently drive cardiac-specific gene expression and mark the cardiomyogenic lineage in human and mouse ESC differentiation systems (2) the cardiac α-actin enhancer confers a further increase in gene-expression specificity of TNNI3-LVVs in hESCs. Although this technique may not be useful in tracking small numbers of cells, data suggested that TNNI3-based LVVs are a powerful tool for manipulating human ESCs and modifying hESC-derived CMCs.
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Introduction

Embryonic stem cells (ESCs) have the potential to differentiate into tissue derivatives of all three embryonic germ layers. Cardiomyocytes (CMCs) have been obtained from all three types of murine embryo-derived stem cells: embryonic carcinoma, embryonic stem and embryonic germ cells.[1]

Human ESCs (hESCs), isolated from the inner cell mass of embryos, can be propagated continuously in the undifferentiated state when grown on top of a mouse embryonic fibroblast (MEF) feeder layer.[2] When removed from these conditions and grown in suspension, they begin to generate three-dimensional differentiating cell aggregates, termed embryoid bodies (EBs), in which a variety of tissue types can be found. hESCs have also been shown to differentiate into the cardiac lineage.[3,4]

The multipotency of ESCs and their potentially unlimited capacity to generate multiple types of cells make them a very promising therapeutic tool for treating genetic diseases. However, manipulation of hESCs using non-viral vectors has proven to be difficult.[5] Lentiviral vectors (LVVs) can transduce both dividing and non-dividing cells and were shown to drive gene expression efficiently in various types of 'stem' cells.[6] LVV-mediated transduction results in permanent integration of new genetic material into the host cell genome with little immunogenic effect.[6] Genetic manipulation of hESCs with LVVs thus represents a promising approach for selecting hESC-derived CMCs. Efficient cardiac-specific transgene expression is fundamental for following and enhancing hESC differentiation toward the cardiac lineage.

Here, we report that short human and mouse cardiac troponin proximal promoters retain cardiac specificity when placed in an advanced third-generation LVV backbone. More importantly, these LVVs drive cardiac-specific gene expression during differentiation of human and mouse ESCs toward the cardiomyogenic lineage.

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Results

A short human cardiac troponin proximal promoter retains specificity for cardiomyocytes

The cardiac isoform of the troponin I gene product (TnIc) is not expressed in skeletal or other muscle types. Human and mouse TnIc promoter (TNNI3) regions have been studied previously in order to identify potential *cis*-acting DNA elements conferring cardiac specificity.[7,8] In the case of mouse TNNI3, a region limited to 0.3 kb upstream of the start codon is sufficient to confer cardiac specificity.[8] Since the mouse and human TNNI3-5′ flanking regions are very similar (Supplementary Figure S1), we speculated that a short proximal promoter (−270/+70) is sufficient to confer cardiac-specific gene expression in human cells also. To prove this, the 340-bp fragment of the hTNNI3-5′ flanking region was cloned upstream of a luciferase reporter gene, generating the plasmid pGL3.hTNNI3_340b.luc (Figure 1a), and was used in a standard dual luciferase assay: different cell types (rat cardiomyocytes (rCMCs), NIH-3T3 mouse fibroblasts, murine C2.C12 myotubes and myoblasts) were transfected with pGL3.hTNNI3_340b.luc or with control vectors in which luciferase gene expression was driven by an unspecific, 'universal' promoter (pGL3.SV40.luc or pGL3.CMV.luc). pGL3.hTNNI3_340-b.luc expression was normalized to pGL3.SV40.luc (Figure 1b 1) or pGL3.CMV.luc (Figure 1b 2) expression and reported as a ratio. Data show that the relative expression of pGL3.hTNNI3_340b.luc in CMCs was at least 15 times higher than that measured in non-cardiac cell lines (NIH-3T3 or C2.C12).

When injected *in vivo*, SV40-normalized luciferase expression of GL3.hTNNI3_340b.luc was approximately 10-fold greater in cardiac muscle compared to skeletal muscle (Figure 1c).

Thus, this set of experiments prove that a 340-bp sequence within the hTNNI3-5′ flanking region is sufficient to confer cardiac-specific expression in transient transfection assays.

LVVs engineered with human or mouse TNNI3 proximal promoters drive cardiac-specific expression in CMCs

We tested whether the advanced third-generation LVV containing either the short human or mouse TNNI3 promoter is capable of transducing rCMCs, and whether they are tissue-specific. A schematic drawing of the LVV used in this study is shown in Figure 1d.

Our group has already shown that this LVV backbone is capable of transducing CMCs efficiently.[9] TNNI3-based LVVs gave similar results: more than 90% of rCMCs could be transduced after only one round of transduction (data not shown).

Tissue specificity of the constructed LVVs was assayed by normalizing enhanced green fluorescence protein (EGFP) expression of TNNI3-LVVs vs phosphoglycerate kinase (PGK)-LVV or EF1-LVV (Figure 1e). Cells (HEK-293 or rCMCs) were transduced with mTNNI3-, hTNNI3-, PGK- or EF1-LVV at multiplicity of infection (MOI) = 1, and mean fluorescence intensity, which grossly correlates with promoter activity, analyzed 52 h later. PGK-normalized hTNNI3_340b-associated EGFP expression in rCMCs was up to 15-fold higher than that measured in HEK-293 (Figure 1e 1). A similar result was obtained when hTNNI3-LVV-induced expression was normalized to that of EF1-LVV (Figure 1e 2).

