A fluorescent reporter gene as a marker for ventricular specification in ES-derived cardiac cells

Nathalie Meyer[a], Marisa Jaconi[b], Angela Landopoulou[c], Philippe Fort[a], Michel Pucéat[a,*]

[a] *CRBM, CNRS UPR 1086, IFR 24, 1919, route de Mende, 34293 Montpellier, France*
[b] *Laboratory of Ageing, University of Geneva, Geneva, Switzerland*
[c] *Molecular Diagnostics Laboratory, I/R-RP, NCSR 'Demokritos', Athens, Greece*

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Abstract We have established a CGR8 embryonic stem (ES) cell clone (MLC2ECFP) which expresses the enhanced cyan variant of *Aequorea victoria* green fluorescent protein (ECFP) under the transcriptional control of the ventricular myosin light chain 2 (MLC2v) promoter. Using epifluorescence imaging of vital embryoid bodies (EB) and reverse transcription-polymerase chain reaction (RT-PCR), we found that the MLC2v promoter is switched on as early as day 7 and is accompanied by formation of cell clusters featuring a bright ECFP blue fluorescence. The fluorescent areas within the EBs were all beating on day 8. MLC2ECFP ES cells showed the same time course of cardiac differentiation as mock ES cells as assessed by RT-PCR of genes encoding cardiac-specific transcription factors and contractile proteins. The MLC2v promoter conferred ventricular specificity to ECFP expression within the EB as revealed by MLC2v co-staining of ECFP fluorescent cells. MLC2ECFP-derived cardiac cells still undergo cell division on day 12 after isolation from EBs but withdraw from the cell cycle on day 16. This ES cell clone provides a powerful cell model to study the signalling roads of factors regulating cardiac cell proliferation and terminal differentiation with a view to using them for experimental cell therapy.

Key words: Embryonic stem-derived cardiac cells; Ventricular myosin light chain 2 promoter; Embryoid bodies

1. Introduction

Heart morphogenesis is among the earliest milestones of organogenesis in vertebrate embryos. Cardiac progenitor cells derive from mesodermal cells shortly after gastrulation. Subsequent cardiac cell differentiation is then a complex cellular process that comprises cell proliferation, migration and differentiation. Once determined, cardiomyocytes form the linear heart tube, so-called primary myocardium, one of the earliest morphogenetic events in the embryo [1]. The cardiac cell is unique in its ability to commit to a terminally differentiated muscle phenotype whilst concomitantly undergoing cellular proliferation. This property is, however, restricted to embryonic cardiac cells. Adult cardiomyocytes are locked in the G0 or G1 phase of the cell cycle [2] and are thus terminally differentiated. Although the latter dogma has been challenged [3], loss of contractile cardiomyocytes during aging, after severe ischemia, or in cardiomyopathies triggers first a compensatory cardiac hypertrophy and, then, a failure of cardiac function, pointing to a limited proliferative capability of adult cardiomyocytes.

Determinants of the cardiogenic phenotype are still unclear. However, three major transcription factors of the Nkx, GATA, and MEF2 families play in concert a preponderant role in cardiac cell differentiation [4,5]. These factors transactivate muscle-specific genes including actin, myosin light chain (MLC), myosin heavy chain (MHC), troponins and desmin [1,6]. Little is known about the regulatory mechanisms including signals of differentiation and growth factors that arise from the endoderm and which specify or promote the cardiogenic phenotype. To address such questions in an 'in vivo-like' environment, pluripotent embryonic stem cells (ES cells) represent a suitable cell system since they recapitulate programmed expression of cardiac genes characteristic of the mouse embryo [7,8]. Furthermore, since the establishment of a human ES cell line [9], engraftment of cardiac-committed stem cells into the heart [10] has become a very promising and challenging therapeutic approach to improve the state of a failing heart. A similar approach with transplanted stem cells has already turned out to be successful for neurons to rescue the function of an injured spinal cord [11]. However, to reach such a goal in cardiology, and to eliminate the risk of uncontrolled cell proliferation or of miscommitment, it is mandatory to study the terminal steps of differentiation of ventricular cells.

In a pioneering work, Metzger et al. (1996) engineered a D3 ES cell clone, which expresses as a reporter the β-galactosidase gene under the transcriptional control of the α-actin promoter. More recently, Kolossov et al. [12] established a similar D3 ES cell clone using green fluorescent protein (GFP) as a reporter gene. Although the α-actin gene is the earliest cardiac marker, it is not specific of ventricles or even of the heart [13,14]. Chamber specification occurs early during embryonic heart development. Atrial and ventricular cells arise from divergent lineages that are determined in the 'primary' myocardium before looping of the heart occurs [15,16]. This 'time window' during which ventricular and atrial cells are already determined but not terminally differentiated can be used to sort out precursors of one cell population from the other.

Here, we have engineered and characterized the potential of a CGR8 ES cell clone in which the enhanced cyan fluorescent protein (ECFP) is exclusively expressed in cardiac ventricular cells. These cells provide a suitable model to study the signal-

*Corresponding author. Fax: (33)-4-67 52 15 59.
E-mail: puceat@crbm.cnrs-mop.fr

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ling pathways of growth factors, which regulate ventricular specification in an embryonic-like developmental context.

