Fine-tuning in Ca\textsuperscript{2+} homeostasis underlies progression of cardiomyopathy in myocytes derived from genetically modified embryonic stem cells

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Mutations of genes encoding contractile proteins are responsible for familial hypertrophic cardiomyopathies. Understanding the process of differentiation of cardiomyocytes carrying a mutated protein is a crucial step towards potential treatments of inherited cardiac disorders. Embryonic Stem (ES) cells which faithfully recapitulate \textit{in vitro} the process of cardiac cell differentiation can be genetically modified to incorporate a mutation mimicking a cardiomyopathy. ES cell lines engineered to express a wild-type (MLC2vGFP) or a mutated form (R58QMLC2vGFP) of ventricular myosin light chain 2 (MLC2v) fused to GFP were differentiated into cardiomyocytes within embryoid bodies (EBs). Visualization of GFP combined with sarcomeric actinin immunofluorescence of EBs revealed that mutated MLC2v dramatically prevented myofibrillogenesis. Cardiomyocytes expressing wild-type MLC2v featured spontaneous Ca\textsuperscript{2+} spiking, but not those harboring the mutation. Expression of cardiac transcription factors Mef2c, GATAs, myocardin and Nkx2.5 was not affected by cell expression of mutated MLC2v. A dramatic decrease in expression of mRNAs encoding \alpha\textsuperscript{-}actin, MLC2a and MLC2v was observed in R58QMLC2vGFP EBs. This event was attributed to a failure of Mef2c to translocate into the nucleus, a Ca\textsuperscript{2+}-dependent process. Expression in mutated cells of a constitutively active Ca\textsuperscript{2+}- and calmodulin-dependent kinase II or treating EBs with ionomycin fully restored translocation of Mef2c into the nucleus and expression of mRNAs encoding sarcomeric proteins partially rescued contractile activity of EBs. Alteration of Ca\textsuperscript{2+} homeostasis in mutated cardioblasts affects the transcriptional program of cardiac cell differentiation leading to a defect in myofibrillogenesis, and, in turn, in contractility. Genetically modified ES cells provide a unique cell model to determine abnormalities in Ca\textsuperscript{2+} homeostasis underlying progression of human cardiomyopathies.

INTRODUCTION

Familial hypertrophic cardiomyopathies and dilated cardiomyopathies are inherited as autosomal dominant diseases (1–3). Primary genetic defects have been identified for these pathologies. These include mutations in genes encoding myosin heavy and light chains as well as troponins, tropomyosin, actin and myosin binding protein C (4). Expression of these mutated proteins in the heart leads to both an impaired contractile function and electrical disturbances resulting, in some cases, in premature sudden death (5–7).

From the perspective of potential treatments of inherited cardiac disorders, it is crucial to comprehend the various molecular steps leading to myocardial defects including sudden death. Deciphering the signals which regulate macromolecular assembly of muscle contractile apparatus during cardiogenesis is specifically important for a better understanding of the myofibrillar myopathies. In addition, it is critical to determine the consequences of a single point mutation on both the processing and the functioning of a protein as well as on the whole cell homeostasis and, in turn, on the mechanical function of the diseased myocardium. So far, to address such an issue, genetic manipulation in transgenic mice has been used as an approach. Targeted expression of a mutated sarcomeric protein into murine myocardium has brought important findings to the pathological consequences of such mutations at

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the molecular, cell and animal levels (8). However, the α–myosin heavy chain (α–MHC) promoter has been used most widely to specifically target expression of the mutated protein into the heart. This strategy suffers from several limitations. First, the α-MHC promoter is only turned on at birth, preventing the investigation of pathological consequences of the mutation during embryogenesis. Indeed, alteration of the process of cardiac cell differentiation during early stages of cardiogenesis may have tremendous consequences on cardiogenesis itself and later on the function of the adult heart. Second, rescuing the phenotype of a mouse bearing a mutation in a sarcomeric protein by cross-breeding transgenic mice is not an easy process, is time consuming and often difficult to interpret because of different genetic backgrounds. Herein, we chose an alternative strategy to address the question. We used the in vitro model of embryonic body to investigate the expression of endogenous MLC2v in cardiomyocytes (9), a three-dimensional model which faithfully recapitulates the early stages of embryonic cardiogenesis (10,11). We engineered an ES cell line which expresses a mutated form of the ventricular myosin light chain 2 (MLC2v) under the control of the early functional cardiac α-actin promoter. As a proof of principle of this approach, we chose to mutate arginine 58 of MLC2v into a glutamine (R58Q). This MLC2v mutation located in the immediate extension of the helices flanking the regulatory light chain Ca2+ binding site is associated with a typical form of hypertrophic cardiomyopathy. Although of low occurrence, (12) this mutation results in dramatic alterations in the calcium binding capacity of the myosin light chain (13). This ES cell line growing inside cardiomyocytes harboring a mutated MLC2v provides a valuable cell model to investigate the impact of alterations in Ca2+ homeostasis on the development of cardiomyopathies (14). This cell line should also help in designing new Ca2+-based therapeutic strategies to alleviate patients affected by cardiomyopathies.

