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Expression and function of α-smooth muscle actin during embryonic-stem-cell-derived cardiomyocyte differentiation

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Summary

Three α-muscle actin isoforms are sequentially expressed during in vivo cardiac development. α-Smooth muscle actin is first and transiently expressed, followed by α-skeletal and finally α-cardiac actin. The significance of these transitions in actin gene expression during myogenesis remains to be determined. To understand whether actin isoforms have specific functions during cardiac development and cardiomyocyte contractility, we have hampered α-smooth muscle and α-skeletal actin expression and organization during embryonic stem cell differentiation towards cardiomyocyte. We show that the sequence of actin isoform expression displays similar pattern in the in vitro model and in mouse heart embryogenesis. Treatment with an interfering fusion peptide containing the N-terminal sequence of α-smooth muscle actin during a time window preceding spontaneous beating, prevents proper cardiac sarcomyogenesis, whereas α-skeletal actin-fusion peptide has no effect. Knockdown of α-smooth muscle actin in embryonic stem cells using RNA interference also affects cardiac differentiation. The application of both fusion peptides on beating embryoid bodies impairs frequency. These results suggest specific functional activities for actin isoforms in cardiogenesis and cardiomyocyte contractility.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/2/229/DC1

Key words: Cardiomyocyte contraction, Antennapedia-fusion peptide, shRNA, Cytoskeleton, Cardiogenesis, Heart, Sarcomyogenesis

Introduction

The six actin isoforms found in mammalians constitute a family of closely related proteins expressed in a tissue-specific way. β- and γ-cytoplasmic actins are ubiquitous, and four muscle actins with very similar primary sequences [α-skeletal (α-SKA), α-cardiac (α-CAA), α-smooth muscle (α-SMA) and γ-smooth muscle actin (γ-SMA)] are found in the different muscle types. Despite their high similarity, we and others have been able to develop specific antibodies for some actin isoforms: α-SMA (Skalli et al., 1986), β-cytoplasmic actin (Gimona et al., 1994), α-SKA (Clement et al., 1999) and α-CAA (Clement et al., 2003; Franke et al., 1996).

In normal myocardium, α-CAA, α-SKA and α-SMA, are co-expressed and the amount of their transcripts has been shown to vary with species, developmental stage, aging and during pathological situations (Carrier et al., 1992; Schwartz et al., 1992; Schwartz et al., 1986; Winegrad et al., 1990). During in vivo cardiogenesis, α-SMA marks the onset of cardiomyocyte differentiation, and as development proceeds, it is sequentially replaced by α-SKA and α-CAA isoforms (Ruzicka and Schwartz, 1988; Woodcock-Mitchell et al., 1988). In the mouse embryo, as the cardiac compartment is formed, the early cardiomyocytes express all α-muscle actin isoforms, with α-CAA being predominantly expressed throughout development (Sassoon et al., 1988). In normal adult myocardium, the two sarcomeric actins, α-CAA and α-SKA, are co-expressed and represent the preponderant actin isoforms (Vandekerckhove et al., 1986). The significance of these transitions in actin gene expression during myogenesis is still an open question. In addition, when newborn and adult cardiomyocytes are cultured in vitro, they re-express fetal proteins such as α-SMA, α-SKA, β-myosin heavy chain (β-MHC), and atrial natriuretic factor (ANF) (Eppenberger-Eberhardt et al., 1990; Schaub et al., 1997; van Bilsen and Chien, 1993). These genes are also re-expressed during cardiac hypertrophy in vivo and represent well-accepted markers of this phenomenon.

It has been hypothesized that muscle actin isoforms may be required to achieve different degrees of myocardial contractility and several approaches have been used to examine the developmental and functional significance of these actins: for instance, the targeted expression of γ-SMA, the only actin isoform normally absent in the myocardium, in the heart of transgenic mice results in a hypodynamic heart (Kumar et al., 1997). Knockout mice for α-CAA usually die in the neonatal period. However, when γ-SMA is expressed under the control of the cardiac α-MHC promoter, these knockout mice survive to adulthood, but their hearts remain highly hypodynamic.

More recently, Martin et al. have shown that the substitution of γ-SMA for α-CAA in isolated cardiac fibers alters the actin interaction with its partners in the myofilament (troponin and myosin) (Martin et al., 2002). In addition, myofilaments containing γ-SMA display a decreased sensitivity to Ca^{2+} (Martin et al., 2002). Thus, even if γ-SMA can substitute α-CAA, it cannot completely rescue the heart function. However, perfused hearts isolated from BALB/c mice, which naturally express high levels of α-SKA (Garner et al., 1986), show increased levels of contractility compared with other strains of mice (Hewett et al., 1994). Altogether, these observations support the assumption that very few amino acid differences between muscle actin isoforms can have major functional consequences. In rat cardiomyocytes, functional heterogeneity between the different actin isoforms has been investigated by monitoring the consequences of their ectopic expression (von Arx et al., 1995). Incorporation of α-SMA was observed in stress fiber-like structures and sarcomeres, contrary to the three other muscle actins, which exclusively displayed a sarcomeric incorporation. Expression of cytoplasmic actins induced dramatic phenotypic changes and cessation of beating of adult rat cardiomyocytes (ARC). Albeit, the beating activity of ARC was not hampered by the γ-SMA, α-SMA effect was not reported. The N-terminus, where the main differences are located, has been directly implicated in the binding of several actin binding proteins such as gelsolin (Sutoh and Yin, 1989) or troponin I (Lehman et al., 2001). The physiological significance of such domain has been recently validated in the case of α-SMA in myofibroblasts. Intracellular delivery of the α-SMA fusion peptide (FP) SMA-FP, containing the N-terminal sequence AcEEED of α-SMA fused to the 16-amino-acid third domain of the Antennapedia homeodomain (pAntp) (Derossi et al., 1994) abolished α-SMA staining in stress fibers (Hinz et al., 2002), leading to a significant decrease of myofibroblast contractility both in vitro and in vivo (Hinz et al., 2002). We therefore decided to expand these investigations to other actin isoforms, in particular to understand their function during cardiac differentiation.

Mouse embryonic stem (ES) cells provide a unique experimental model to study the regulation of cardiomyocyte growth and differentiation in vitro. ES cells are derived from the inner cell mass of the blastocyst and can be maintained in culture as a self-renewing pluripotent population in the presence of leukemia inhibitory factor (LIF) (Robertson, 1987; Smith et al., 1988). ES cells differentiate in vitro in a broad range of specific cell types of all three germ layers including cardiomyocytes. Cultured within embryoid bodies (EBs), ES cells recapitulate the development of cardiomyocytes from early cardiac precursors to terminally differentiated cells. The appearance of spontaneously beating cardiomyocytes is observed after 1 week of culture.

Using the ES cell differentiation model and combining different analytical and technical approaches (e.g. specific antibodies, fusion peptides), we provide here the first clues in the understanding of the specific functions of α-SMA and α-SKA, the two 'non heart-typical' actin isoforms expressed during heart development.