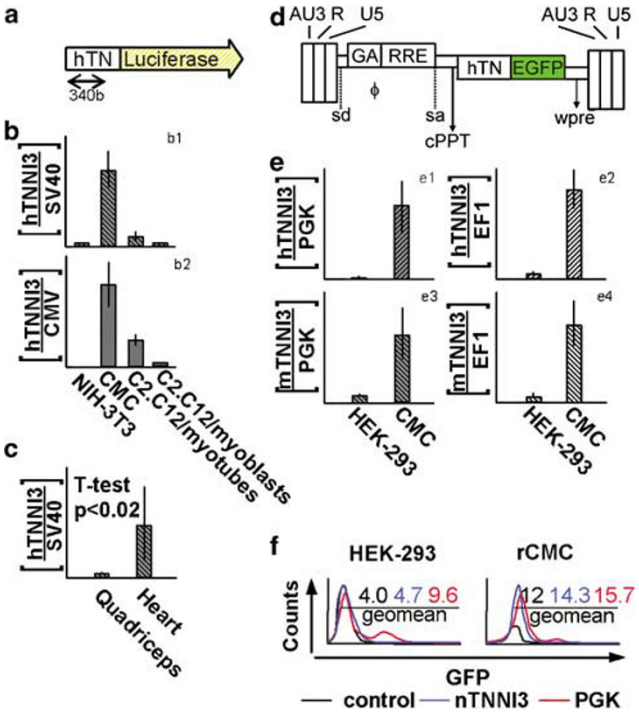

Figure 1 (**a**) Schematic representation of the structure of pGL3.hTNNI3_340b.luc, the TNNI3-luciferase plasmid used in co-transfection experiments: 340 bp at the 5′ flanking region of the human cardiac troponin promoter (hTN) was cloned upstream of a luciferase reporter gene (Luciferase). (**b**) Graphs of the data form luciferase assays in murine fibroblasts (NIH-3T3), neonatal rat cardiomyocytes (CMC) and mouse myoblasts and myotubes (C2.C12) transfected transiently with pGL3.hTNNI3_340b.luc (hTNNI3) and normalized either with pGL3.SV40.luc (b1), a plasmid containing the simian virus 40 promoter (SV40) or with pGL3.CMV.luc (b2), a cytomegalovirus (CMV) reporter-plasmid. Values are presented as mean ± s.d. of three independent experiments. (**c**) Luciferase assays were conducted on tissues after injection of pGL3.hTNNI3_340b.luc (hTNNI3) or GL3.SV40.luc (SV40) into the ventricular wall of the heart and the quadriceps of mice. Graph of mean ± s.d. of SV40-normalized hTNNI3-driven luciferase ($n = 6$/group). (**d**) Schematic drawing of pRRLcPPT.hTNNI3_340b.EGFP.WPRE, the vector used for transduction experiments. AU3 R U5, the LTR regions, with a deletion of 400 bp, including the enhancer and promoter from U3; s.d., major splice donor site; ϕ, encapsidation signal including the 50 bp portion of the gag gene (GA); RRE, Rev-response element; sa, splice acceptor sites; cPPT, nuclear import sequence; hTN, human cardiac troponin I 5′ flanking region (340 bp); EGFP, enhanced green fluorescent protein; WPRE, regulatory element of woodchuck hepatitis virus. The other vectors used differ only in their respective promoters: mouse cardiac troponin (mTNNI3)-5′ flanking region drives EGFP expression in pRRLcPPT.mTNNI3_427b.EGFP.WPRE; human phosphoglycerate kinase (hPGK) promoter for pRRLcPPT.hPGK.EGFP.WPRE; elongation factor 1α (EF1a) promoter for pSINF.EF1a.GFP.SAR/HS. (**e**) Graphs of mean fluorescence intensity evaluated by standard flow cytometry of neonatal rat cardiomyocytes (rCMCs) and human embryonic kidney-293 cells (HEK-293) transduced for 52 h with hTNNI3-LVV (e1 and e2) or mTNNI3-LVV (e3 and e4) and normalized to PGK-LVV (e1 and 3) or EF1-LVV (e2 and 4). Values are presented as mean ± s.d. from three independent experiments. Background samples were included in all experiments. (**f**) Representative histogram of the EGFP signal in HEK-293 cells and rCMCs which were untreated (control, black) or transduced with hTNNI3-LVV (blue) or PGK-LVV (red). The geometric mean (geomean) is given for each.

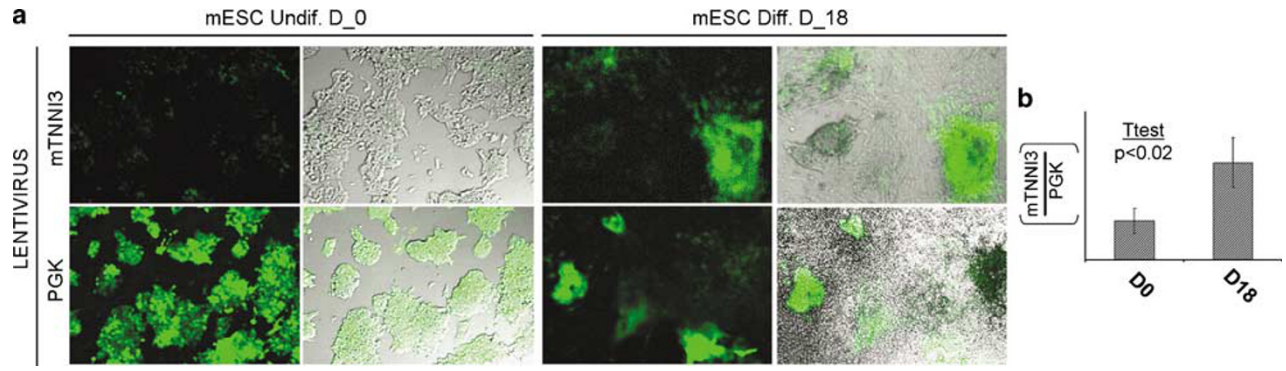

Figure 2 (**a**) Fluorescence microscopy of enhanced green fluorescence protein (EGFP) expression in undifferentiated mouse embryonic stem cells at day 0 (mESC undiff. D_0) and 18 days later, when pulsating cardiomyocytes become visible (mESC diff. D_18). Transduction (MOI = 2) was performed with mTNNI3- or PGK-LVV. (**b**) Graph of data from FACS analysis of EGFP expression. The percentage of EGFP-positive cells obtained after transduction with mTNNI3 or PGK were calculated and are reported as a ratio. Values are presented as mean ± s.d. of two independent experiments (done in triplicate). EGFP, enhanced green fluorescence protein; mESC, mouse embryonic stem cells; mTNNI3, mouse cardiac troponin; PGK, phosphoglycerate kinase.

Representative histograms of EGFP expression in HEK-293 and rCMCs are shown in Figure 1f.

To evaluate prolonged expression in rCMCs, similar analyses were performed 2 weeks after LVV transduction (is not possible to maintain rCMCs properly in culture for longer); similar data were obtained to those above (not shown).

Thus, the result of experiments with LVV is in accordance with that of the above-described plasmid luciferase transfection experiments.

Data similar to those generated with hTNNI3-LVV were obtained with mTNNI3-LVV (Figures 1e 3 and 4). In fact, the PGK- or EF1-promotor-normalized mTNNI3_427b-induced EGFP expression levels were approximately eight times higher in rCMCs than in the control cell type. This result is analogous to that obtained by Ausoni *et al*.[8] using the 307-bp fragment of the mTnIc promoter in transient transfection experiments in CMCs compared to fibroblasts. Moreover, experiments using different MOIs (0.5 and 2), viral preparations, control cell types and length of time after transduction (2 weeks), gave analogous results (not shown).

Fluorescence microscopy observations were also performed. EGFP expression was stronger in rCMCs than in HEK-293 when TNNI3-LVVs were used; when CMCs and HEK-293 were transduced with PGK- or EF1-LVVs, strong expression was obtained in both cell types (Supplementary Figure S2).