RESULTS

Myosin light chain fused to GFP integrates the sarcomeric units and replaces endogenous MLC2v

To visualize the process of myofibrillogenesis in differentiating ES cell-derived cardiomyocytes, we engineered an ES cell line expressing MLC2v fused to GFP under the transcriptional control of the cardiac α-actin promoter. We then allowed this cell line to differentiate into cardiomyocytes within embryo bodies (EBs). Real-time PCR was used to quantify mRNAs encoding total MLC2v. The content of MLC2v mRNAs was seven times (6.9 ± 0.3, n = 3) higher in MLC2vGFP ES cell-derived cardiomyocytes than in wild-type ES cell-derived myocytes differentiating within days 5–9 EBs. This suggests that the transcriptional activity of the α-actin promoter could overcome one of the endogenous MLC2v promoters. EBs expressing MLC2vGFP at differentiation day 10 harbored vigorously beating cells with sarcomeric units having incorporated the MLC2vGFP (Fig. 1A) (see Supplementary Material). Observation at high magnification of these sarcomeric units, featuring a size of 1.8 ± 0.2 μm (Fig. 1B), showed that MLC2vGFP mostly replaced endogenous MLC2v.

Mutated MLC2v is expressed and impairs beating activity of EBs

Mutation of a single base in the MLC2v cDNA allowed for the substitution of amino acid arginine 58 (R) by a glutamine (Q). The mutated cDNA was then subcloned downstream of the cardiac α-actin promoter and a stable ES cell line carrying this cDNA construct was generated. To quantify the content of mutated MLC2v expressed by differentiating cardiomyocytes, we used real-time PCR and fluorescent labeled hybridization probes targeted at the mutated site. Quantification was calculated from the area under the melting curves of the wild-type or mutated MLC2v amplicon. Wild-type and mutated cDNA expression plasmids were used to determine the melting temperature of each amplicon (Fig. 2A). Quantification of mRNA extracted from EBs, by RT–PCR revealed that 78.6 ± 4.4% (n = 5) of MLC2v was mutated in day 7 or day 9 EBs (Fig. 2B).

EBs expressing wild-type MLC2vGFP in cardiomyocytes started beating at day 7 and maximal contractile activity was observed at days 10–12 as previously demonstrated in wild-type EBs (9,15). In contrast, poor beating activity was observed in ES cell-derived cardiomyocytes expressing a mutated MLC2v. Maximal beating activity reached a plateau at day 9 and at day 12, only 20 ± 7.2% (n = 4) of EBs were beating (Fig. 3).

Defect in expression of sarcomeric proteins and in myofibrillogenesis of ES cell-derived cardiomyocytes harboring mutated MLC2v

To understand the origin of impaired beating activity of ES cell-derived mutated cardiomyocytes, mRNA level of expression of genes encoding cardiac-specific transcription factors and constitutive sarcomeric proteins was quantified by real-time PCR in mutated and wild-type MLC2vGFP ES cell-derived cardiomyocytes within EBs. Expression of cardiac transcription factors, Nkx2.5, MeF2c, GATA4, GATA6 and myocardin was not significantly different in mutated and in wild-type EBs (Fig. 4A). On the contrary, α-actin mRNA content was dramatically decreased in mutated EBs as early as day 7, when the α-actin promoter attained full activity (16,17). Similarly, expression of both total sarcomeric proteins MLC2v and MLC2a was inhibited in day 7 and day 9 EBs featuring mutated cardiomyocytes (Fig. 4B).

The sarcomeric protein actinin is predominantly expressed in ES cell-derived cardiomyocytes at an early stage of EB differentiation (18). Therefore, we then looked at the extent of actinin-positive areas and the state of myofibrillogenesis of ES cell-derived cardiomyocytes within day 12 EBs. Observation at low magnification of immunostained α-actinin revealed extensive areas of cardiomyocytes within the wild-type EBs (Fig. 5A). Images acquired at high magnification showed well-structured sarcomeric units in wild-type (Fig. 5B). Observation of MLC2vGFP in wild-type EBs immunolabeled with an anti-actinin antibody further confirmed co-expression of the z-line protein α-actinin and MLC2vGFP in sarcomeres (Fig. 5C).
In EBs carrying the MLC2v mutation, actinin-expressing cardiomyocytes were spread out and in a more limited number (Fig. 5D). These mutated cardiomyocytes featured disrupted sarcomeres (Fig. 5E). In fact, α-actinin was clustered along the membrane (as shown by the yellow arrow), where sarcomeric units normally start to set (19) and showed sets of periodic dots in primitive myofibrils (as shown by white arrows). GFP fused to R58QMLC2v was only observed as diffuse patches in cells and was not incorporated into sarcomeric structures (inset).