Results

Actin isoform expression during mouse development

Since most of the information available in the literature on α-actin expression during cardiogenesis resulted from mRNA analyses, we have carried out careful examination of the three muscle actin isoforms expression at the protein level. We performed immunochemistry using specific anti-actin antibodies during embryogenesis and post natal development. Fig. 1 shows that, at day 9.5 post coitum (E9.5), all α-actin isoforms are present in the heart, α-SKA being expressed only in a few cells (Fig. 1Ab, arrows). At E13, the expression levels of α-SMA, α-SKA and α-CAA were comparable (Fig. 1Ad-f); the area of positive staining represented approximately 65% (Fig. 1B). At E17, α-SMA started to be downregulated (Fig. 1Ag; Fig. 1B) and 2 weeks after birth, it was only expressed in smooth muscle cells within the vessels (Fig. 1j; Fig. 1B). At this time, α-SKA was present in a subpopulation of cardiomyocytes (10.3±2.5%), as previously shown in other species (Clement et al., 1999; Clement et al., 2001; Suurmeijer et al., 2003).

Actin isoform expression during ES cell differentiation

It has been extensively shown that ES cell differentiation mimiks in vivo cardiac development (Sachinidis et al., 2003). To ensure that this was true for actin isoform expression and to validate ES cell differentiation as an appropriate model to study their function, we established the temporal expression of actin isoforms by triple staining immunofluorescence on embryoid bodies (EB) at day 6, 8, 12 and 15. Western blot as well as triple staining immunofluorescence (Fig. 2) illustrate that the sequence of isoactin expression closely correlated with the one previoulsy observed at mRNA level (Ng et al., 1997). Indeed, α-SMA is the first actin isoform, appearing at day 6 in the cells of the outer layer of EBs (Fig. 2Aa; Fig. 2C). At day 8, α-SKA and α-CAA started to be expressed within the beating areas (Fig. 2Ae-h; highlighted by dashed white lines on overlay pictures). At high magnification, we observed that all three isoforms were organized in striations but that their expression appeared heterogeneous within the beating area (Fig. 2B, inset). α-SMA is also highly expressed in the fibroblast-like cells that border the EB, as previously described (Ng et al., 1997). From day 12, α-SMA is markedly downregulated in the cardiomyocytes (Fig. 2Ai,m) but not in the fibroblast-like cells. This downregulation, however, was only visible by immunofluorescence, because western blotting analyses were performed with protein extracted from all types of cells present in EBs and it was impossible to discriminate between cell types (e.g. smooth muscle cells or myofibroblasts for α-SMA, or skeletal muscle cells for α-SKA).

Thus, the expression of actin isoforms in ES-cell-derived cardiomyocytes closely follows the timing of expression observed by immunohistochemistry during cardiac development in vivo. These results validate the ES cell differentiation system as a suitable in vitro model to study the function of actin isoforms in cardiogenesis.

Analysis of actin isoform function during ES cell differentiation

To understand how the expression of α-SMA and α-SKA is important to achieve correct terminal differentiation of ES-cell-derived cardiomyocytes, we have inhibited their function with fusion-peptides (FPs); SMA-FP and SKA-FP contain Ac-EEED and Ac-DEDE, respectively, at the N-terminus of the cell-penetrating vector pAntp-Pro50. As FPs precipitated in

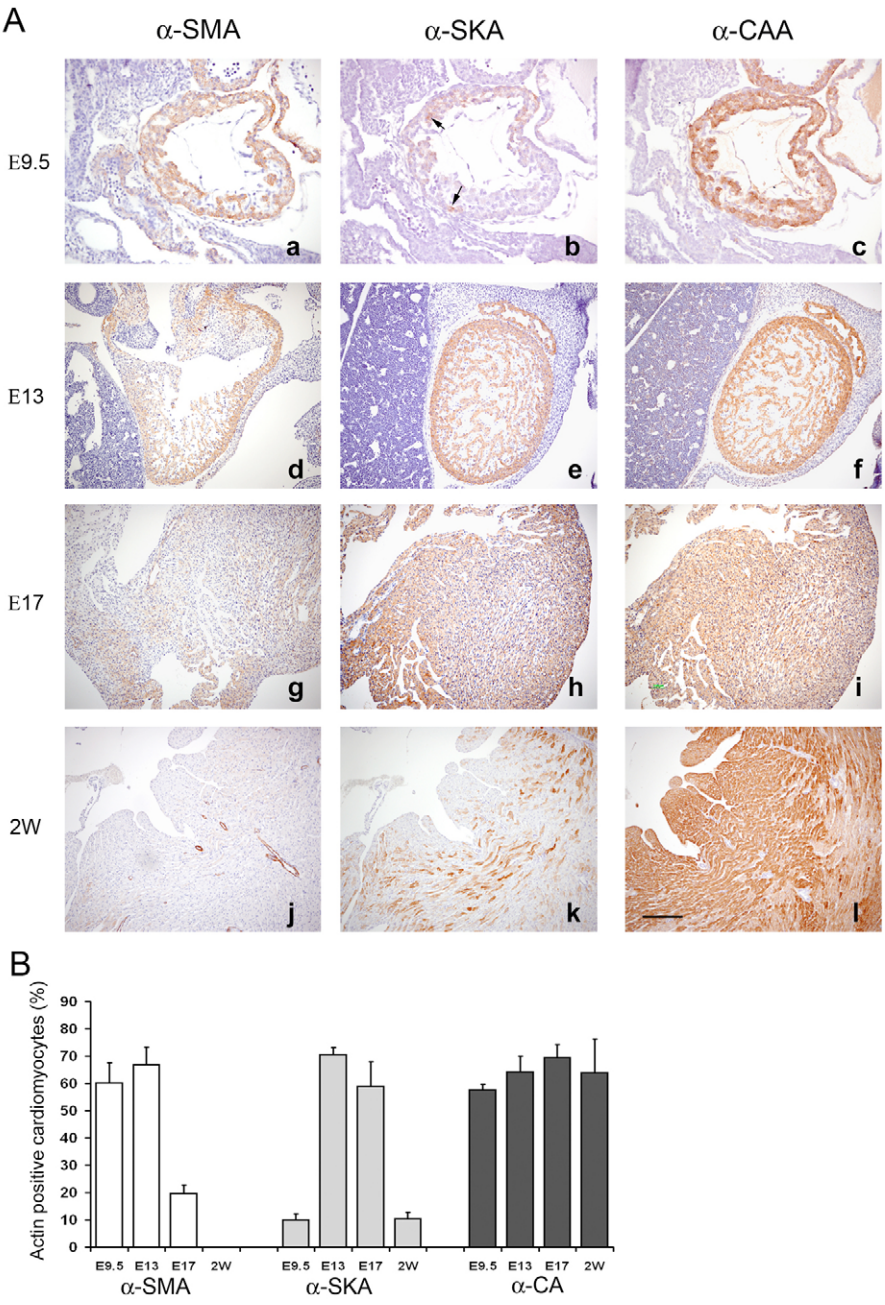

Fig. 1. Distribution and quantitative evaluation of actin isoforms during heart development. (A) 4-μm sections of embryos at E9.5 (a-c), E13 (d-f) and E17 (g-i), and hearts from 2-week-old mice (j-l) were used for immunochemistry with anti α-SMA (a,d,g,j), anti αSKA (b,e,h,k) and anti α-CAA (c,f,i,l) antibodies. Bar, 100 μm. (B) The percentage of α-SMA-positive, α-SKA-positive and α-CAA-positive cardiomyocytes was calculated using the software KS400.

presence of fetal calf serum (FCS), which is usually present in ES cell differentiation media, we have established culture conditions in the presence of knockout serum (KO serum, Invitrogen; serum replacement with defined formulation initially designed to support the growth of undifferentiated ES cells). Using such culture conditions, we could confirm that cardiac differentiation – identified by the appearance of spontaneous beating – proceeded normally (59.9±4.2% beating EBs at day 8 when cultured in presence of FCS compared with 58.3±4.1% beating EBs cultured in presence of KO serum). To ensure that FP efficiency was preserved in KO serum, we have successfully tested these culture conditions on myofibroblasts (cells expressing a high level of α-SMA; data not shown).