2. Materials and methods

2.1. Plasmid construction

The 250 bp fragment of the MLC2 promoter was excised from a luciferase expression vector pMLCLΔ5′ [17] and was subcloned into the *Hin*dIII/*Eco*RI sites of the promoterless pECFP vector (Clontech). After linearization using the unique *Xho*I restriction site, the plasmid was electroporated into CGR8 ES cells (generated from the pluripotent inner cell mass of the preimplantation 129J mouse embryos at the Centre for Genome Research in Edinburgh) according to the standard protocol. The ES clones (MLC2ECFP) were propagated in the presence of leukemia inhibitory factor (LIF) and selected for 10 days using G418 (250 μg/ml). Seven neomycin-resistant colonies of ES cells showing the brightest fluorescence after 9 days of differentiation were further selected.

2.2. ES cell differentiation

ES CGR8 cells were propagated in BHK21 medium supplemented with pyruvate, non-essential amino acids, mercaptoethanol, 10% fetal calf serum [18] and LIF conditioned medium obtained from pre-confluent 740 LIF-D cells that are stably transfected with a plasmid encoding LIF [19]. The cells were trypsinized and replated every second day. Under these conditions, the ES cells feature an undifferentiated phenotype (i.e. tightly packed cells forming colonies) as assessed by daily microscopic observation. Differentiation was carried out using the hanging drop method [7]. Briefly, embryoid bodies (EBs) were formed in hanging drops of differentiation medium (BHK21 medium supplemented with pyruvate, non-essential amino acids, mercaptoethanol and 20% fetal calf serum without LIF) for 2 days (D0–2). Then, the EBs were incubated for 3 days in suspension (D2–5) and for 7–15 days on gelatin-coated dishes or glass coverslips (D6–12).

2.3. Cell imaging

Images of EBs or isolated cells were acquired with a Leica epifluorescence microscope equipped with a 20×, 40× or 100× objective mounted on a piezo-electric device (LVPZT position servo controller, Physik Instrumente, Waldbronn, Germany) driven by Metamorph software (Universal Imaging, USA). To visualize in situ immunostaining of MLC2v, optical *z*-sectioning of the EBs was carried out using a 0.4 μm step. To detect ECFP fluorescence, the EBs or isolated cells were illuminated with a mercury lamp at 400 ± 20 nm and the ECFP fluorescence recorded with a X114-2 CFP Leica filter cube that consists of a dichroic mirror DM 455 and an emission filter at 480 ± 30 nm. Images were acquired with a micromax 1300YHS CCD camera (Princeton Instruments, USA) and stored as single tiff images or as a volume file ('stack' of *z*-section images) using the Metamorph. To improve the resolution and the signal-to-noise ratio of the volume data, digital deconvolution was applied to 'stacks' of images. The images were restored using Huygens software (Huygens 2.2.1, Scientific Volume Imaging, The Netherlands) and visualized using Imaris (Bitplane, Switzerland). All calculations were performed using an Octane workstation (Silicon Graphics).

2.4. Cell immunostaining

EBs of 12–14 days were fixed in 3% paraformaldehyde for 45 min and permeabilized for 30 min with 1% Triton X-100. In some experiments, EBs were dissociated on day 9 with trypsin (0.05% in phosphate-buffered saline, EDTA 1 mM) for 5 min at 37°C or with collagenase for 20–30 min at 37°C (Worthington CLSII, 1 mg/ml) in HEPES buffer adjusted to pH 7.35, containing NaCl 117 mM, HEPES 20 mM, NaH_2PO_4 1.2 mM, KCl 5.4 mM, $MgSO_4$ 1 mM, glucose 5 mM. The isolated cells were plated on gelatin-coated glass coverslips. After 3 days, cells were fixed in 3% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunostaining was performed as previously described [20]. Polyclonal anti-MLC2a and anti-MLC2v antibodies were previously characterized [21]. The monoclonal anti-ryanodine antibody was from Affinity Bioreagents [22]. The anti-mouse and anti-rabbit IgG secondary TRITC-conjugated antibodies were from Sigma (La Verpillère, France).