Electronic microscopy demonstrated the alignment of the myofibrils in ES cell-derived wild-type cardiomyocytes but not in cardiomyocytes expressing the mutated myosin (Fig. 5F and G).

Altogether, these results showed in mutated cells a dramatic decrease in expression of mRNAs encoding constitutive proteins and their lack of integration into myofibrils.

**Myosin light chain kinase is functional and phosphorylates R58QMLC2v**

Phosphorylation of MLC2v by its specific myosin light chain kinase (MLCK) is required for its integration into sarcomeric units (20,21). As R58QMLC2v did not integrate the sarcomeric units, it was important to measure MLCK activity in cardiomyocytes expressing the mutated protein.

Cardiomyocytes, isolated from the hearts of E9–11 mouse embryos, a stage of development which reflects the stage of cardiac cell differentiation in day 10–12 EBs (22) were transfected with MLC2v or R58QMLC2v cDNA together with the MLCK-biosensor cDNA (23). FRET energy transfer efficiency of the MLCK-biosensor was measured. Values of energy transfer efficiency were positive in wild-type cells and negative in mutated cells indicating a stronger MLCK activity in

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**Figure 1.** Confocal fluorescence microscopy of ES cell-derived cardiomyocytes within day 12 EBs. (A) MLC2v fused to GFP integrated the sarcomeric units. (B) The image acquired at higher magnification showed that MLC2vGFP mostly replaced endogenous MLC2v.
cardiomyocytes expressing a mutated MLC2v than in myocytes expressing the wild-type myosin (23) (Fig. 6A). In line with this finding, the anti-MLC2v antibody specifically recognizing the phosphorylated form of MLC labeled both wild-type MLC2vGFP incorporated in sarcomeres where MLCK was localized (inset Fig. 6B) and R58QMLC2v GFP remained at the periphery of cells (Fig. 6B). These results show that the impaired integration of R58QMLC2v cannot be attributed to a deficient MLCK activity.

Impaired Ca\(^{2+}\)-signaling and mislocalization of Mef2c in MLC2v mutated cardiomyocytes

To determine the origin of low \(\alpha\)-actin and MLC2v/\(\alpha\)-expression in mutated ES-derived cardiomyocytes, we investigated the localization of Mef2c within both wild-type and mutated ES cell-derived cardiomyocytes. We surmised that expression of R58QMLC2v which lost or increased its capability to bind Ca\(^{2+}\) depending upon its phosphorylation state (13) disturbed intracellular Ca\(^{2+}\) homeostasis. Indeed, Mef2c integrates many Ca\(^{2+}\)-dependent signaling events (calcineurin, CaMK, MKK6/p38, etc.) (24–27). The transcription factor Mef2c which regulates expression of both \(\alpha\)-actin and MLC2v was located in the nucleus in wild-type cardiomyocytes but remained around the nucleus in mutated cardiomyocytes (Fig. 7A).

To directly evaluate the consequences of mutated MLC2v on Ca\(^{2+}\) homeostasis, wild-type MLC2vGFP and R58QMLC2v EBs were loaded with Fluo-4 in order to monitor spontaneous Ca\(^{2+}\) spiking. As expected, rhythmic Ca\(^{2+}\) oscillations were observed in wild-type MLC2vGFP expressing cardiomyocytes. No calcium spikes were observed in R58QMLC2vGFP EBs (Fig. 7B).

Expression of a Ca\(^{2+}\)-calmodulin-dependent kinase partially rescued the phenotype of mutated MLC2v cardiomyocytes

We hypothesized that the impaired nuclear translocation of Mef2c was due to a lack of Ca\(^{2+}\)-dependent phosphorylation of the protein (28). An ES cell clone expressing both R58QMLC2vGFP and a constitutively active form of Ca\(^{2+}\)- and calmodulin-dependent kinase II (CaCamKII) both under the transcriptional control of the \(\alpha\)-actin promoter was generated and differentiated into cardiomyocytes within EBs.