We first investigated the effects of 10 and 50 μg/ml SMA-FP or SKA-FP on differentiating EBs that were treated twice a day during a window of time, preceding the appearance of spontaneously beating cardiomyocytes – namely from day 6 to day 8 (Fig. 3A). At day 8, SMA-FP significantly decreased the percentage of beating EBs in a dose-dependent manner (Fig. 3B). Viability of cells within EBs estimated by Trypan Blue dye exclusion technique assay was not affected by FP treatment (10.5±2.1% dead cells in untreated EBs vs 9.9±3.4% dead cells in SMA-FP-treated EBs). Interestingly, SMA-FP specifically impaired the formation of myofibrils as observed by the non-organized α-actinin pattern (Fig. 3Da compared with b, inset). Although α-SKA was expressed in EBs during this developmental stage, SKA-FP did affect neither beating (Fig. 3B) nor sarcogenesis (Fig. 3Ea compared with b, inset). As expected, the protein expression of the different actin isoforms was not affected by the treatment with FPs (Fig. 3C). This observation is in accordance with our previous reports (Clement et al., 2005; Hinz et al., 2002) showing that FPs interfere with actin isoform organization but not with their expression; the disappearance of immunostaining being explained by the fact that soluble actin (not organized into structures such as myofibrils) is not stained by the antibody because it is diffusely distributed throughout the cytoplasm.

To confirm the role of α-SMA during cardiac differentiation, we have designed small interference RNAs (siRNAs; see Materials and Methods and Fig. 4A) specific to this isoactin. We first tested the efficacy of these siRNAs on mouse lung fibroblasts, known to differentiate in culture into myofibroblast-like cells containing high levels of α-SMA (Dugina et al., 1998; Xu et al., 1997). As shown in Fig. 4B, all three siRNAs (siSMA1-3) reduced α-SMA expression as visualized by immunofluorescence, whereas control siRNA had no effect. None of the other actin isoforms (β- and γ-cytoplasmic actins) expressed in these cells were affected by siSMAs (not shown). All results were similarly reproduced with the two other siSMAs, with siSMA3 showing the most

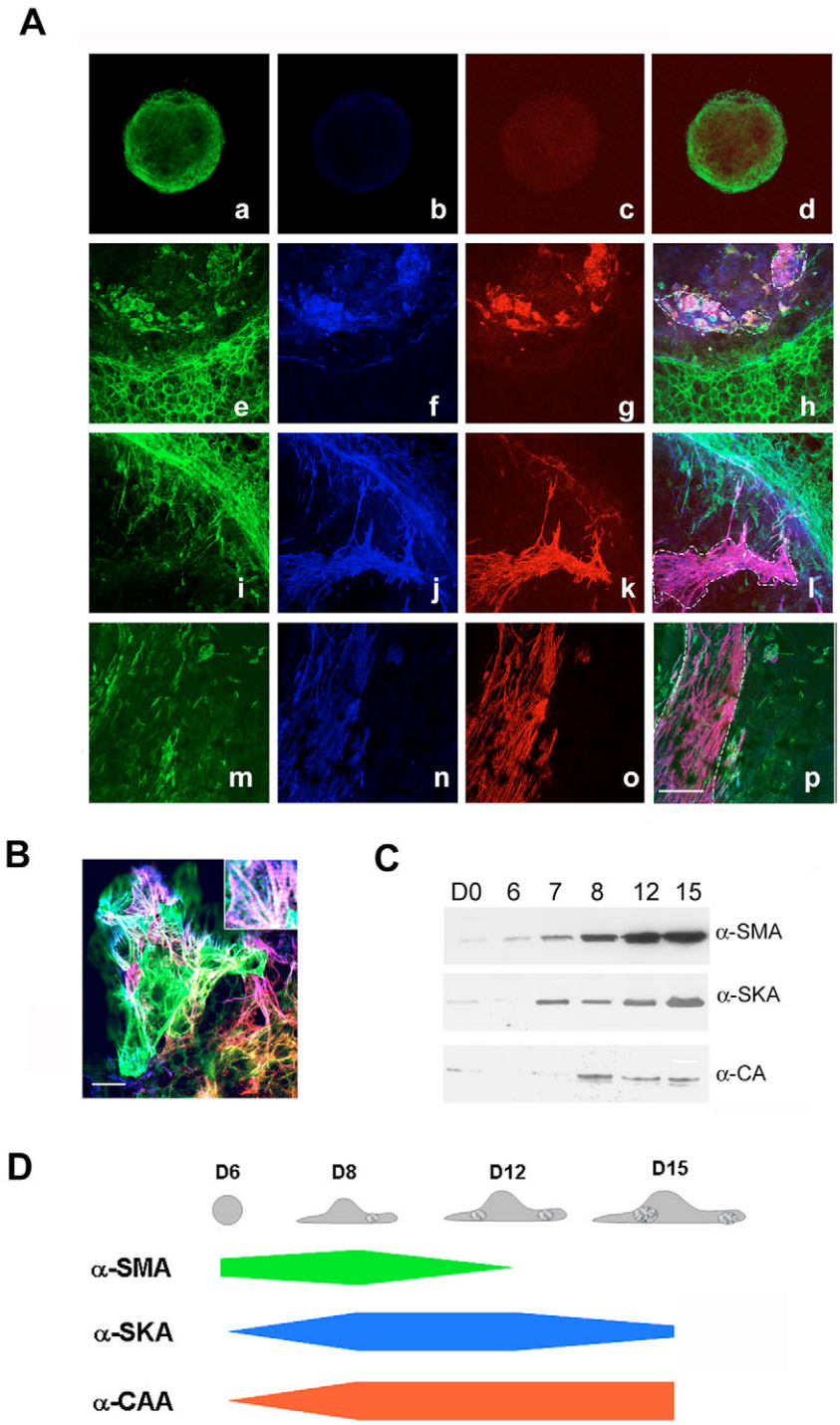

Fig. 2. Temporal and spatial distribution of actin isoforms during ES cell differentiation. (A) EBs fixed at different time points (6, 8, 12 and 15 days) were triple-stained with antibodies against all three anti-actin isoforms (α-SMA, green; α-SKA, blue; α-CAA, red). In overlay images (d, h, l and p), beating areas are highlighted by a dashed white line. Bar, 200 μm. (B) Overlay image of a magnified beating area at day 8 co-stained with α-SMA (green), α-SKA (blue) and α-CAA (red) antibodies. Inset, magnified part of the beating area. Bar, 20 μm. Images were acquired with a confocal microscope using either 10× (A) or 63× oil immersion objectives (B). (C) Whole-cell extracts of undifferentiated ES cell (D0) and EBs at days 6, 7, 8, 12 and 15 were analyzed by SDS-PAGE and immunoblotted with anti-SMA, anti-SKA and anti-CAA antibodies. (D) Schematic representation of expression of actin isoform during EB differentiation. Width of each bar correlates with the qualitative changes of the three actins over time.

pronounced knockdown effect (Fig. 4Be). Therefore, we only showed experiments performed with siSMA3.