2.5. Cell division

Cells isolated from 9- or 13-day-old EBs with collagenase, as described above, were plated on gelatin-coated glass coverslips. On day 12 (9+3), or 16 (13+3), bromodeoxyuridine (BrdU) was added for 30 min. After washing and fixing, incorporation of BrdU was revealed using a monoclonal anti-BrdU antibody (Amersham) and a secondary TRITC-conjugated anti-mouse IgG antibody. Ventricular cells undergoing DNA synthesis were detected by both ECFP fluorescence and nuclear BrdU staining. To investigate whether ES-derived ventricular cells were still dividing, cells isolated on day 9 from EBs, and plated on glass coverslips set in small steel cuvettes, were transferred on day 12 to the stage of the microscope in a small (10 cm diameter) plexiglass culture chamber. To maintain a temperature of 37°C, both the stage of the microscope and the objective were temperature-controlled. Moreover, heated gas (95% O_2/5% CO_2) was pulsed by a minipump into the chamber. ECFP fluorescent images were captured with the CCD camera every 4 h for 3 days using the time-lapse mode of the Metamorph software.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by a modified phenol–chloroform method from 10 EBs lysed in guanidinium thiocyanate-containing buffer [23,24]. After reverse transcription, 100–300 ng RNA was used for PCR to stay within the linear range of amplification. PCR was carried out using a set of gene-specific primers (GATA4, sense 5′-CGAGATGGGACGGGACACT-3′, antisense 5′-CTCACCCTCGGCCATTACGA-3′; Nkx2.5, sense 5′-TGCAGAAGGCAGTGGAGCTGGACAAGCC-3′, antisense 5′-TGCACTTGTAGCGACGGTTCTGGAACCAG-3′; MEF2C, sense 5′-AGCAAGAATACGATGCCATC-3′, antisense 5′-GAAGGGGTGGTGGTACGGTC-3′; βMHC, sense 5′-GCCAACACCAACCTGTCCAAGTTC-3′, antisense 5′-CTGCTGGAGAGGTTATTCCTCG-3′; MLV2v, sense 5′-GCCAAGAAGCGGATAGAAG G-3′, antisense 5′-CTGTGGTTCAGGGCTCAGTC-3′; tubulin, sense 5′-TCACTGTGCCTGAACTTACC-3′, antisense 5′-GGAACATAGCCGTAAACTGC-3′) as previously described [24]. Tubulin, studied as a housekeeping gene to check the amount of RNA used in each RT-PCR, βMHC, GATA4 and MLC2v cDNA were amplified for 30 cycles. Nkx2.5 and MEF2 cDNA were amplified for 30–36 cycles. The PCR products were analyzed in agarose gel electrophoresis and the ethidium bromide-stained bands were quantified by scanning photographs of gels using a CCD camera and the Image J 1.01 software (NIH, USA). The nature of PCR products was checked by size and in some cases by restriction analysis or sequencing.

3. Results

We established a CGR8 cell line by transfecting ES cells with a construct composed of the 250 bp MLC2v promoter upstream of the ECFP cDNA. The clones were selected for 10 days with G418. To ensure that ECFP was stably expressed under the control of a tissue-specific promoter, MLC2ECFP ES cells were allowed to differentiate for 5 days within EBs. EBs were then transferred into a small culture chamber, set on the stage of an inverted microscope. The differentiation process (EB beating and ECFP expression) was recorded on live cells. Images were acquired for 4 days with a CCD camera in transmitted light every hour and using the epifluorescence light every 3 h. Fig. 1 shows selected images captured on days 5, 6, 7 and 8. No blue background fluorescence was detected in round EBs on day 5 (Fig. 1a,g). On day 6, only a weak ECFP fluorescence was detected (Fig. 1b,h). A diffuse fluorescence could already be observed on day 7 (Fig. 1c,i). Patches of fluorescent cells were clearly detected on day 8 (Fig. 1d,j). They overlapped with beating areas of the EBs recorded in both transmitted and epifluorescence lights by video microscopy. This is better seen in higher magnification images (Fig. 1e,f,k,l). The time courses of expression of ECFP and MLC2v were well correlated. MLC2v mRNA was already expressed on day 7, reaching a maximal level on day 12 (Fig. 2a). In order to compare the kinetics of differentiation of the