We first looked at the localization of Mef2c within the ES cell-derived cardiomyocytes expressing both CaCamKII and R58QMLC2v. The transcription factor was translocated into the nucleus in the rescued cells (Fig. 8A). The time course and extent of expression of genes targets of Mef2c and encoding sarcomeric proteins (i.e. \(\alpha\)-actin, MLC2a, MLC2v) did not differ between wild-type and CaCamKII expressing R58QMLC2v EBs (Fig. 8B).
In contrast to what was observed in R58QMLC2v EBs, actinin labeled cells with a fully organized contractile apparatus were found in CaCamKII R58QMLC2v EBs (Fig. 8C). These cells represented 8% of the mesodermal area of EBs to compare with 3 and 12% in R58QMLC2v and wild-type EBs, respectively (Fig. 8D). However, the R58Q MLC2GFP was still not incorporated into sarcomeric units. Ca\(^{2+}\)-transients were also recorded in day 12 EBs generated from CaCamKII R58QMLC2v ES cells (Fig. 8E) and beating activity of cardiomyocytes derived from this clone was partially rescued at the later stage of cell differentiation when CaCamKII reached a high level of expression (Fig. 8F).

Altogether, these findings show that expression of a constitutively active CaCamKII is able to partially rescue the phenotype of ES cell-derived cardiomyocytes expressing a R58Q mutant of MLC2v. In the same line, a brief challenge of R58QMLC2v EBs at day 6 with 0.1 \(\mu\)M ionomycin to increase cytosolic Ca\(^{2+}\) transiently rescued expression of cardiac constitutive genes, Mef2c intranuclear localization, Ca\(^{2+}\) transients and partially beating activity and myofibrillogenesis (Supplementary Material, Fig. S1).

DISCUSSION

The development of transgenic animal models (8,29–32) paved the way to study the consequences of genetic defects responsible for human cardiomyopathies. These models mainly showed a failing myocardial contractility associated with hypertrophy, fibrosis and myofibril disarrays, mostly...
recapitulating the clinical spectrum of human patients (3). Mutated proteins are thought to act through a dominant negative mode resulting in the impairment of myocardial function. However, in transgenic animals, mutated genes are often expressed under the transcriptional control of the α-MHC promoter, a promoter which in mice is only active at birth (33). Thus, these models do not allow investigating the molecular mechanisms underlying the dominant negative effect of the mutated protein. These animals are not helpful to understand the deleterious consequences which originate from expression of the mutated protein early during embryogenesis. Here, we used ES cells differentiated in vitro into cardiomyocytes and we provide evidence that a single point mutation in the gene encoding MLC2v has dramatic consequences on myofibrillogenesis and contractility of ES cell-derived cardiomyocytes. The mutation, which leads to a significant change in Ca²⁺ affinity of the myosin light chain (13) inhibits spontaneous Ca²⁺ spiking of cells and affects the translocation of Mef2c into the nucleus, preventing expression of constitutive proteins. This suggests that perturbations in whole cell Ca²⁺ homeostasis impair the genetic program of cardiogenesis.

To visualize the process of myofibrillogenesis in both wild-type and mutated ES cell-derived cardiomyocytes, we fused GFP to MLC2v. Addition of GFP to wild-type MLC2v associated with its overexpression under the transcriptional control of the α-actin promoter did not affect the process of cardiac differentiation. Indeed, cardiac transcription factors Nkx2.5, GATAs, mycardin and Mef2c (34) and constitutive myofibrillar proteins, such as α-actin and myosin light chains were normally expressed in cells expressing MLC2vGFP. In addition, MLC2vGFP was readily incorporated into sarcromeric units featuring a normal length and a regular assembly. Within a single ES cell-derived cardiomyocyte, all sarcomeric units displayed GFP fluorescence suggesting that most of MLC2vGFP expressed under the transcriptional control of the α-actin promoter replaced the endogenous form of MLC2v. This was expected from the earlier transactivation of the α-actin promoter compared with activation of endogenous MLC2v promoter in both embryogenesis and ES cell-derived EBs (9,16,17,35,36). Diffraction of fluorescence light limiting the spatial resolution of confocal microscopy does not, however, allow us to definitively conclude that exchange of myosin light chains was complete at the molecular level. In any case, contractility of EBs with cardiomyocytes expressing MLC2vGFP was comparable with EBs generated from non-genetically modified ES cells (9,15) demonstrating the lack of incidence of GFP or overexpression of MLC2v on the process of cardiac differentiation or myofibrillogenesis. Altogether, these findings definitively exclude the possibility that the phenotype of cardiomyocytes expressing the mutated R58QMLC2vGFP could be attributed to addition of the fluorescent protein or of its overexpression.