To investigate the capacity of α-SMA-knockdown ES cells to differentiate in functional cardiomyocytes, stable siSMA-expressor ES cells were generated (referred to as siSMA3-ESC). Previous work by Tang et al. has proven the feasibility of U6-promoter-driven shRNA expression in ES cells (Tang et al., 2004). Undifferentiated siSMA3-ESC continued to maintain typical ESC morphology (growth in compact colonies; data not shown). Oct-4 expression, a marker of stem cell pluripotency (Fig. 5A), as well as cell proliferation assessed by FACS analysis (Fig. 5B) in undifferentiated siSMA3-ESC was comparable with those of wild-type and siControl-ES cells. At day 6 of culture, α-SMA content within EBs was markedly reduced when compared with controls, whereas α-SKA and β-cytoplasmic actin expression (Fig. 5C, lanes 4-6) was not affected. Thus, siSMA3 could selectively repress α-SMA expression. During cardiac differentiation of ES cells into EBs, α-SMA downregulation resulted in an impaired activity of EB beating (Fig. 5D). Approximately 80% of EBs expressing siSMA3 did not feature any contracting foci at day 8 of culture. Similar results were obtained with the two other siSMAs described above (data not shown). At day 12, however, such effect on beating became undetectable (data not shown). The reason for this loss of siSMAs effect remains to be determined. Several hypotheses can be raised. The effect could be due to (1) a silencing of the U6 promoter beyond day 6, (2) a limiting amount of siRNA compared with the amount of endogenous α-SMA expressed in the cells or, (3) the lack of α-SMA in cardiomyocytes induces a delay in the differentiation process from which cardiomyocytes can eventually recover.

To assess whether the blockade of differentiation was due to a decreased commitment of the cardiac progenitor cells, we measured by real-time reverse transcriptase (RT)-PCR the expression of early transcription factors involved in cardiac determination, namely Nkx-2.5 and MEF2C. When compared with controls, downregulation of α-SMA led to a 1.6-fold and 2.2-fold decrease of Nkx-2.5 and MEF2C, respectively, at day 5 (Fig. 5E).

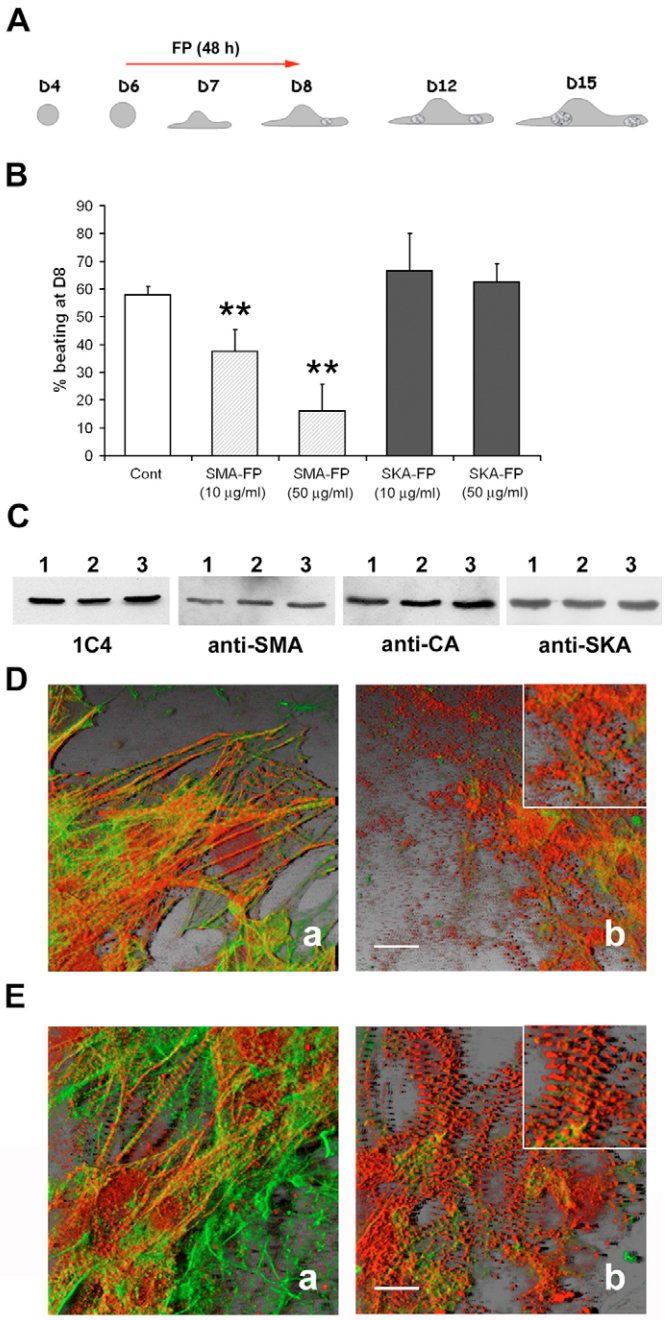

Fig. 3. Differential effect of SMA-FP and SKA-FP on beating and myofibril organization. (A) Schematic representation of the protocol used to treat EBs with FPs twice a day with 5 or 10 μg/ml of SMA-FP, or SKA-FP from days 6 to 8. At day 8, the capacity of the treated ES cells to differentiate into fully differentiated cardiomyocytes was assessed by determining the appearence of spontaneous beating. (B) Percentage of beating EBs at day 8 in untreated EBs (white bars), SMA-FP-treated (striped bars) and SKA-FP (black bars) treated EBs. Error bars represent s.e.m. of a total of five independent experiments (**$P \leqslant 0.001$). (C) Western blot analysis of untreated EBs (lanes 1) and EBs treated with 10 μg/ml SMA-FP (lanes 2) or 10 μg/ml SKA-FP (lanes 3). Proteins were immunoblotted with anti-total actin (1C4), anti-SMA, anti-CAA and anti-SKA antibodies. (D) 3D reconstruction of confocal microscopy images of untreated EBs (a) and SMA-FP-treated EBs (b) fixed at day 8 and double-stained with anti-α actinin (red) and anti-SMA (green) antibodies. (E) 3D reconstruction of confocal microscopy images of untreated (a) and SKA-FP treated (b) EBs fixed at day 8 and double-stained with anti-α actinin (red) and anti-SKA (green) antibodies. Bars, 10 μm. Insets in b, magnification of the myofibrils.