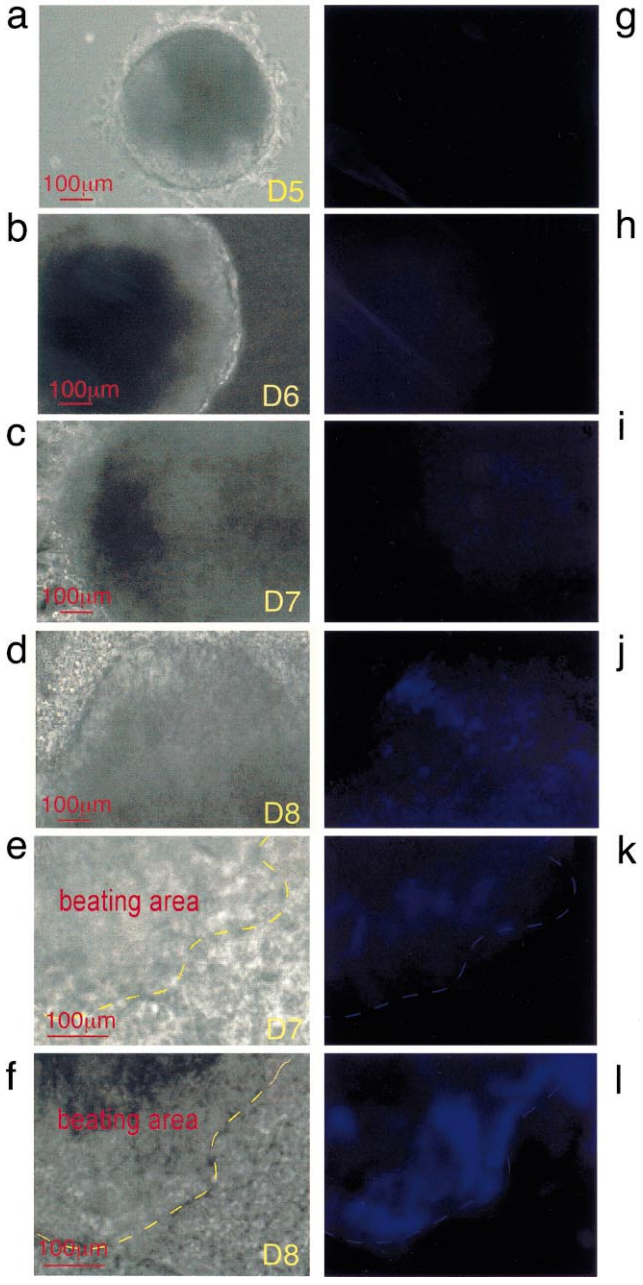

Fig. 1. Expression of ECFP under the transcriptional control of the MLC2v promoter in EBs generated from CGR8 ES cells. EBs generated from the MLC2ECFP CGR8 ES cells were transferred on day 5 to a culture chamber on the stage of an inverted microscope. Transmitted light (a,c,e,g,i,k) and fluorescence images (b,d,f,h,j,l) were captured with a CCD camera on days 5, 6, 7 or 8 at different magnifications as shown by the scale bar on each image. Beating areas were observed by video microscopy illuminating the cells with transmitted light or epifluorescence. Images e,f,k,l are selected from the videos. The experiment was repeated four times with similar time courses.

MLC2ECFP cells with that of wild type CGR8 cells (mock cells), several mRNAs of cardiac-specific genes were amplified by RT-PCR. In undifferentiated CGR8 cells, none of the cardiac-specific genes GATA4, Nkx2.5, MEF2C, βMHC or MLC2v was expressed (Fig. 2b). In 5-day-old EBs, GATA4, Nkx2.5, and MEF2C, three cardiac transcription factors, were all detected in both wild type (mock) and MLC2ECFP EBs (Fig. 2b). Expression of transcription factors reached a plateau on day 5 for at least 15 days. Transcripts of βMHC and MLC2v genes were detected on days 9 and 7, respectively, and maximal expression was observed on day 12 in both mock and MLC2ECFP EBs (Fig. 2c).

Spontaneous beating activity of EBs, another indication of cardiac differentiation, was assessed daily by visual observation. Spontaneously beating areas were observed in at least 25% of both mock and MLC2ECFP EBs on day 6 (Fig. 3a). Spontaneous Ca^{2+} spiking was recorded within Fluo3-loaded EBs on the same day (data not shown). The beating activity (i.e. number of beating EBs) increased over the next days to reach a plateau on day 9 (Fig. 3a). Beating areas overlapped with blue ECFP fluorescent regions in MLC2ECFP EBs (Fig. 3b). The beating frequency of MLC2ECFP EBs on day 9 (1.1 ± 0.2 beats/s, $n = 20$) was not significantly different from that of mock EBs (1.2 ± 0.2 beats/s, $n = 20$).

To further assess whether fluorescent areas were specifically constituted of ventricular myocytes, cells were isolated from 9-day-old EBs and plated for 3 days on glass coverslips. Immunostaining using an anti-MLC2v antibody was then carried out. Fig. 4a shows a merged image of cells expressing blue ECFP and red MLC2v, visualized by the primary anti-MLC2v and secondary TRITC-conjugated antibodies. Clearly, these distinct fluorescence patterns are seen in the same cells (125 counted cells from three differentiation experiments) showing that ECFP-positive cells also express MLC2v. On the other hand, an anti-MLC2a antibody combined with a secondary TRITC-conjugated antibody labelled the myofilaments of many cells. None of them, however (88 counted cells from three differentiation experiments), expressed ECFP (Fig. 4b). Fig. 4c shows a merged image of ECFP fluorescence and of red ryanodine receptor 2 antibody recognized by a specific antibody and a secondary TRITC-conjugated antibody. The distribution of ryanodine receptors in ECFP-positive cells was still not well organized as expected from the poor development of the sarcoplasmic reticulum at this stage of development. In situ immunostaining in a 9-day-old EB showed that the MLC2v-positive cells were organized as clusters in which myofibrils of individual cells formed an interconnected network (Fig. 4d). Furthermore, a detail of a single cell within such a network reveals that the regular sarcomeres reached a length (2 μm) similar to that of sarcomeres of an adult cardiac cell, indicating that cells are fully organized at this stage of development (Fig. 4d, right image).

The exit from the cell cycle is indicative of terminal differentiation of cardiac cells and occurs on day 16 in mouse EBs [31]. An early exit would suggest a possible long-term toxicity of ECFP. To address this issue, we cultured for 3 days cells isolated from EBs on day 9 or 13. Incorporation of BrdU into nuclei revealed that most (72%, out of 105 counted cells in two separate experiments) of ECFP fluorescent cardiac cells still underwent DNA synthesis on day 12 (Fig. 5a,c). However, only a low percentage of ECFP-positive cells (12%, out of 95 counted cells in two separate experiments) featured BrdU staining on day 16 (3 days after isolation from EB on day 13) (Fig. 5c). Most of these cells were multinucleated, an indicator of cell cycle withdrawal (Fig. 5b). Cells isolated from 9-day-old EBs were also cultured for 3 days in a small culture chamber on the stage of an inverted microscope. Direct observation of living cells within 72 h also showed proliferation of ECFP-positive cells (Fig. 5d).