Overexpression of R58QMLC2v, expected to replace endogenous MLC2v, induced dramatic molecular and cellular consequences in early cardiogenesis. Myofibrillogenesis was severely impaired in mutated cardiomyocytes. Indeed, R58QMLC2v was not capable to incorporate the sarcomeric structure. Possible explanations for this defect include the lack of phosphorylation of this mutant, a condition required prior to the assembly of the light chain into sarcomeric units (20,21) and/or a structural change in the protein.

Several of our findings point to severe disturbances in Ca²⁺ homeostasis of mutated cardiomyocytes. First, we observed a lack of spontaneous Ca²⁺ spiking in embryonic cardiomyocytes expressing mutated R58QMLC2v. Such a Ca²⁺ signaling defect if homogenous within the cell should in principle inhibit the Ca²⁺-calmodulin dependent MLCK or other kinases preventing phosphorylation of mutated MLC2v, as well as non-muscle myosin at early stages of myofibrillogenesis (37,38). However, experiments designed to monitor MLCK activity in embryonic cardiomyocytes and direct evidence of in situ phosphorylation of MLC2v rather demonstrate that MLCK is fully and even more active in cells expressing R58QMLC2v than in cells expressing the wild-type myosin. Thus, it is conceivable that R58QMLC2v acquiring a higher Ca²⁺ affinity than wild-type MLC2v following its phosphorylation (13) traps Ca²⁺ in the vicinity of MLCK bound to myofibrils, structures
that already contributed to a major Ca\(^{2+}\) buffer during Ca\(^{2+}\) transients in cardiac cells (39). Thus, deficient integration of R58QMLC2v into sarcomeres is not due to impaired phosphorylation of the light chain but most likely to a change in the mutated protein structure as it might be expected from an increased content of \(\alpha\)-helices in phosphorylated R58QMLC2v compared with phosphorylated wild-type MLC2v (13). Nevertheless, the failure of R58QMLC2v to integrate the sarcomeric units prevents the assembly of the myofibrils which remained at the stage of pre-myofibrils featuring membrane-associated actinin and aligned dots, typical of these pre-sarcomeres (18,37). Given the impaired myofibrils in mutated cardiomyocytes, the weak contractility of EBs is not surprising.

A second Ca\(^{2+}\)-dependent phenomenon, namely the translocation of transcription factor MeF2c into the nucleus did not occur in ES cell-derived cardiomyocytes expressing R58QMLC2v also pointing to a displacement of Ca\(^{2+}\) from the cytosol to the pre-myofibrils. MeF2c is located within a cardiac transcriptional network at a key Ca\(^{2+}\)-dependent crossroad (24–27) and thus serves as a sensor in Ca\(^{2+}\)-dependent transcriptional pathways. MeF2c, unable to cross the nuclear envelope could not exert its transcriptional function on genes encoding \(\alpha\)-actin and MLC2s (40,41). Thus, mislocalization of MeF2c within the cell accounts for the decrease in expression of constitutive genes in R58QMLC2v cardiomyocytes.

Interestingly, part of the phenotype of cardiomyocytes expressing the mutated MLC2v can be rescued by the

Figure 7. Ca\(^{2+}\) homeostasis is disturbed in R58Q MLC2v expressing cardiomyocytes. (A) Mislocalization of transcription factor MeF2c in ES cell-derived cardiomyocytes. Anti-MeF2c immunostaining of nuclei of ES cell-derived cardiomyocytes in day 12 MLC2vGFP or R58Q MLC2vGFP EBs. Z-optical sections of immunolabeled EBs were acquired at 63x magnification. The stacks of images were restored using Huygens software and visualized using Imaris software as a shadow projection. Inset show intranuclear localization of MeF2c visualized as the summed fluorescence distribution across the nuclei. (B) Fluo-4 loaded ES cells derived cardiomyocytes expressing R58Q MLC2vGFP do not feature Ca\(^{2+}\) spikes, while myocytes expressing wt MLC2vGFP integrated into sarcomeric units shows spontaneous and rhythmic Ca\(^{2+}\) oscillations. Recordings show Ca\(^{2+}\) spikes within selected ROI and are expressed as \(\Delta F/F_0\), where \(F_0\) is the lowest basal fluorescence recorded at the beginning of the experiment. The figure is representative of three experiments with similar results.
expression of a constitutively active Ca\(^{2+}\)-independent Calmodulin-dependent kinase II or by treating EBs by the Ca\(^{2+}\) ionophore ionomycin.