Analysis of actin isoform function on cardiomyocyte contractility

We then investigated the effect of the peptides on cell contractility by assessing beating frequency in untreated versus treated EBs. As previously shown for other cell types (Chaponnier et al., 1995; Hinz et al., 2002), SMA-FP and SKA-FP when applied on differentiated EBs for 2 hours at day 8 (Fig. 6A and supplementary material Fig. S1A), specifically lead to the almost complete disappearence of α-SMA and α-SKA immunodetection, respectively (supplementary material Fig. S1B). We have recently established that this lack of immunostaining is due to the blocking of actin incorporation into filamentous structures by fusion peptides (Clement et al., 2005). Regarding cardiomyocyte contractility, we have found that application of SKA-FP for 2 hours induced a 1.5-fold decrease of the beating frequency (Fig. 6B), whereas a 2-hour treatment with SMA-FP did not change beating frequency (Fig. 6B) but, rather recurrently, affected the regularity of beating, i.e. occurrence of temporary pauses characteric of arrhythmia (Fig. 6Cb compared with a). To quantify these observations, we recorded 20-second-long movies and obtained plots directly representative of the beating activity (Fig. 6C, see Materials and Methods). Using this method, we could visualize the decrease of frequency (Fig. 6Cd compared with c) and chaotic rhythms (Fig. 6Cb compared with a) induced by SKA-FP and SMA-FP, respectively. Fourier transformation was carried out with the objective to analyze these spectral data. The integration of the peaks representative of irregular beating clearly showed that SMA-FP treatment increased the index of arrhythmia fourfold compared with control conditions (Fig. 6D).

Discussion

Our results suggest that α-muscle actin isoforms that are sequentially expressed during cardiac differentiation play different functional roles in this process. The reliability of our model is supported by the observation that the expression of α-muscle actin isoforms exhibits a similar temporal sequence during in vitro ES cell differentiation and in vivo heart development. Owing to our newly developed specific antibodies, we have extended the knowledge concerning the expression of the three α-isoactins. Previously, investigations have been carried out mostly at the mRNA level during mouse heart development (Lyons et al., 1991; Sassoon et al., 1988) and ES cell differentiation (Ng et al., 1997).

α-SMA is the first isoform to be expressed in the peripheral cells of the spread EBs and, at day 8, all three isoforms are simultaneously present in beating areas. Nevertheless, the expression of these three proteins appeared to be irregular within the beating area indicating such that, at this point, all cardiomyocytes did not have the same expression pattern of actin isoforms. The notion of cardiomyocyte heterogeneity is in agreement with the fact that, in EBs, different cardiac cell types can be produced (Maltsev et al., 1993).

Here, we have focused on the physiological relevance of the tight regulation of α-SMA during cardiac differentiation.

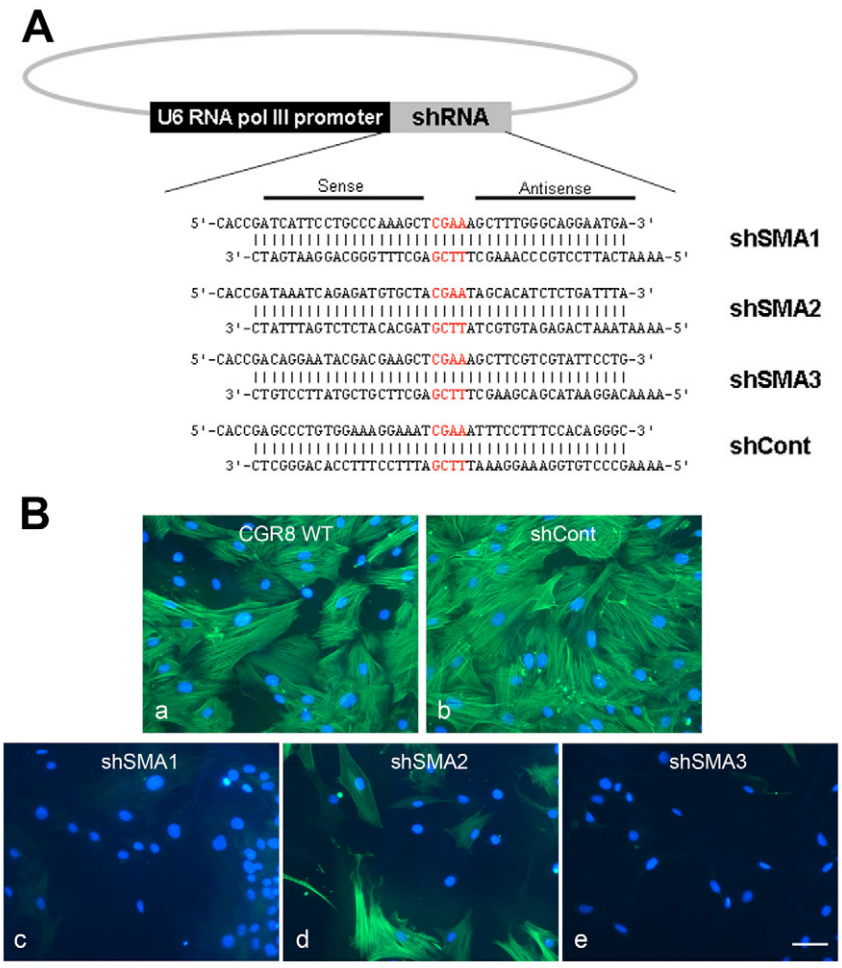

Fig. 4. Design of shRNAs and their effect on α-SMA expression in lung fibroblasts (A) Schematic representation of shRNA constructs targeting α-SMA gene (shSMA1-2-3). A shRNA sequence inactive against all actin isoforms was used as control (shCont). The resulting entry vectors were then recombined into pLenti6/BLOCK-iT™ RNAi Vector. The loop sequence CGAA is indicated in red. (B) Untransduced mouse lung fibroblasts (a), and mouse lung fibroblasts transduced with shCont (b), shSMA1 (c), shSMA2 (d) and sh-SMA3 (e) were stained for α-SMA (green). DAPI (blue) was used to stain nuclei. Bar, 20 μm.

Our results, using both SMA-FP and siRNAs underline the importance of transient α-SMA expression during cardiac differentiation. Hindrance of expression and organization of this protein blocked the differentiation process, as reflected by the decrease in the percentage of beating EBs. This effect may be explained by three non-exclusive mechanisms. (1) α-SMA could constitute a scaffold for contractile protein organization during myofibrillogenesis. The fact that SMA-FP specifically impairs the formation of myofibrils is an argument in favor of this notion, as previouly hypothesized (Clement et al., 2001; Ehler et al., 2004). (2) α-SMA could be a major contributor to the production of cellular tension. The implication of α-SMA in cell tension production is well accepted (Hinz et al., 2001; Hinz and Gabbiani, 2003; Hinz et al., 2002). In addition, it has been described that mechanical stimuli profoundly affect cardiomyocyte differentiation (Heng et al., 2004). Factors responsible for ES cell commitment to a cardiovascular fate are still poorly understood; nevertheless, hemodynamic fluid forces have been shown to play an important role during cardiomyogenesis, and loss of shear stress results in the formation of an abnormal cardiac chamber and valve formation (Illi et al., 2005). It is conceivable that the absence of α-SMA leads to reduced cell tension and, consequently, to a blockade of cardiogenesis. (3) We showed that a downregulation of cardiac transcription factors known to be implicated in cardiac differentiation (Nkx2.5 and MEF2-C) correlates with the expression of siSMAs. Growing evidence in the literature suggest a relationship between the perturbation of actin cytoskeleton organization and altered gene expression (Mack et al., 2001; Posern et al., 2002; Sotiropoulos et al., 1999). In our model of cardiac differentiation, such a role in the control of gene transcription may be attributed to α-SMA expression and polymerization.