TNNI3-LVVs track CMC differentiation in ESCs

The next series of experiments was aimed at proving that TNNI3-LVVs transduce ESCs and that EGFP reporter gene expression is activated during cardiomyogenic differentiation. We first performed experiments in a mouse ESC (mESC) line, which does not require a feeder layer (see Materials and methods section). To induce CMC differentiation, medium was supplemented with dimethylsulfoxide after the appearance of EBs. Spontaneously contracting areas appeared within 16–18 days.

Undifferentiated mESCs were transduced with mTNNI3- or PGK-LVV at an MOI of 2; at this MOI, the transduction efficiency was $\leqslant$10% once cells were differentiated, so within the linearity range for quantitative measurement of EGFP expression. EGFP was assayed 18 days before and after the start of differentiation.

mESCs transduced with mTNNI3-LVV presented with very low EGFP signals at fluorescence microscopy when in the undifferentiated state (Figure 2a, D_0), but remarkably increased expression when differentiated (Figure 2a, D_18). This was confirmed by fluorescence-activated cell sorting (FACS) analysis; an increase in percentage of EGFP-positive cells was detected in differentiated cells (Figure 2b). Transgene expression was found to be stable during prolonged cultivation (data not shown).

We then determined whether TNNI3-LVVs could be used to transduce and follow CMC differentiation of human ESCs. We used cell lines 7 and 8 of the 17 hESC lines generated by Dr D Melton's laboratory.[10] hESC differentiation was addressed through quantitative real-time PCR (qRT-PCR) gene-expression measurements of one marker of the undifferentiated stage (human alkaline phospatase, *ALP*), and one marker of cardiac differentiation (cardiac troponin, *TnIc*) (Figure 3). As a reference marker, mRNA levels of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were measured. MEF, END2 and human skeletal muscle mRNAs were used as controls for specificity of the relative variations of ALP and TnIc mRNAs.

It has been demonstrated that hTnIc expression increases up to fourfold in spontaneously differentiated hESCs at day 10 compared to the undifferentiated condition (day 0). Moreover, cardiomyogenic differentiation of hESCs can be enhanced by treatment with 5-aza-2′-deoxycytidine (AZA), presumably via DNA demethylation.[11] We therefore cultured hESC 7 with or without AZA and measured hTnIc mRNA levels. As described previously,[11] AZA treatment increased CMC differentiation as assessed by qRT-PCR measurements of hTnIc mRNA levels (Figure 3, Student's *t*-test $P < 0.05$, hESC on MEF D10 vs D10+AZA).

Mummery *et al*.[12] demonstrated that co-culture of hESC lines on END2 cells in a specific differentiation medium enhances cardiomyogenesis within 2 weeks, particularly in those hESC lines which do not differentiate into CMCs spontaneously. We therefore determined whether CMC differentiation of hESC 7 is

increased by pre-culturing them with END2 rather than MEF.[13] hESC 7, previously amplified in the undifferentiated condition on MEF cells, were switched onto END2. Three days later, hESCs were dispersed into suspension to allow EB formation, and cultured with or without AZA. hTnIc mRNA (Figure 3) was increased up to threefold at day 10 (hESC on END2 D_10) compared to day 0 (hESC on END2 D_0). Coherent with the result of Xu C *et al*.[11], AZA treatment increased hTnIc expression up to ninefold (hESC on END2 D_10+AZA vs D_0). Intriguingly, hTnIc expression was similar when pre-culturing hESC 7 on END2 or MEF cells at day 10 when AZA was used. This observation may be explained by the fact that we used a different protocol to that of Mummery's group.

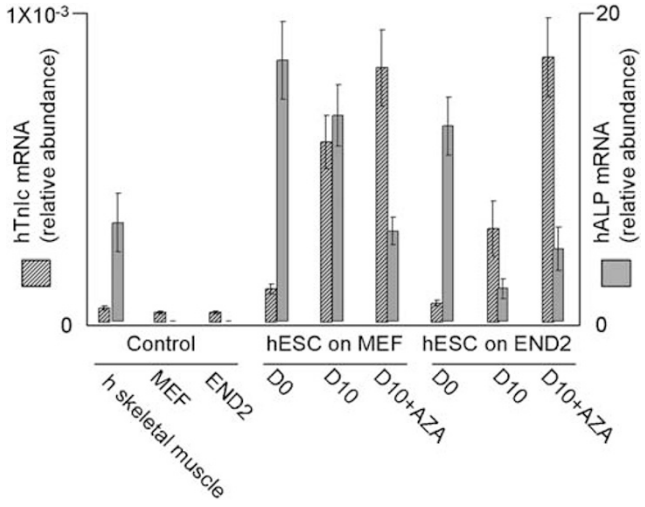

Figure 3 The relative abundance of human cardiac troponin mRNA (hTnIc, value reported on the left axis and marked by diagonal bars) and human alkaline phosphatase mRNA (hALP, value reported on the right axis and marked by stippled bars) were evaluated by qRT-PCR during hESC differentiation. Values are reported as mean ± s.d. of three independent experiments. RNA was collected before dispersing hESCs—grown on MEF or END2—into suspension to permit embryoid body formation (hESC on MEF D_0 and hESC on END2 D_0, respectively). hESCs were then cultured with or without 5-azacytidine (AZA) and RNA collected 10 days later (hESC on MEF D_10 or hESC on END2 D_10, and hESC on MEF D_10+AZA or hESC on END2 D_10+AZA, respectively). RNA from MEF, END2 and from a human skeletal muscle sample was collected and used as oligo-specificity controls; RNA from human heart was used as calibrator. hESCs, human embryonic stem cells; MEF, mouse embryonic fibroblast.

It is worth noting that hALP mRNA levels were inversely related to hTnIc expression. A similar result was obtained using the hESC 8 line (not shown).

Microscopic observations and FACS analysis are shown in Figures 4 and 5, respectively. hESCs growing on MEF were transduced (MOI = 1–2) with hTNNI3- or PGK–LVV; at this MOI, the transduction efficiency was $\leqslant$10% after differentiation, hence within the linearity range for quantitative measurement of EGFP expression. On MEF, hESC expression of hTNNI3_340b-driven EGFP at day 0 was lower in terms of both fluorescence intensity and numbers than that driven by hPGK, with increased non-homogeneity in EGFP expression within the colonies (Figures 4c vs d). A similar result was obtained using END2 (Figures 4a vs b). Representative images of differentiating EBs deriving from hTNNI3-LVV-transduced cells, are also given in Figures 4e and f.