Expression of CamKII relocalized Mef2c into the nucleus and restored in part myofibrillogenesis and contractile activity but not incorporation of mutated MLC2v into sarcomeric units. This suggests that constitutively active CamKII by-passed the depletion of cytosolic Ca\(^{2+}\) following Ca\(^{2+}\) sequestration by pre-myofibrils and thus restored endogenous MLC2v expression overcoming expression of exogenous R58QMLC2v. This hypothesis is further supported by the fact that ionomycin which dramatically increases cytosolic Ca\(^{2+}\) in EBs (28) also partially restored the phenotype of R58QMLC2v EBs.

Altogether, our findings uncover that a single mutation in MLC2v which disturbs its Ca\(^{2+}\) affinity may have dramatic consequences on whole cell Ca\(^{2+}\) homeostasis at the subcellular level compromising the Ca\(^{2+}\)-dependent cardiac transcriptional network. The resulting defects in the process of cardiac differentiation at early stages of embryogenesis lead to an impaired myofibrillogenesis and, in turn, a failing contractility of mutated cardiomyocytes. The phenotype observed in the ES cell-derived EB model is thus similar to the one of the transgenic mice carrying mutated sarcomeric proteins affecting their Ca\(^{2+}\)-binding capability (32) which validates the cellular model. In addition, the ES cells allow to delineate the Ca\(^{2+}\)-dependent molecular mechanisms responsible for the defect in cardiac cell differentiation, at very early stages of cardiogenesis. This suggests that Ca\(^{2+}\) equilibrium between the cytosol, the myofibrils and intracellular Ca\(^{2+}\) stores (i.e. sarcoplasmic/endoplasmic reticulum, mitochondria) is crucial for an appropriate function of cardiomyocytes and the development of the heart. Once phosphorylated, the mutated MLC2v sequesters Ca\(^{2+}\) in the myofibrillar environment which depletes the intracellular Ca\(^{2+}\) stores including the ER/SR necessary for spontaneous Ca\(^{2+}\) spiking and perinuclear Ca\(^{2+}\) regulating intranuclear transport of transcription factors (42). How mislocalization of Ca\(^{2+}\) within a cell affects the process of cardiac cell differentiation is currently under investigation.

Besides restoring the expression of a wild-type protein, therapeutic strategies based on manipulation of the Ca\(^{2+}\)-homeostasis (14) may be of high value to relieve some symptoms of cardiomyopathies specifically due to the negative effect of the mutated protein. Thus, here we provide a proof of the principle that genetically modified ES cells represent a precious model in order to design such strategies to rescue the phenotype of the mutated cardiomyocytes specifically affecting MLC2v, a key protein in regulation of cardiac contractility (43–45).

**MATERIALS AND METHODS**

**Mutation of MLC2v and generation of ES cell clones**

The cDNA encoding MLC2v was excised from the plasmid \(\alpha\)-MHC clone 26 (given by J. Robbins) using the restriction enzymes SalI and BamH1 and subcloned in frame in the pEGFP-C2 plasmid (Clontech). The cDNA encoding MLC2v fused to GFP was then excised using AgeI and StuI and subcloned downstream of the \(\alpha\)-actin promoter using the plasmid \(\alpha\)-actinECFP (46). MLC2v cDNA was mutated using the Quick change kit (Stratagene) using the following primers, forward atttgctgccctaggacaagtgaacgtgaaaaatg, and reverse cattttcagctactgcttcgagcaaat to replace arginine 58 by glutamine. Both wild-type MLC2vGFP and MLC2vR58QGFP DNA constructs were separately transfected into CGR8 ES cells and the cell clones selected by G418 for 7–10 days. Three separate cell clones were used throughout the study. To rescue the phenotype of mutated