Our results also show that activities of α-SKA and α-SMA differentially influence cardiomyocyte rythmicity. Once the cardiomyocytes spontaneously contract, SKA-FP decreases cardiomyocyte beating frequency whereas SMA-FP induces arrhythmia. The effect of SKA-FP is in accordance with reports suggesting that α-SKA increases the contractile property of these cells (Clement et al., 2005; Hewett et al., 1994; Suurmeijer et al., 2003). More complex is the interpretation of the SMA-FP effect. Given that ES cells can form an organized, functional cardiac conduction system in vitro (White and Claycomb, 2005), a tempting explanation for this arrhythmia induction would be that pace-maker cells are preferentially affected by SMA-FP. An observation favoring this idea is that, during rat heart development, expression of α-SMA persists longer in the ventricular conduction system, making it a convenient marker for the ventricular conduction system in the fetal heart (Ya et al., 1997). Nevertheless, given that not all cardiac cells within beating areas in EBs express α-SMA, it is possible that hindrance by cell contractility in only the α-SMA-positive fraction of cells contributes to the disorganization of the electrical conduction of the signal.

An interesting area for future investigations would be to enlarge this approach to an in vivo model. α-SMA-null mice have been produced (Schildmeyer et al., 2000) and even though they apparently did not suffer from cardiac problems leading to premature death (they appeared to have no difficulty feeding or reproducing), further and specific investigations on heart functionality (in particular possible arrhythmia) would be of great interest.

In conclusion, our results shed some light on α-SKA and α-SMA functions during cardiomyocyte differentiation and on cell rythmicity. In addition, this work confirms that the isoactin N-terminus is functionally crucial, as recently suggested for α-

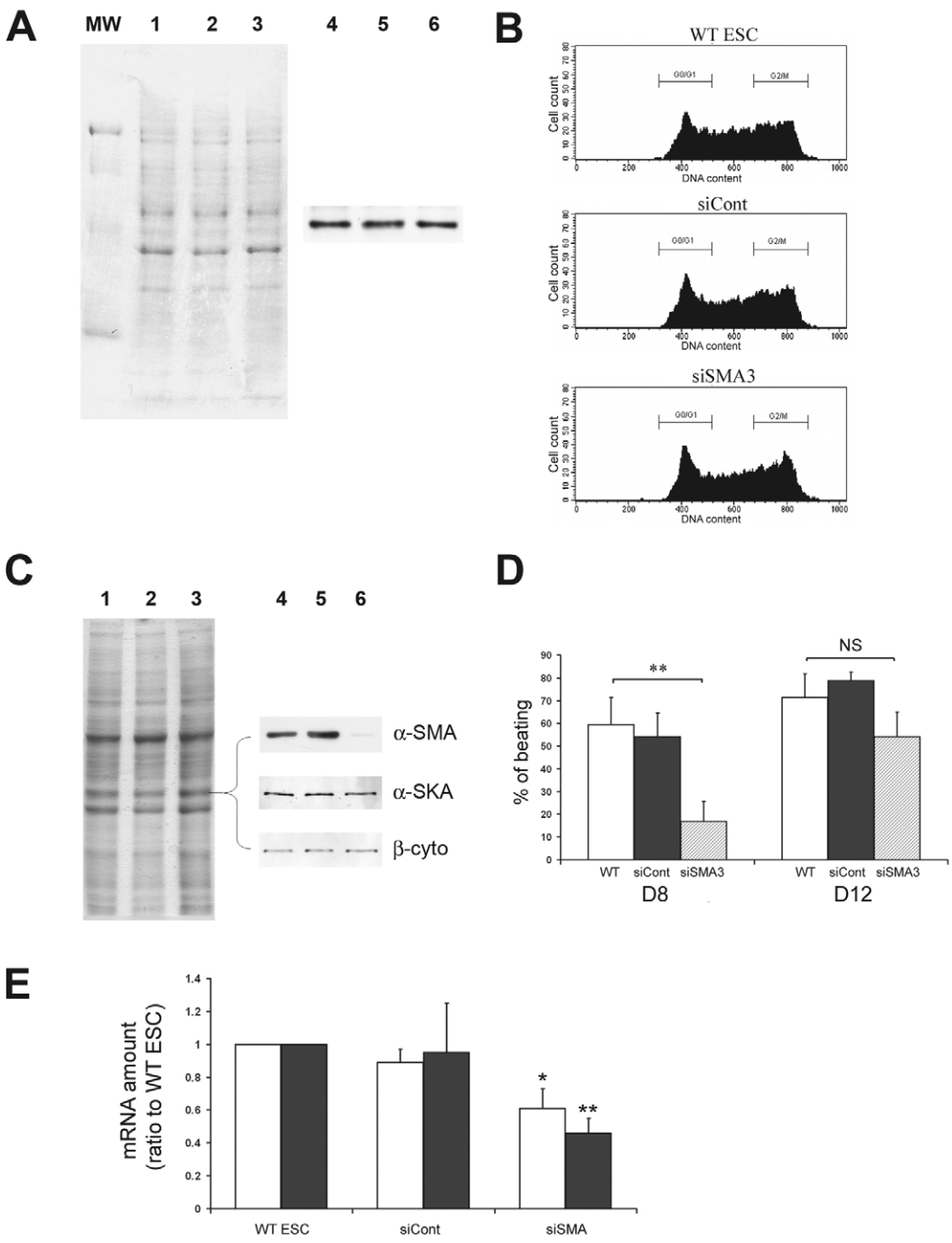

Fig. 5. Effect of shRNA targeting α-SMA in ES cells. (A) Proteins of undifferentiated cells (lanes 1 and 4, untransduced ES cells; lanes 2 and 5, ES cells transduced with lentivectors expressing shCont; lanes 3 and 6, ES cells transduced with lentivectors expressing shSMA3) were submitted to SDS-PAGE (Coomassie Blue, lanes 1-3), transferred onto nitrocellulose and blotted with anti-Oct-4, a marker of pluripotenciality (lanes 4-6). (B) Cell cycle analysis by FACS cytometry of propidium iodure labeled untransduced (top), shCont (middle) and shSMA3 (bottom) ESC. (C) Proteins of EBs at day 6 (lanes 1 and 4, untransduced ESC; lanes 2 and 5, transduced with lentivectors expressing shCont; lanes 3 and 6, ES cells transduced with lentivectors expressing shSMA3) were submitted to SDS-PAGE (lanes 1-3; Coomassie Blue staining) and immunoblotted with anti-α-SMA, anti-α-SKA and anti-β-cytoplasmic actin antibodies (lanes 4-6). (D) The percentage of beating EBs at day 8 was assessed under all three conditions. Bars represent s.e.m. of five independent experiments (**$P \leqslant 0.001$). (E) Impact of α-SMA downregulation on the expression of cardiac transcription factors Nkx2.5 (black bars) and MEF2C (white bars) was evaluated by real-time RT-PCR. Bars represent s.e.m. of three independent experiments.*$P \leqslant 0.05$, **$P \leqslant 0.001$.

SMA (Hinz et al., 2002) in myofibroblats. The results shown here further demonstrate that the N-terminal sequences of α-SMA and α-SKA isoforms have a major and specific effect on their function.