Quantification of cardiac-specific expression was performed by FACS at days 0, 10 and 17 (representative dot plots are shown in Figure 5a). To reduce the percentage of MEF expressing EGFP within the pool of cells analyzed by FACS, hESCs were amplified after transduction in the undifferentiated condition on MEF for three days, then split onto a fresh feeder layer (either MEF or END2) on which hESCs were left to amplify for an additional 3 days before inducing them to form EBs. hESCs were distinguished from any transduced MEFs

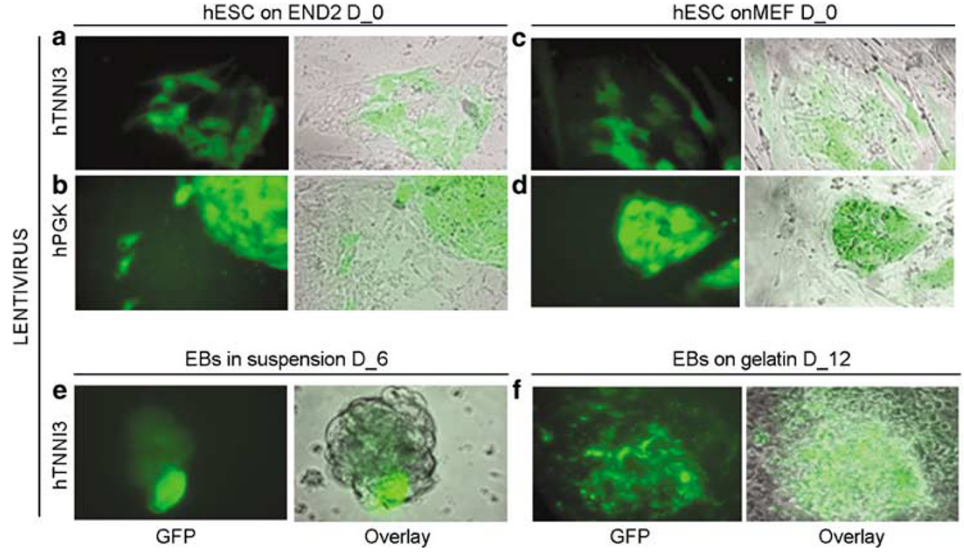

Figure 4 hESCs growing on MEF were transduced (MOI = 1) with hTNNI3- or PGK -LVV. Three days after transduction, hESCs were split onto END2 or MEF (referred to as hESC on END2 or on MEF, respectively) and observed 3 days later (termed day 0) (× 400 magnification) (**a–d**). hTNNI3-LVV-transduced hESCs grown on MEF were allowed to form EBs; pictures were taken at day 6 (× 400 magnification) (**e**). EBs plated on gelatin-coated plates; pictures were taken at day 12 (12 days after dispersion in suspension— × 200 magnification) (**f**). Direct fluorescence of EGFP (GFP) or phase-contrast overlays (overlay) are shown. EB, embryoid body; EGFP, enhanced green fluorescence protein; hESCs, human embryonic stem cells; LVV, lentiviral vector; MEF, mouse embryonic fibroblast; PGK, phosphoglycerate kinase.

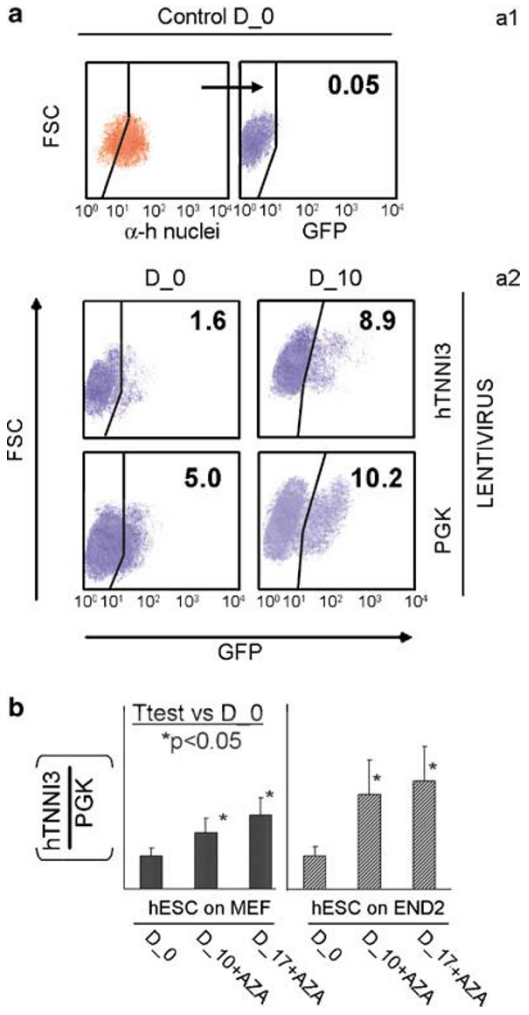

Figure 5 hESCs growing on MEF were transduced (MOI = 2) with hTNNI3- or PGK-LVV. Three days later, plates were trypsinized and the cells split onto either MEF or END2; after a further 3 days, hESCs were induced to differentiate through the formation of EBs in suspension and treated with or without 5-aza-2′-deoxycytidine. Seven days later, EBs were plated onto gelatin. FACS analysis was performed on days 0 (when hESCs were still undifferentiated), 10 and 17. (**a**) Representative dot plots of gate-in of hESCs with an antihuman nucleus antibody in non-transduced cells (a1) and hTNNI3/PGK-LVV EGFP expression in the gated hESC population at day 0 (D_0) and 10 (D_10) (a2). (**b**) Percentage of hESCs expressing EGFP under the control of hTNNI3_340b was normalized to percentage of hESCs expressing EGFP under the control of hPGK. Values are presented as mean ± s.d. of three independent experiments. EB, embryoid body; GFP, green fluorescence protein; hESCs, human embryonic stem cells; LVV, lentiviral vector; MEF, mouse embryonic fibroblast; PGK, phosphoglycerate kinase

still present within the cell suspension analyzed by gating-in the hESC population with an antihuman nuclei antibody (Figure 5a 1). Due to promoter leakage and hESC inhomogeneity, there was a certain degree of hTNNI3-LVV EGFP expression in undifferentiated hESCs and in feeder cells (representative density dot plots are shown in Supplementary Figure S3). hPGK-normalized values show that when transduced with hTNNI3-LVV, the percentage of cells expressing EGFP increases significantly during differentiation (Student's *t*-test $P < 0.05$ using either MEF or END2) (Figure 5b).