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**Figure 8.** Expression of a CaCamKII partially rescued the phenotype of R58QMLC2v cardiomyocytes. (A) Immunostaining of Mef2c in CaCamKII and R58QMLC2v expressing ES cell-derived cardiomyocytes with EBs. (B) \(\alpha\)-Actin, MLC2a and MLC2v gene expression was monitored by RT–quantitative PCR of RNA extracted from CamK MLC2aGFP and CamKR58QMLC2vGFP EBs on days 5, 7 and 9. Results are expressed as a ratio of gene expression in mutated to wild-type EBs. The graph represents the mean ± SEM of three experiments. (C) Immunostaining of sarcomeric \(\alpha\)-actinin in CaCamK and R58QMLC2v expressing ES cell-derived cardiomyocytes with EBs. (D) Extent of cardiac area within wt, R58QMLC2v and CaCamKIR58QMLC2v EBs revealed by anti-actinin staining. (E) Ca\(^{2+}\) spiking in CaCamKIR58QMLC2v expressing ES cell-derived cardiomyocytes with EBs loaded with fluo-4. Recordings show Ca\(^{2+}\) spikes within selected ROI and are expressed as \(\Delta F/F_0\), where \(F_0\) is the lowest basal fluorescence recorded at the beginning of the experiment. The figure is representative of three experiments with similar results. (F) Contractile activity of wt (filled square), R58QMLC2v (filled circle) and CaCamKIR58QMLC2v (filled triangle) expressing ES cell-derived cardiomyocytes with EBs. Asterisk indicates values significantly different from wild-type EBs. ○ indicates values significantly different from mutated R58QMLC2v EBs (\(P < 0.01\)).
cardiomyocytes, a constitutively active Ca\textsuperscript{2+} calmodulin-dependent kinase II (47) was subcloned downstream the α-actin promoter in the pIRESGFP vector (Clontech) using PstI and KpnI restriction sites, linearized and electroporated together with a linearized pcDNA−hygromycin vector in MLC2vR58QGFP ES cells. The cell clones were selected by hygromycin for 10 days.

**Culture and differentiation of stem cells into EBs**

The pluripotent murine embryonic stem cell line CGR8 was propagated in BHK21 medium supplemented with sodium pyruvate, non-essential amino acids, mercaptoethanol, 7.5% fetal calf serum and leukemia inhibitory factor. To maintain the undifferentiated phenotype, CGR8 cells were split every 2 days. Differentiation was carried out using the ‘hanging drop’ method (9,48).

**Antibodies and immunocytochemistry**

For immunostaining, EBs were fixed with 3% paraformaldehyde or cooled (−20 °C) in methanol–acetone (50/50, v/v) solution, permeabilized with 1% Triton X-100 and labeled with mouse anti-sarcomeric α-actin, MLCK, antibodies (1/500) (Sigma) (9). Alexa 546 conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR) (dilution 1:300) was used as a secondary antibody. Embryonic cardiomyocytes were stained with an anti-phosphorylated MLC2 antibody (45) and secondary Alexa 464 conjugated goat anti-rabbit antibody.

Images were acquired using a confocal (Ultraview, Perkin Elmer) microscope equipped with a 63× objective-mounted piezo-electric controller (Physik Instrumente) driven by the Methamorph software (Visitron Universal Imaging). Images were recorded with a coolsnap CCD camera (Princeton). Three-dimensional reconstruction was carried out in 0.3 μm-steps in the z-axis using Huygens software (Scientific Volume Imaging), and visualized by Imaris (Bitplane) software running on a DELL Precision 450 workstation.

**Transmitted electron microscopy**

For transmitted electron microscopy, EBs were fixed in phosphate-buffered 1% OsO\textsubscript{4}, stained en bloc with 2% uranyl acetate, dehydrated in ethanol and propylene oxide, and embedded in low viscosity epoxy resin. Thin (90 nm) sections were cut on an ultramicrotome (Reichert Ultracut E), placed on 200 μm mesh copper grids, and stained with lead citrate. Micrographs were taken on a JEOL 1200 EXII electron microscope operating at 60 kV.

**RNA extraction and reverse transcription reaction**

Total cellular RNA was isolated from EBs using a RNA extraction Kit (Zymo Research, CA, USA). One microgram of RNA was reverse-transcribed using the MLUV reverse transcriptase (In Vitrogen, Cergy, France). The cDNA was diluted 5-fold prior to PCR amplification.

**Real-time quantitative PCR by SYBR green detection**

The nucleotide sequences of the PCR primers used were: Mef2C forward 5′-AGATAACCAACAACACACCGCGC C-3′ and reverse 5′-ATCCCTCAGAGTGCATGCCTT T-3′; Nkx2.5 forward 5′-TGCAGAAGCCATGGACCTG TACAGGCC-3′ and reverse 5′-TGCATTGTAGCAGCGT TTCGGGACAG-3′; MLC2v forward 5′-GCACAAGACCG GATAAGAGG-3′ and reverse 5′-CTGTTGGTCAAGGCTCA GTC-3′; MLC2a forward 5′-TCTCTTAAATGCTTCTCAT AT T-3′ and reverse 5′-TCTACTCTCTTCTTCTCATCC CCGT-3′; β-tubulin forward 5′-CCGGA-CAGTGTGTCG CACCAGATCGG-3′ and reverse 5′-TGCCCA AAAAGG CCTGAGCGACCGG-3′; GATA4, forward 5′-GGTTT CCAAGCCTCTTGCAATGCCG-3′, reverse 5′-AGTGCCAT TGCTGAGTACCCTGGT-3′; GATA6, forward 5′-CCCGG AGTGCCTGAAT-3′, reverse 5′-CGCTCTGGTGTG CTTG ATGAG-3′; myocardin, forward 5′-CATGTTTTCCCA AGG AGATTC-3′, reverse 5′-CGATGTTCACCTCCCTAA-3′.