Materials and Methods

ES cells culture and differentiation

Mouse ES cells CGR8 (European Collection of Cell Cultures Salisbury, Wiltshire, UK) were cultured in BHK21 medium (Gibco, Invitrogen, Basel, Switzerland) supplemented with non-essential amino acids, pyruvate, β-mercaptoethanol, glutamine, penicillin-streptomycin, 10% fetal calf serum (FCS, Gibco) and LIF-conditioned medium in a humidified 5% CO_2 atmosphere at 37°C, and maintained at less than 70% confluency to keep an undifferentiated phenotype (Li et al., 2002; Meyer et al., 2000). The differentiation of ESC was performed by the hanging drop method (Maltsev et al., 1994). In brief, EBs were formed for 2 days in hanging drops (450 cells/20 μl) in differentiation medium (BHK21, as described above), containing 20% FCS (Hyclone, Logan, UT) and lacking LIF. After 4 days in suspension, cultured EBs were plated on gelatin-coated 24-well plates or coverslips (Meyer et al., 2000). The number of EBs that contained beating cardiomyocytes was counted under a phase-contrast microscope at day 8 of differentiation.

Treatment with fusion-peptides (FPs)

The fusion peptides SMA-FP and SKA-FP – containing Ac-EEED and Ac-DEDE, respectively, at the N-terminus of the cell-penetrating vector pAntp-Pro50 (Derossi et al., 1994) – were synthesized to a purity of 95% (UCB Bioproducts, Belgium). The FPs were administrated either to differentiating EBs before the onset of beating or to contracting EBs. In the first case, FPs were added twice a day (9 am and 6 pm) at concentrations of 10 or 50 μg/ml at day 6 and 7 of differentiation. Percentage of beating EBs was estimated at Day 8 (9 am). In the second set of experiments, beating EBs were treated for 2 hours with 50 μg/ml FPs at day 8.

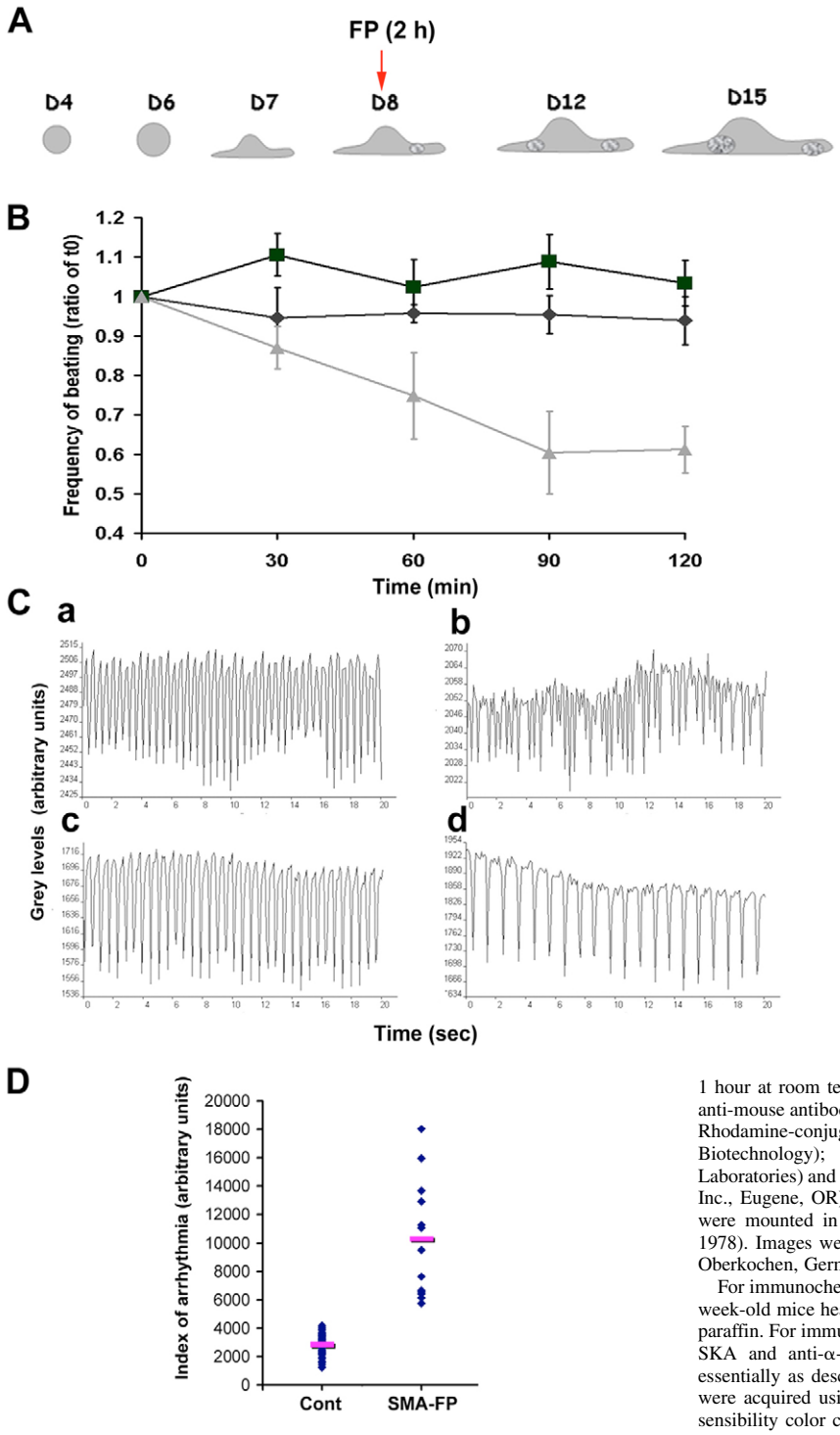

Fig. 6. Effect of FPs on cardiomyocyte beating activity. (A) SMA-FP and SKA-FP (50 μg/ml) were applied on differentiated EBs at day 8 for 2 hours. (B) The frequency of beating was estimated by counting under the microscope the number of beats per 30 seconds of about 50 EBs during a 2-hours period without any treatment (■) or following a treatment with SMA-FP (◆) or SKA-FP (△). (C) 20-second movies of beating EBs were recorded and the variation of grey level in regions at the periphery of EB was calculated over time. Plots are representative of the EBs beating activity before (a and c) and after 2 hours treatment with 50 μg/ml of either SMA-FP (b) or SKA-FP (d). (D) To obtain beating rate spectrums of the frequency plots, Fourier transformation was performed on plots shown in C (see Materials and Methods for details). Numbers on the *y*-axis represent an 'index of arrhythmia'.

Electrophoretic and immunoblot analysis

For immunoblotting, cells were thoroughly scraped from culture dishes in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 50 mM DTT, 0.01% Bromophenol Blue). Total cell lysates were run on 10% SDS-minigels (Bio-Rad Laboratories, Glattbrugg, Switzerland) (Laemmli, 1970) and electroblotted onto nitrocellulose (Towbin et al., 1979). Nitrocellulose membranes were incubated with anti-SMA [αsm-1 (Skalli et al., 1986)], anti-SKA (Clement et al., 1999), anti-α-CAA (Clement et al., 2003), anti-total actin [Mouse mAb (clone 1C4; Chemicon International, Temecula, CA)], anti-Oct4 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted in Tris-buffered saline (TBS) containing 3% BSA and 0.1% Triton X-100 for 2 hours at room temperature. After three washes with TBS, a second incubation was performed with horseradish-peroxidase-conjugated affinity-purified goat anti-rabbit IgG or anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1:10,000 in TBS, containing 0.1% BSA and 0.1% Triton X-100. Peroxidase activity was developed using the ECL western blotting system (Amersham, Rahn AG, Zürich, Switzerland), according to the manufacturer's instructions and blots were scanned (Arcus II; Agfa, Mortsel, Belgium).