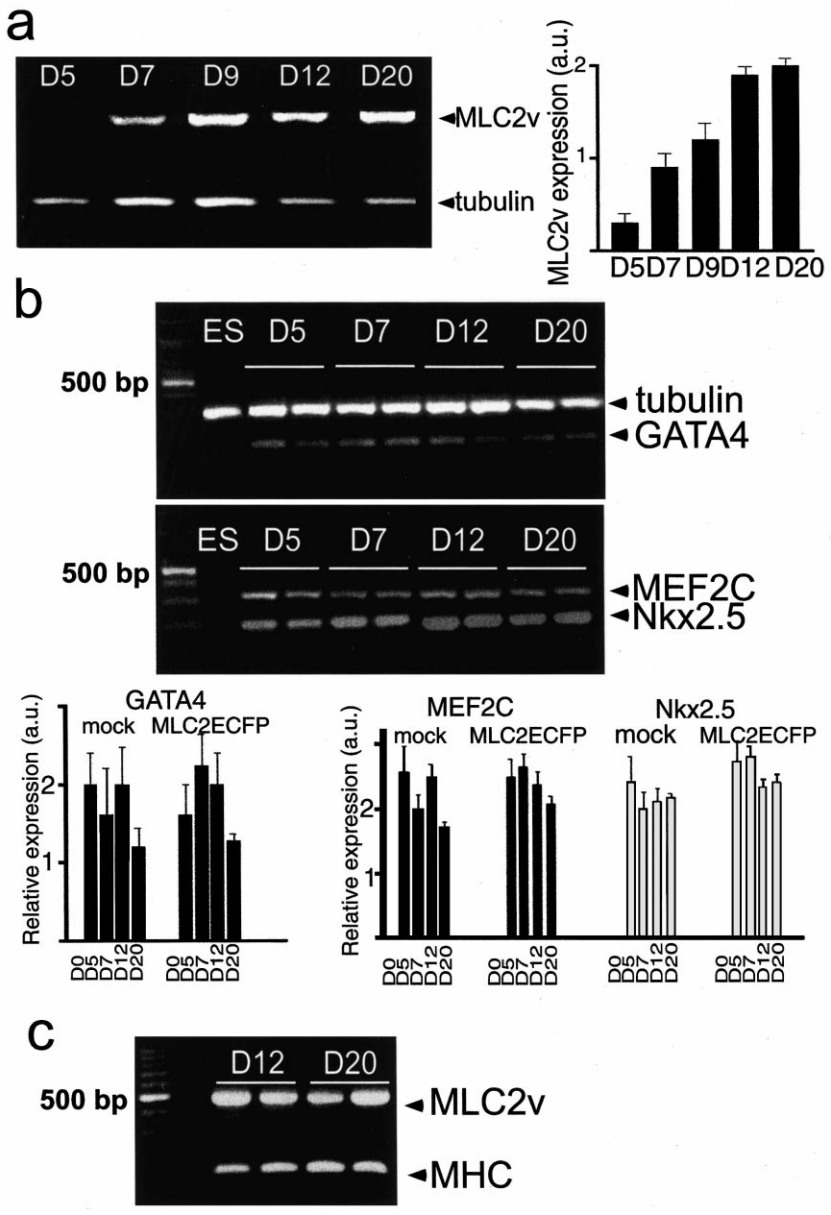

Fig. 2. a: RT-PCR of cardiac-specific genes. RNA was extracted from 10 MLC2ECFP EBs on days 5, 7, 9, 12 and 20, and MLC2v mRNA was amplified by PCR. The graph on the right presents data from three experiments, performed with three different cell clones. b,c: Cardiac-specific genes (GATA4, Nkx2.5, MEF2C, βMHC and MLC2v) were amplified by RT-PCR from total RNA extracted from ES cells, mock (left lanes) or MLC2ECFP (right lanes) EBs, on days 5, 7, 9, 12 or 20. The graphs present data from three experiments performed with three different cell clones. Data were normalized to the level of expression of tubulin. The DNA 100 bp size marker shows the size of the amplified fragments.

4. Discussion

We employed CGR8 ES cells to generate a cell clone expressing ECFP, a variant of GFP, under the transcriptional control of the MLC2v promoter. CGR8 cells are derived from the pluripotent inner cell mass of the preimplantation 129J mouse embryos. In comparison with other mouse stem cells, CGR8 cells feature several advantages as an experimental cell model. First, they do not require feeder cells and are propagated in the presence of LIF under established culture conditions [18]. Second, they differentiate earlier into cardiac cells than the widely used D3 ES cell line as assessed by the time course of expression of the MLC2v gene. Therefore, we report that this ES cell type, which can colonize the germline, when injected in a blastocyst [25] is suitable to study cardiac cell differentiation.

MLC2v is the earliest ventricle-specific marker [15]. The 250 bp fragment of the MLC2v promoter used in this study has been previously characterized [26]. This promoter confers cardiac ventricular specificity in vivo when used to target gene expression in the ventricle of transgenic mice [27,28]. Although it has been suggested that low levels of transcript are produced by this promoter fragment [29], we observed a bright ECFP fluorescence in the EBs and isolated ES-derived cardioblasts even after 15 passages of the ES MLC2ECFP cell clone. This suggests that a high number of copies of the reporter gene were inserted into the ES genome by cell electroporation. This further demonstrates that ES cells have been