Real-time quantitative PCR was performed using a LightCycler rapid thermal cycler (Roche). Amplification was carried out as recommended by the manufacturer. A 12 μl reaction mixture contained 10 μl of LightCycler-DNA Master SYBR Green I mix (FAST Start Kit, containing Taq DNA polymerase, reaction buffer, deoxynucleoside triphosphate mix and SYBR Green I dye), 3 mM MgCl\textsubscript{2} and 0.5 μM concentration of appropriate primer and 2 μl (16 ng) of cDNA. Results were expressed as a function of the level of expression of the gene of interest in control undifferentiated stem cells using a previously described mathematical model (49). Data were normalized by PCR analysis of β-tubulin. The amplification program included the initial denaturation step at 95°C for 8 min, and 40 cycles of denaturation at 95°C for 3 s, annealing at 65°C for 10 s and extension at 72°C for 10 s. The temperature transition rate was 20°C/s. Fluorescence was measured at the end of each extension. Melting curves were used to determine the specificity of PCR products, which was further confirmed using conventional gel electrophoresis.

To detect and quantify the amount of mutated MLC2v, hybridization probes conjugated to LCred 640 (anchor probe, LCred640-5′-CAGCAAATG TGTCCCTTAGGTCA TCTTT-3′) or to fluorescein (sensor probe, 5′-CTTTCCAGGCTCTCTCAGAG-3′-fluorescein spanning the sequence where the mutation occurs and fully matching the wild-type sequence) combined with a sense (AAGGAAGGCTTTCACAATCATG) and an antisense (TCTTCAGGATG AGCCCTTTTA) primers to amplify the MLC2v mRNA were used. The amplification program included the initial denaturation step at 95°C for 8 min, and 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 20 s. Fluorescence was measured at the end of the annealing step when both anchor and sensor probes bind cDNA to allow for the phenomenon of FRET. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C, cooling it at 20°C/s to 45°C, keeping it at 45°C for 60 s, and then slowly heating it at 0.1°C/s to 95°C. Fluorescence was measured through the slow heating phase. The point mutation resulted in a mismatch between the sensor probe and the target shifting the temperature peak of
the melting curve from 64 to 56°C. The extent of mutated mRNA was calculated from the areas under the melting curve (Fig. 2).

Transfection and cell Ca$^{2+}$ imaging in embryonic cardiomyocytes and EBs

Cardiomyocytes were isolated from ventricles of E9 mouse embryos, a differentiation stage close to day 12 EBs, using collagenase and pancreatin (50). These proliferative cells were chosen because contrary to ES-derived cardiomyocytes isolated from EBs, they are easy to isolate and to transfect. They were transfected with MLC2vGFP or R58QMLC2vGFP using Effectene (Qiagen, France). EBs cells were loaded 24 h later for 10 min with 5 μM fluo-4 AM for Ca$^{2+}$ measurement and transferred onto a heated stage of an Ultraview Perkin Elmer confocal microscope, to be superfused with ADS buffer including 20 mM HEPES, 117 mM NaCl, 5.7 mM KCl, 1.2 mM Na$_2$PO$_4$, 4.4 mM NaHCO$_3$, 1.7 mM MgCl$_2$ and 1.8 mM CaCl$_2$.

Expression of the biosensor MLCK and FRET experiments

Cardiomyocytes, isolated from hearts of E9–11 mouse embryos were transfected using Effectene with MLC2vGFP or R58QMLC2vGFP cDNA together with the biosensor MLCK cDNA (23) to monitor activity of the kinase. Fluorescence measurements in single cells were acquired with a LSM510 confocal microscope. FRET was measured using the acceptor photobleaching method (51). Cells were illuminated at 405 nm using the laser diode 405 nm and fluoroesences of the donor ECFP and of the acceptor EYFP were measured using bandpass filters (420–480 nm for ECFP and 530–600 nm for EYFP) after light going through a dichroic mirror HFT405/514. A region of interest (ROI) was bleached using the 514 nm beam Arg/Xe laser. Fluorescence of the acceptor EYFP was recorded before and after photobleaching. The FRET energy transfer efficiency was calculated as described by Karpova et al. (52).

Statistics

Results are expressed as mean ± SEM. Statistical analysis was carried out by the Student’s t-test. Significant difference was accepted at the p < 0.05 level.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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