Indirect immunofluorescence, immunohistochemistry and confocal laser scanning microscopy

For immunofluorescence staining, cells were fixed in 3% paraformaldehyde in PBS for 10 minutes at room temperature followed by three washes with PBS and then permeabilized with 0.3% Triton X-100 in PBS for 10 minutes. Subsequently, cells were stained with the following primary antibodies: anti-αsm-1 (IgG2a), anti-SKA (rabbit polyclonal), anti-CAA [rabbit polyclonal (Clement et al., 2003) or mouse mAb (IgG1), developed in C.C.'s laboratory (unpublished data)] and mouse anti-α-actinin (IgG1, Sigma), diluted in PBS-Tween 0.1% for 1 hour at room temperature. Samples were then incubated with FITC-conjugated anti-mouse antibodies (IgG2a specific; Southern Biotechnology, Birmingham, AL); Rhodamine-conjugated anti-mouse antibodies (IgG1 specific; Southern Biotechnology); CY5-conjugated anti-rabbit Ig (Jackson ImmunoResearch Laboratories) and a 1:1000 dilution of the nuclear dye TOTO-III (Molecular Probes, Inc., Eugene, OR), for 1 hour at room temperature. After washing in PBS, cells were mounted in polyvinyl alcohol (PVA) as described by Lennette (Lennette, 1978). Images were acquired using a confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany, using either a 10× or a 63× oil immersion objective).

For immunochemistry, embryos from BALBc mice (at stage E9.5, 13, 17) and 2-week-old mice hearts were fixed in 10% neutral buffered formol and embedded in paraffin. For immunochemistry, 4-μm sections were used with anti-α-sm-1, anti-α-SKA and anti-α-CAA antibodies. Immunoperoxidase staining was performed essentially as described previously (Clement et al., 1999). After staining, images were acquired using an Axiophot microscope (Carl Zeiss) equipped with a high sensibility color camera (Axiocam, Carl Zeiss). A set of images was analyzed to assess the percentage of the isoactin-positive cardiomyocyte area using the KS400 software (Kontron System, Zeiss Vision, Oberkochen, Germany) as previously reported (Clement et al., 1999).

Recording and analysis of cell contractility

Recording of 20-second-long phase-contrast movies of beating EBs at day 8 was with a Nipkow microscope equipped with Ultraview software (PerkinElmer, Boston, MA). The effect of FP treatment was evaluated on EBs with a frequency of approximately 100 beats/minute. Regions of interest were drawn at the periphery of beating clusters. Beating of these regions generated movements detectable by variations in the gray level, which were recorded over time with Ultraview software. The resulting frequency plots accurately represent the beating activity. Beating-rate spectra were obtained by fast Fourier transformation of the frequency plots. Lowest and highest frequencies were excluded in favor of the middle range frequencies,

which are the most representative for beating rates. The main peak of the spectrum, corresponding to the basal frequency, was removed so that only the information about arrhythmic beating remained. Spectral analyses and beating-rate variability were compared between untreated and FP-treated EBs. A similar power spectral analysis of rate fluctuations has been established by Akselrod et al. (Akselrod et al., 1981).

Short hairpin RNA and Gateway lentiviral system

We designed siRNA for α-SMA using the siRNA selection program developed at the Whitehead Institute (custom AAN19). Best candidates were selected according to the criteria associated with siRNA functionality identified by Reynolds et al. (Reynolds et al., 2004) and submitted to a BLAST search against the mouse genome to ensure the selective targeting of the SMA gene. The chosen siRNAs, 21-base-pair (bp) homologs to either the coding region (siSMA3, bp 1135-1157) or the 3′ UTR sequence (siSMA1, bp1310-1332 and siSMA2, bp 1306-1328) of mouse α-SMA (accession number BC064800) were converted to short hairpin RNAs (shRNAs) using the BLOCK-iT^{TM} RNAi designer (Invitrogen). They were then cloned in the pENTR^{TM}/U6 vector (Invitrogen) according to the manufacturer's instructions. A shRNA sequence inactive against all actin isoforms was used as control. The resulting entry vectors were then recombined with pLenti6/BLOCK-iT^{TM} RNAi vectors (Invitrogen) using the Gateway® LR plus clonase enzyme mix (Invitrogen).

Lentivector production and transduction

The lentivector particles were produced by transient transfection in HEK 293T cells as previously described (Dull et al., 1998). The lentivector-containing supernatant was collected after 72 hours, filtered through a polyethersulfone membrane (pore size 0.45 μm) and concentrated 120-fold by ultracentrifugation (25,000 ***g***, for 90 minutes at 4°C). The pellet was resuspended in complete cell culture medium and subsequently added to the target cells. Estimated titers of the concentrated lentivector were between 5×10^7 and 1×10^8 transducing units per ml. ES cells (10^4 cells/well in six-well plates) or mouse lung fibroblasts (10^4 cells/well; kindly provided by C. Barazzone) (Pagano et al., 2005) were seeded on gelatin-coated six-well plates and transduced the next day. Two days later, cells were split into gelatin-coated culture dishes. Three days after transduction, 7.5 μg/ml blasticidin was added to the culture medium of ES cells and the selection was maintained for 6 days.

RNA isolation, reverse transcription and real-time quantitative PCR

Total RNA was isolated from EBs at day 5 using TRIzol reagent (Invitrogen). Reverse transcription (RT) was performed in a 20-μl-mixture containing 1 μg of total RNA, 50 μM of random hexamers and 200 units of Superscript II (Invitrogen). The reaction was incubated at 42°C for 90 minutes and the volume was then adjusted to 30 μl. The nucleotide sequences of the PCR primers were: MEF2C forward, 5′-CCTACATAACATGCCGCCATCT-3′; MEF2C reverse, 5′-GTGGTACGGTCTCC-CAACTGA-3′; Nkx2.5 forward, 5′-GGATAAAAAAGAGCTGTGCGC-3′; Nkx2.5 reverse 5′-GGCTTTGTCCAGCTCCACTG-3′; β-tubulin forward, 5′-AGA-CAACTTCGTTTTCGGTCAGT-3′; β-tubulin reverse, 5′-CCTTTAGCCCAGTT-GTTGCCT-3′.

To avoid amplification of genomic DNA, primers were designed to be intron-spanning. Real-time quantitative PCR was performed using a TaqMan rapid thermal cycler (ABI Prism 7700) in 25 μl reaction mixtures containing 12.5 μl of SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA), appropriate primer concentration and 1 μl of cDNA. The relative cDNA concentrations were established from a standard curve using sequential dilutions of corresponding PCR fragments. The amplification program included the initial denaturation step at 95°C for 10 minutes, and 40 cycles of denaturation at 95°C for 10 seconds, annealing and extension at 60°C for 1 minute. Fluorescence was measured at the end of each extension step. After amplification, melting curves were acquired and used to determine the specificity of PCR products, which were further confirmed using conventional gel electrophoresis. The results were normalized against β-tubulin.

Statistical analyses

Quantitative results are presented as the mean ± s.e.m. Differences between mean values of percentage of beating were calculated by using the Student's *t*-test. Real-time PCR data were compared using analysis of variance (ANOVA) followed by a Newman-Keuls post-hoc test. Differences were considered significant when $^*P<0.05$ and $^{**}P<0.001$.

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