A cardiac-specific enhancer improves the performance of hTNNI3-LVV

It has been shown that expression of the mouse cardiac actin gene in skeletal and cardiac muscle is regulated by enhancers upstream of the proximal promoter;[14] the enhancer is included between base pairs −2354 and −1360 of the gene and is active only in CMCs, and not in C2 skeletal muscle cells.[14] *In vivo*, it directs reporter gene expression in the adult heart, where the proximal promoter alone is inactive.[14] An 85-bp region within the enhancer is highly conserved between human and mouse and contains a central AT-rich site essential for its activity. We cloned the human sequence containing this conserved region (which we named hEnAct) upstream of the hTNNI3_340b promoter, obtaining an LVV (hEnAct_TNNI3-LVV) where EGFP expression is under the control of this promoter chimera (Figure 6a).

Experiments similar to those reported in Figure 1e were performed to evaluate the cardiac specificity of hEnAct_TNNI3-LVV in comparison to hTNNI3-LVV (Figure 6b). The inclusion of the hEnAct sequence determined a further two- to threefold increase in cardiac specificity as compared to hTNNI3_340b alone. We then constructed an LVV in which hEnAct was replaced by a fragment of the hCMV promoter, annotated as a potential enhancer (hEnCMV_TNNI3-LVV); the contribution of this constitutive *cis* element in our setting was, as expected, detrimental for tissue specificity (data not shown).

We, therefore used hEnAct_TNNI3-LVV to prove further the potential of TNNI3-based LVVs to track cardiac lineage differentiation of hESCs. hESCs growing on MEF were transduced with hEnAct_TNNI3- or PGK-LVV and induced to differentiate through EB formation in the presence of AZA. After 10 days, samples were sorted on the basis of EGFP expression. The resulting subpopulations were used immediately for qRT-PCR to evaluate relative abundance of hTnIc mRNA (Figure 6c). The subpopulation of hEnAct_TNNI3-LVV-transduced hESCs expressing EGFP had an increased amount of hTnIc mRNA when compared to EGFP-negative cells. Immunofluorescence conducted on EBs at day 10 with an anti-cTnI antibody revealed areas associated with EGFP in hEnAct_TNNI3-LVV-transduced hESCs (Figure 6d, top panel). EGFP was more diffuse in PGK-LVV-transduced hESCs compared to those transduced with hEnAct_TNNI3-LVV.

Experiments similar to those reported in Figure 2 were performed in mouse ESCs using hEnAct_TNNI3-LV, and similar data were obtained (not shown). Here also, immunofluorescence showed that in contracting EBs (12 days after differentiation; Supplementary Figure S4) there were cTnI-positive areas associated with EGFP (Figure 6d, bottom panel). In accordance with the result obtained in hESCs, EGFP was more diffusely expressed when cells were transduced with PGK-LVV.

The above data suggest that hEnAct_TNNI3-LVV can be used to tag and select a subpopulation of cells deriving from hESCs that express cardiac-specific markers.

Discussion

Recent evidence demonstrated that CMCs obtained from hESCs can be used to replace sino-atrial nodal cells in an