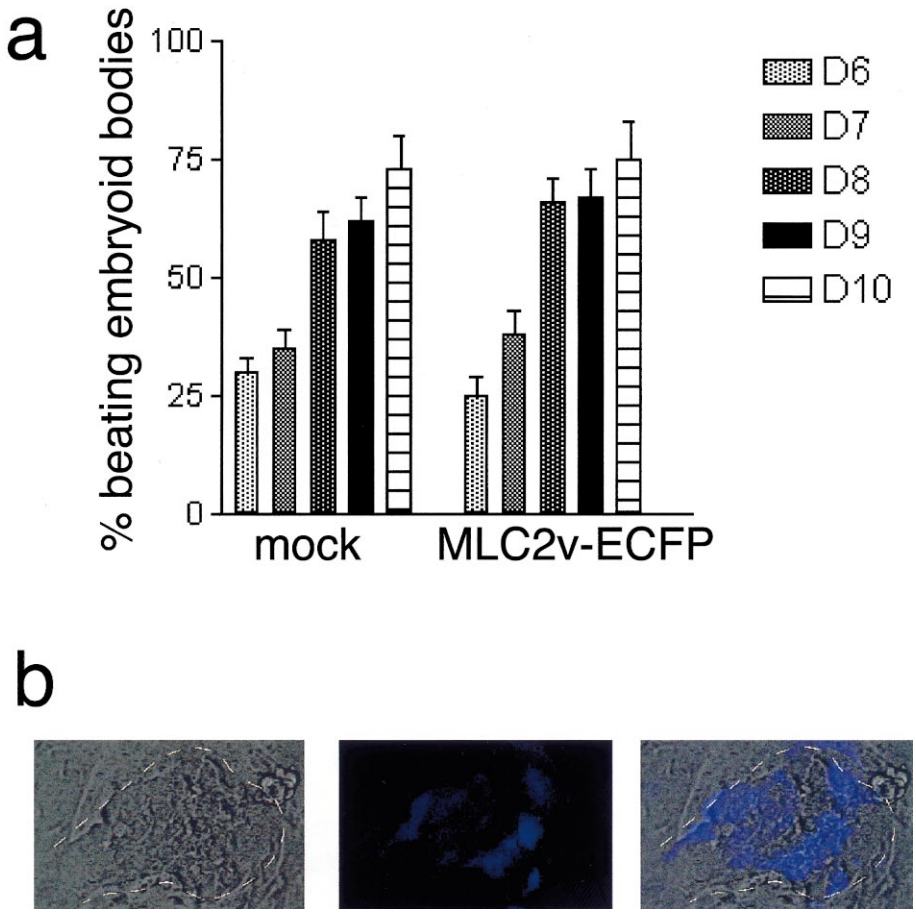

Fig. 3. ECFP expression does not affect beating activity. a: In each experiment, 25 mock and 25 MLC2ECFP EBs were observed each day under the microscope and beating EBs were counted. The figure shows the average (±S.E.M.) of four experiments. Data were analyzed with Student's *t*-test. b: Beating activity of a MLC2ECFP EB (day 8) was observed and captured by a CCD camera under transmitted light (left panel), or epifluorescent (middle panel). The right panel is the merged picture.

stably transfected. However, we did notice a slight progressive decrease in ECFP expression in EBs derived from later passages of ES cells, which encouraged us to use stocks of ES cells from earlier passages for our further experiments.

To evaluate the effects of ECFP expression on cardiac differentiation of ES cells, we first used RT-PCR to screen expression of cardiac-specific genes. The earliest markers of cardiac cell determination (i.e. Nkx2.5, GATA4, MEF2C) were all turned on in the MLC2ECFP cell clone and appeared with the same kinetics and to the same extent as in mock cells (Fig. 2). Similarly, the gene of the constitutive contractile protein MLC2v was expressed as early as day 7. Both MLC2v and MHC mRNAs were strongly expressed later on day 12. This is in line with the early detection of ECFP fluorescence, on day 7 (Fig. 1). That confirms that the MLC2v promoter is turned on at this stage of development in CGR8-derived cardiac precursors as previously found in ES5 and ES14 cell lines [30]. It should, however, be noted that expression occurs earlier than in the D3 cell line, in which MLC2v is only detected on day 9 [16,31]. Furthermore, expression of the ventricular gene in CGR8-derived cardiac cells in vitro correlates well with the expression pattern observed in 8-day post-coitum embryos in vivo [15]. Also, both the extent and the rate of spontaneous beating activity were similar in ECFP-expressing EBs and in wild type EBs (Fig. 3). Altogether, these findings demonstrate that the ECFP protein does not affect early or late cardiac cell differentiation. ECFP can thus be used as a suitable specific marker for cell fate and cardiac autonomic activity.

In order to determine whether expression driven by the MLC2v promoter is strictly confined to ventricular precursors in the EBs, we used RT-PCR and specific antibodies to study expression of the ventricular- and atrial-specific MLC2v and MLC2a, respectively. We found that both mRNA of MLC2v and MLC2a (our unpublished data) and proteins were expressed in ES-derived cardioblasts within the EBs. However, only MLC2v but not MLC2a was found in ECFP-positive cardiac cells derived from the MLC2ECFP ES cells. MLC2v was seen in organized sarcomeric units, suggesting that ECFP does not impair myofibrillogenesis. The MLC2v-expressing cells seemed to be clustered together forming a network within the EBs (Fig. 4d). This spatial organization is similar to that observed in embryonic cardiac explants [32], which further validates these EBs as a model to study early cardiac development.