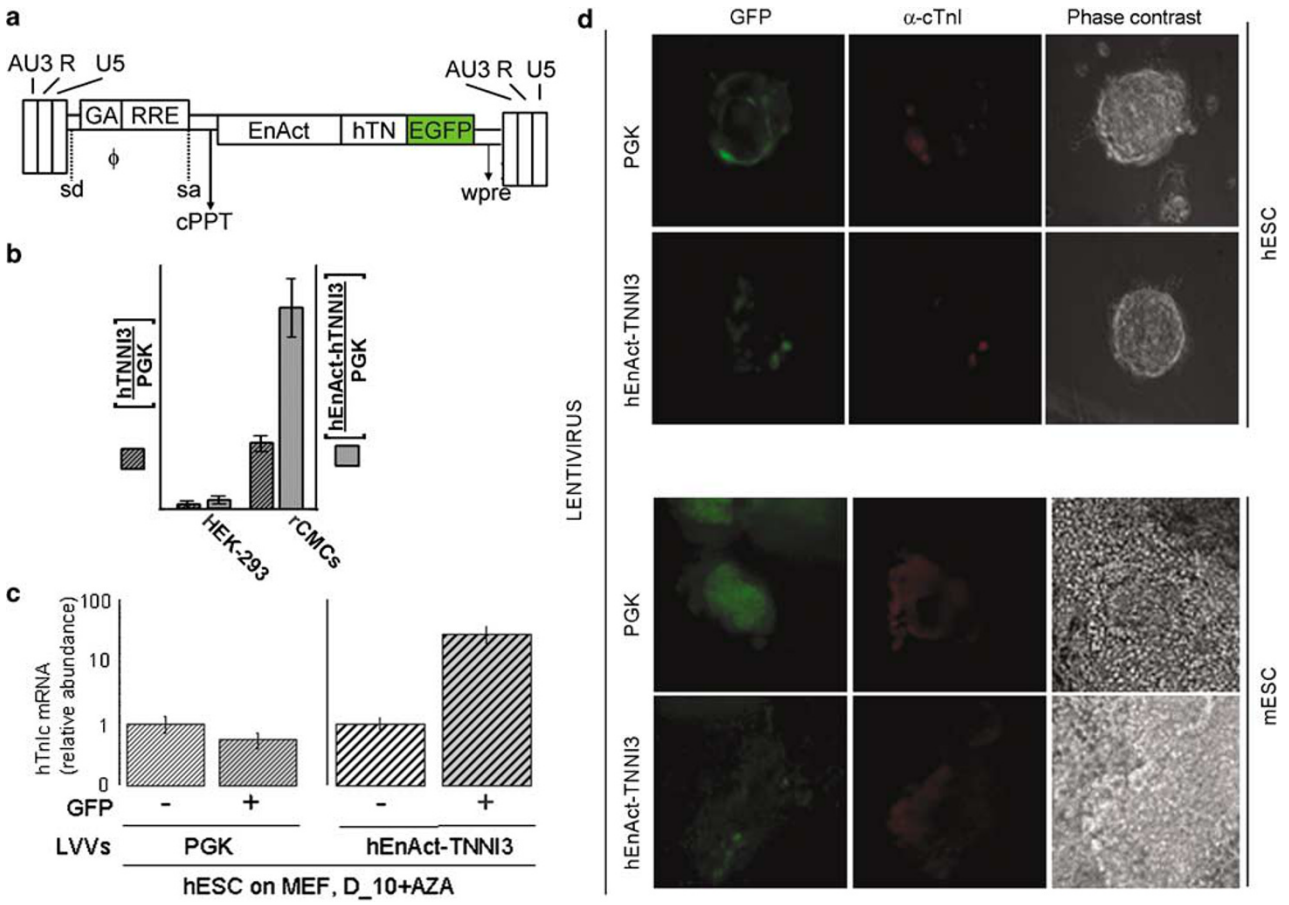

Figure 6 (**a**) Schematic drawing of the pRRLcPPT.hEnAct-hTNNI3_340b.EGFP.WPRE vector used in the transduction and sorting experiments. hTN, human cardiac troponin (hTNNI3)-5′ flanking region (340 bp). EnAct, human α-cardiac actin enhancer (850 bp). LVV backbone already described above in Figure 1d. (**b**) Mean fluorescence intensity of neonatal rat cardiomyocytes (rCMCs) and human embryonic kidney-293 cells (HEK-293) after transduction (MOI = 1) with hEnAct_TNNI3- or hTNNI3-LVV and normalized with PGK-LVV. (**c**) hESCs grown on MEF were transduced (MOI = 4) with hEnAct_hTNNI3- or PGK-LVV, amplified through two rounds of subculture on MEF, then induced to differentiate through EB formation in the presence of AZA. After 10 days, EBs were collected with collagenase/trypsin to obtain single-cell suspensions (hESC on MEF, D_10+AZA). Samples were sorted on the basis of EGFP expression by standard procedures. RNA was collected from the subpopulations for qRT-PCR. Fold increases in human cardiac troponin (hTnIc) mRNA, normalized to glyceraldehyde-3-phosphate dehydrogenase, are shown. Values are reported as mean ± s.d. of the experimental duplicate. (**d**) EBs at day 10 transduced with either PGK-LVV or hEnAct_TNNI3-LVV and immunostained with an anticardiac troponin I (α-cTnI) antibody (red). EGFP is shown in green. Top: human ESCs; bottom: mouse ESCs. ×200 magnification. AZA, 5-aza-2′-deoxycytidine; EB, embryoid body; EGFP, enhanced green fluorescence protein; hESCs, human embryonic stem cells; LVV, lentiviral vector; MEF, mouse embryonic fibroblast; PGK, phosphoglycerate kinase.

experimental swine model of supraventricular bradycardia.[15] In addition, several reports demonstrated that mESC-derived CMCs can integrate with host myocardium, generating electromechanical coupling capable of substituting the loss in myocardial contractile force;[16] more recently, similar therapeutic effects were demonstrated using mouse ESC-derived CMCs in a sheep model of heart failure.[17] While bulk cultures of ESC-derived differentiated CMCs can be obtained from mouse ESCs using transfection of plasmids with tissue-specific promoters and marker selection,[16] similar results have not yet been obtained using human ESCs. One major obstacle is represented by the low yield of CMCs deriving spontaneously from hESCs; as a consequence, therapeutic application of hESC-derived CMCs to myocardial disease can be envisioned at the moment only for disorders that require a small number of cells for repair, such as atrio-ventricular (AV) block.[15] Therefore, the use of exogenous cytokines and growth factors is necessary to enhance cardiomyogenic differentiation of hESCs. Several cytokines and growth factors have already been studied in mouse rather than human stem cell systems. Of particular interest as inducers of cardiomyogenic differentiation are members of the transforming growth factor-β1 family and Wnt-11 protein.[18,19] Thus, it is critical to set up a system that permits quick and easy screening of conditions capable of inducing cardiac differentiation of ESCs, through generic quantification. To this aim, the transduction of ESCs with an LVV constructed with a tissue-specific promoter driving a reporter gene could be useful. In this manuscript, we describe LVVs constructed around the advanced third-generation HIV/SIN-lentiviral backbone[9] and containing a short human or mouse cardiac troponin proximal promoter[7,8] with or without an upstream human cardiac α-actin enhancer.[14] These TNNI3-based LVVs efficiently transduce neonatal rat CMCs, and more importantly, we demonstrate that it is possible to use these vectors to

manipulate hESCs and track the cardiac fate of the cells during differentiation.

The transduction of hESCs with LVVs is still a technique hampered by a certain degree of variability, however, due to the lack of standardized protocols. We found that LVV transduction efficiency was inversely associated to hESC colony size and directly associated to the colony's confluence, as previously observed.[20] Moreover, transduction may also be inhomogeneous due to intra- and inter-lineage variability. It is necessary, however, to underline that obtaining stable hESC clones expressing genes under a cardiac-specific promoter is not trivial in that the ideal clone should express the transgene (for example, EGFP) solely after differentiation. Amplification (cloning) would be carried out during the undifferentiated stage of ESCs, so screening of the right clone is not easily performable. Moreover, a pool of ESCs transduced with an LVV would be more useful than a stably transduced ESC clone in that the later is potentially subject to bias: the reasons why a clone could result unsuitable include possible specific genetic side effects induced by the transgene inserted into the genome at a specific site, or those inherent to the specific subline selected from the ESC population. We think that the above considerations argue against the use of a stably transduced clone as a screening tool. Moreover, the setting up of methodologies utilizing LVV_hEnAct_TNNI3 on a pool of stem cells would permit easier use subsequently in non-embryonic stem cells. To this end, as a proof of principle, we tested hEnAct_TNNI3-LVV in the cardiosphere-derived cell (CDC) model. Recent reports have described the presence and properties of stem cells within the adult heart.[21–24] Messina *et al*.[25] were the first to isolate CDCs from murine and human hearts without a stem-cell marker selection step. Cells are spontaneously shed from human surgical specimens (open heart surgery or percutaneous endomyocardial biopsies) and murine heart samples in primary culture. These cells are self-renewing, clonogenic and multipotent giving rise spontaneously to CMCs, smooth muscle cells and endothelial cells. In suspension culture, they form multicellular clusters, dubbed cardiospheres, composed of clonally derived cells consisting of proliferating c-Kit positive cells primarily in the center and differentiating cells on the surface.[25–27] To follow expression of our cardiac-specific promoter, we used the cardiosphere-derived cell isolation method after having transduced murine cardiac tissue with LVVs at the earliest stage of the process. We found that explants transduced with hEnAct_TNNI3-LVV showed strong green fluorescence initially only in a few cells ($\approx$5%) compared to those transduced with PGK-LVV. During differentiation, an increase in fluorescence was evident within the cardiospheres that had derived from fragments transduced with hEnAct_TNNI3-LVV. In fully differentiated cells, cTnI positivity was apparent in cells with a strong green fluorescence protein (GPF) signal (Supplementary Figure S5).

An 'ideal' transgene-expressing vector, useful for ESC-based applications, should have very tight expression control and should be able to sustain transgene expression after genome reprogramming, proliferation, transcriptional level reduction and necrosis, all of which are associated with *in vitro* induction of ESC differentiation. Moreover, a tissue-specific promoter should be switched on just when it is in the specific differentiated lineage or in the specific cell type. This is an issue still to pursue for CMCs derived from human ESCs. At the present stage, a more realistic goal would be to select a promoter showing differential expression. The data we have shown suggest that promoter leakage of TNNI3-LVVs, or their non-specific activation, is of a level that can be tolerated for this specific application.

In spite of the fact that this technique may not be useful in tracking small numbers of cells, the development of a cell-selection strategy based on the use of TNNI3-LVVs is a pursuable tool. It may be the quickest way to select and multiply the subpopulation of cells that form cardiac cells from within the heterogeneous pool of hESC-derived cell types obtained by the currently available, unspecific differentiation schemes. Other important potential uses of these vectors include the genetic manipulation of hESCs for tracking cells after transplantation, anyway and for curing single gene disorders.

Materials and methods

An expanded version of the Materials and methods section is available as a Supplementary Data file online.

Cell lines

All cell lines were cultured in an incubator at 37 °C with a mixture of air and 5% CO_2. Reagents were purchased from Invitrogen (Milan, Italy) unless otherwise stated.

Neonatal rCMCs were obtained with a modification of an original protocol.[28]

The mouse myoblast C2-C12 cell line (ATCC) is a striated muscle cell model. Differentiation into myotubes was induced at $\sim$70% confluence by replacing the high-serum medium with Dulbecco's modified Eagle's medium containing a mixture of bovine insulin, human transferrin and sodium selenite (ITS, Sigma, Milan, Italy).

The human embryonic kidney cell lines, HEK-293 and 293T, were kindly provided by Dr FL Graham (Istituto di Ricerche di Biologia Molecolare-IRBM, Rome, Italy).

Mouse fibroblast NIH-3T3 cells (ATCC) and mouse embryo fibroblasts (MEF, Specialty Media) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), L-glu and pen/strep.

Visceral endoderm-like cells (END2) were kindly provided by Professor C Mummery and cultured as described.[13]

The mESC lines[29,30] used in this study are feeder-independent and were cultured on gelatin-coated plates. Cells were maintained in Glasgow MEM/BHK medium containing 10% fetal bovine serum, 0.23% sodium bicarbonate, modified Eagle's medium (MEM) essential amino acids, 1 mM sodium pyruvate, 100 mM 2-mercaptoethanol and L-glu. This medium is referred to as cultivation medium. Leukemia-inhibitory factor (LIF, 1000 U ml^{-1}, Chemicon International, Temecula, CA, USA) was added to maintain the pluripotent, undifferentiated state.[31] To induce differentiation, cultivation medium without LIF was used. When EBs were formed, they were transferred to bacteriological plates and maintained in suspension for 3 days in cultivation medium supplemented with 0.1% dimethylsulfoxide. EBs were maintained in suspension 2 days more with

cultivation medium, then were allowed to settle onto gelatin-coated plates in the presence of cultivation medium without LIF or dimethylsulfoxide. Medium was changed every 2 days. Contracting areas appeared within 16–18 days.

hESC line 7 (hESC7, purchased at passage 11) and 8 (hESC8, purchased at passage 18) were obtained from Professor D Melton. Maintenance, passaging and differentiation were performed following the supplied protocols (http://www.mcb.harvard.edu/melton/hues).[10] Briefly, hESCs were cultured on MitC-mitotically inactivated mouse embryonic fibroblasts in knockout-Dulbecco's modified Eagle's medium supplemented with 10% knockout-serum replacement (KO-SR), 10 ng ml^{-1} basic fibroblast growth factor 12 ng ml^{-1} recombinant human LIF, Glutamax and pen/strep. Cells were adapted to trypsin passaging before being used in experiments. Differentiation was induced by culturing the cells in suspension (starting on what is referred to as day 0) in the absence of hLIF and basic fibroblast growth factor; this allowed them to form cystic EBs.[10] When specified, 5 μM AZA was added at each media change (that is, every third day) starting from day 1. After 7–10 days, EBs were passaged onto plates coated with gelatin and cultured in differentiation medium supplemented with 1% KO-SR.

In all experiments using mouse or human ESCs, the day on which ESCs were dispersed into suspension was considered day 0. This follows the nomenclature given by Kehat,[3] which considers the day of suspension as the day when differentiation starts.

Processing, isolation and immunostaining of cardiosphere-forming cells from murine heart biopsies were performed as extensively described previously.[25]

Transient transfection and dual luciferase assay

Plasmids (pGL3.SV40.luc and pGL3.CMV.luc) expressing luciferase under the control of strong non-tissue-specific promoters (that is, simian virus 40 or cytomegalovirus, respectively) were obtained commercially (Promega, Milan, Italy).

pGL3.hTNNI3_340b.luc was obtained by PCR amplification of residues 837–1174 (−270/+70) of GenBank accession no. X90780.[7]

rCMCs, C2.C12 (myoblasts and myotubes) and NIH-3T3 cells were grown in their respective milieus and transfected in parallel. Subconfluent cell cultures were co-transfected using Lipofectamine, following the supplied instructions: DNA/lipid complexes were prepared by mixing 1 μg of a luciferase reporter plasmid (pGL3.hTNNI3_340b.luc, pGL3.SV40.luc or pGL3.CMV.luc) and 0.1 μg of *Renilla luciformis* luciferase-reporter plasmid (pRL.TK, Promega). All transfections included a background sample. Fifty hours later, cells were harvested and luciferase activity determined with the Dual Luciferase Reporter Assay (Promega) in a TD20 luminometer (Turner Designs, Sunnyvale, CA, USA), following the manufacturer's instructions. The data obtained for each sample was used in the specified ratios.

Intra-myocardial/quadriceps injection of plasmid-DNA and luciferase assay

Mice were anesthetized with a ketamine (100 mg kg^{-1})–xylazine (2.5 mg kg^{-1}) mixture, administered intraperitoneally, and connected to a rodent ventilator, after tracheal intubation. Hearts were exposed and injected twice with 10 μl of 3 μg μl^{-1} plasmid-DNA solution in phosphate-buffered saline (pGL3.hTNNI3_340b.luc or pGL3.SV40.luc). A total of 60 μg was injected. Injection into the free wall of the left ventricle was performed with a 32-gauge needle while the heart was beating, under visual guidance.

Intra-quadriceps injection was performed without surgery. A total amount of 60 μg per leg^{-1} of plasmid DNA was injected (in two injections of 30 μg per 50 μl pGL3.hTNNI3_340b.luc or pGL3.SV40.luc). All experiments included a phosphate-buffered saline injection as negative control.

Mice were killed 3 days after surgery. Heart and quadriceps from each mouse were excised and used for luciferase assay. Mice were treated in accordance with European guidelines.