Finally we report that most ES-derived cardioblasts expressing ECFP still undergo DNA synthesis on day 12 and still divide as revealed by supravital microscopy of cells They withdraw from the cell cycle on day 16 and shift to multinucleation, a characteristic of terminal differentiation (Fig. 5). The latter events occur with the same kinetics as in mock ES cells [33]. Therefore, we conclude that expression of ECFP for

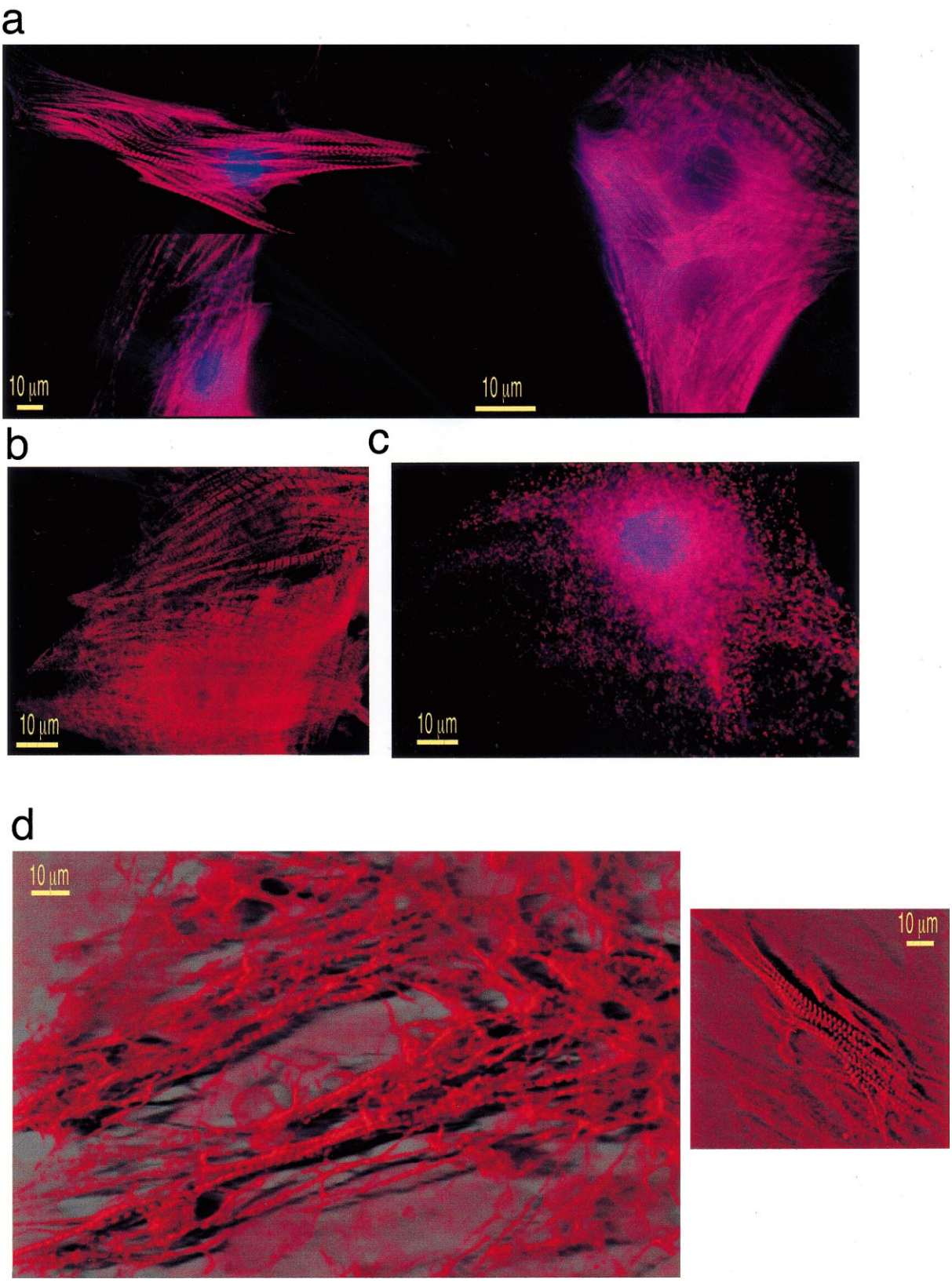

Fig. 4. ECFP fluorescent cells express MLC2v and ryanodine receptors. a: Merged images of blue image of ECFP-expressing cells and red image of myofilaments labelled with an anti-MLC2V and secondary TRITC-conjugated anti-rabbit IgG antibody (left picture: 40× objective; right picture: 100× objective, cells from different microscope fields). b: Image of an ECFP-negative cell, labelled with a specific anti-MLC2a antibody combined with a secondary TRITC-conjugated anti-rabbit IgG antibody. c: Merged image of a cell expressing ECFP and labelled with an anti-ryanodine receptor 2 antibody used with a secondary TRITC-conjugated antibody. d: Immunostaining of cells within a MLC2ECFP EB using an anti-MLC2v antibody and a TRITC-conjugated secondary antibody. The main picture shows an image restored by digital deconvolution from 23 successive optical sections. This figure is representative of at least three similar experiments. The right image shows a single cell inside a network of MLC2v-positive cells. Both images were visualized as shadow projections (Imaris software).