Tissue extracts were prepared and normalized for protein concentration by Bradford assay (BIORAD, Milan, Italy). The luciferase activity of tissue extracts was measured using a Luciferase Assay Kit (Promega) and a TD-20/20 luminometer (Turner Designs) according to the manufacturer's instruction and was calculated by subtracting the level measured in the negative control samples.

Lentiviral vector production and titration

The three-plasmid expression system used to generate LVVs by transient transfection was performed as described previously.[9,32]

The transfer vector plasmid backbone containing the enhanced green fluorescence protein reporter gene driven by the human phosphoglycerate kinase (hPGK) or cytomegalovirus promoters (pRRLcPPT.hPGK.EGFP.WPRE and pRRLcPPT.CMV.EGFP.WPRE, respectively) have been described before for assembling advanced third-generation lentiviruses.[9]

The mouse cardiac troponin (mTNNI3_427b) proximal promoter sequence was amplified by PCR from an original genomic clone. A 427-bp PCR fragment, corresponding to nucleotides −301/+126 of the previously published TnIc promoter (GeneBank accession no. Z22784)[8] was cloned to obtain pRRLcPPT.mTNNI3.EGFP.WPRE.

The human cardiac troponin (hTNNI3_340b) proximal promoter sequence was amplified by PCR from pGL3.hTNNI3.luc and cloned to obtain the plasmid pRRLcPPT.hTNNI3_340b.EGFP.WPRE.

PCR primers were chosen to amplify residues 100 322–101 238 of GenBank accession no. AC087457.5 harboring the cardio-specific enhancer in the cardiac muscle α-actin proprotein promoter on human chromosome 15.[14] The PCR product was cloned upstream from the hTNNI3 promoter to generate pRRLcPPT.hEnAct_846b-TNNI3_340b.EGFP.WPRE.

The fragment containing residues 611–899 of GenBank accession no. X03922, annotated as a potential enhancer in the human cytomegalovirus (hCMV) IE1 gene promoter region was cloned to obtain pRRLcPPT.hEnCMV_288b_TNNI3.EGFP.WPRE.

pSINF.EF1a.GFP.SAR/HS was kindly provided by Professor GR Hawley and has been already described.[33]

Lentiviral vector stocks were obtained from the supernatants of 293T cells co-transfected with the three

plasmids necessary for viral production. These were: the packaging plasmid, pCMVΔR8.74, designed to provide the HIV proteins needed to produce the viral particles; the envelope-coding plasmid, pMD.G, for pseudotyping the virion with VSV-G, and; one of the self-inactivating (SIN) transfer vector plasmids described above (pRRLcPPT.hPGK.EGFP.WPRE, pSINF.EF1a. GFP.SAR/HS, pRRLcPPT.mTNNI3_427b.EGFP.WPRE, pRRLcPPT.hTNNI3_340b.EGFP.WPRE, pRRLcPPT. hEnAct_846b-TNNI3_340b.EGFP.WPRE). The LVVs obtained were denominated PGK-LVV, EF1-LVV, mTNNI3-LVV, hTNNI3-LVV and hEnAct_TNNI3-LVV, respectively.

To determine the transducing unit concentration of the supernatants from 293T cell cultures, experiments were performed with rCMCs with serial dilutions of supernatant. In a typical titration experiment, only dilutions yielding 0.2–20% GFP-positive cells were considered for titer calculations: in this concentration range, we found a linear correlation between MOI and EGFP expression.

Lentiviral transduction

A total of 10^5 cells (CMCs, NIH-3T3 or HEK-293) were plated in 24-well plates. On the day of infection, the medium was removed and replaced with viral supernatant (at the specified MOI) to which polybrene had been added. At 48 h from the start of experiments, cells were harvested and analyzed by FACS.

Undifferentiated mESCs were plated on gelatin-coated plates the day before lentiviral transduction was performed overnight at an MOI of 2. After a media change, cells were cultured as described above.

hESC7 or 8 (at passages 19 and 21, respectively) were amplified on inactivated mouse embryonic fibroblasts feeder layers in six-well plates. Transduction was performed (at the specified MOI) when hESC colonies reached 30% confluence. After an overnight transduction, cells were washed, fresh medium added and cells cultured as described above.

Flow cytometric analysis

Sorting experiments and cytometric analyses were performed using an FACSVantange SE and a FACSCalibur with CellQuest software (Becton Dickinson Immunocytometry Systems), respectively. For all FACS analyses, at least 10 000 events were recorded.

Transduced cells (rCMCs, NIH-3T3 or HEK-293) were analyzed by FACS with standard procedures to evaluate mean fluorescence intensity. Transduced mESCs were collected using trypsin and %EGFP-expressing cells was evaluated with standard procedures. Transduced hESC samples were collected and stained by a standard indirect intracellular staining procedure (Beckton Dickinson, Milan, Italy) using a mouse monoclonal antihuman nuclei antibody (Chemicon, Temecula, CA, USA) followed by R-phycoerythrin (RPE)-conjugated goat antimouse IgG (DakoCytomation, Milan, Italy), according to the manufacturer's instructions. The percentage of hESCs expressing EGFP was calculated.

The protocol used for dispersing hEBs into suspension for FACS was developed following the indications of ES Cell International (ESI_http://www.escellinternational.com). Plates containing EBs were treated with collagenase IV in phosphate-buffered saline (200 U ml^{-1}) for 10 min in the incubator. Vigorous pipetting was used to dislodge cell clumps from the dish. Cells were then transferred to a 15 ml tube and spinned down at 600 *g* for 2 min. The supernatant was removed and the pellet resuspended in 0.05% trypsin (Gibco, Milan, Italy). A single-cell suspension was obtained by vigorous pipetting. Just before sorting, the cell suspension was filtered through a 70 μm cell strainer (Becton Dickinson).

RNA extraction and real-time PCR

Total RNA was extracted from cells with the RNeasy Mini Kit (Qiagen, Milan, Italy) according to the manufacturer's instructions. RT-PCRs were performed using Superscript III reverse transcriptase, random primers and TaqMan oligonucleotides (Assay on Demand, Applied Biosystems, Monza, Italy) for *cardiac troponin I* (TnIc) (code Hs00165957_m1) and *alkaline phosphatase* (ALP) (code Hs00758162_m1), as recommended by the manufacturer, in an ABI PRIS 7000 instrument (Applied Biosystems). All reactions were performed in duplicate. The amount of target, normalized to the values obtained for amplification of *GAPDH* and relative to a calibrator, was determined by $2^{-\Delta\Delta CT}$.

Immunofluorescence staining

Standard indirect immunofluorescence techniques were used to stain paraformaldehyde-fixed cells. Anticardiac troponin I (Babco, Berkeley, CA, USA) and phycoerythrin-conjugated anti-mouse (DakoCytomation) antibodies were used.

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Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)