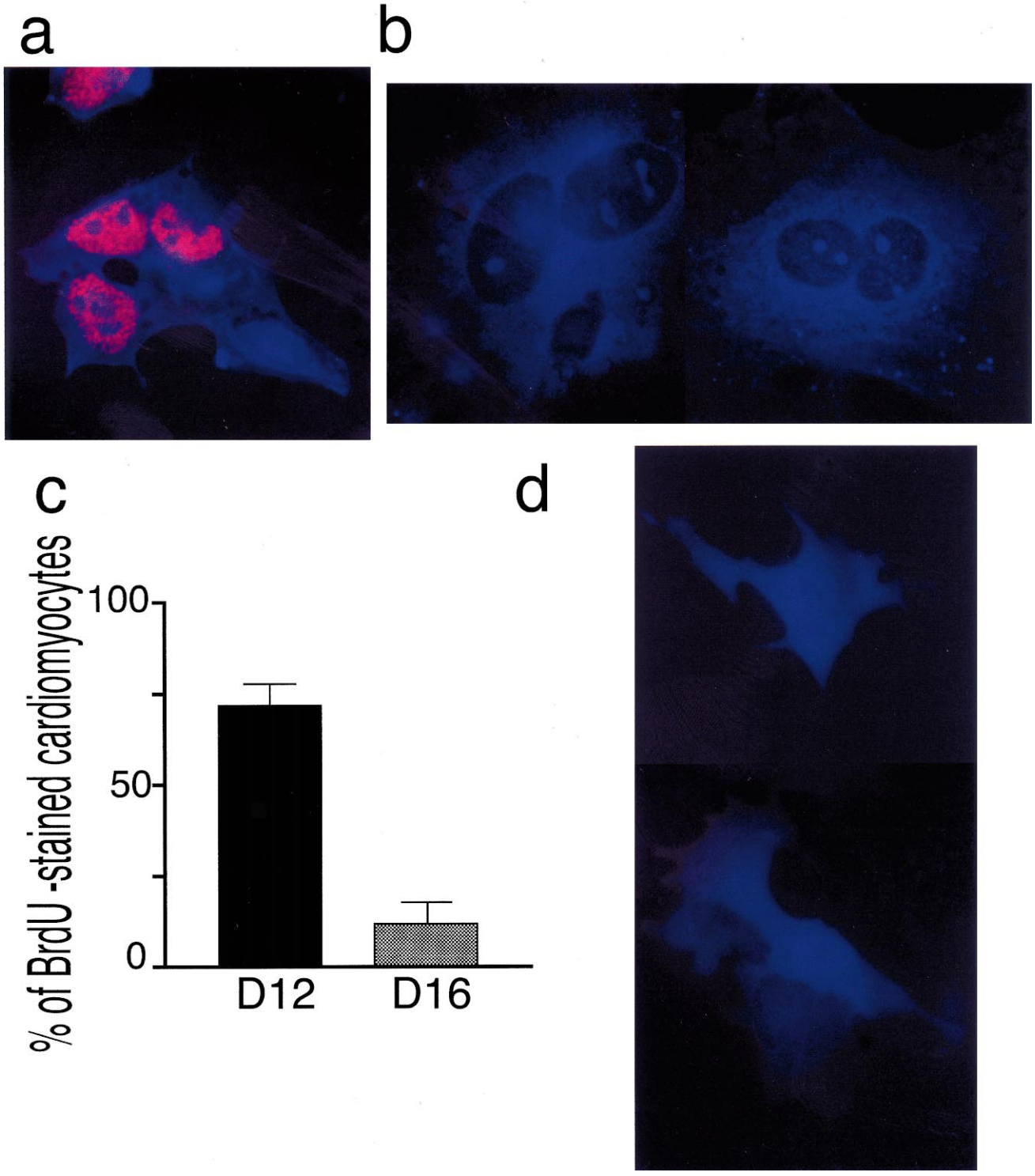

Fig. 5. Proliferative state of ECFP-expressing cells. EBs were dissociated on day 9 (a,d) or 13 (b) and isolated cells were plated in differentiation medium for 3 days. a,b: BrdU was added to cells for 30 min and then cells were fixed. BrdU incorporation was revealed by a monoclonal antibody, and a secondary TRITC-conjugated anti-mouse IgG antibody (magnification 35× (a) and 55× (b)). ECFP fluorescence allowed the detection of ventricular myocytes. c: The bar graph shows the number of ECFP-positive cells showing BrdU staining 3 days after cell isolation from 9- (D12) or 13-day-old EBs (D16). d: Cells isolated on day 9 were transferred to the stage of an inverted microscope and cultured for 3 days. Two ECFP fluorescence images of the same microscope field were captured at $T=0$ (top image) and $T+72$ h (bottom image).

as long as 8 days (days 7–15) does not affect viability or mitotic capability of ES-derived ventricular cells.

ES cells with a fluorescent protein as a reporter under the transcriptional control of a specific ventricular promoter provide a valuable tool to track the ventricular progenitor cells in EBs. Since these fluorescent cells still divide on day 12, they may be easily sorted by FACS analysis after isolation from the EBs on day 9 and used for experimental cell therapy. The

cell model can also be used to address specific questions on how mouse embryonic ventricular cells exit the cell cycle. This is a crucial situation in the mouse in which cell division dramatically decreases after birth [34], a situation similar to that in the human heart [35]. Our findings further encourage the use of a genetically manipulated parental ES cell line to delineate the signalling roads of proliferation, growth and differentiation factors that determine the cardiac ventricular phenotype. Furthermore, the use of the MLC2ECFP gene reporter in ES cells derived from transgenic mice may be of help to understand the causes of lethality in embryos which die of heart failure after transgenesis.

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