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

CLEMENT, Sophie, et al.

Abstract

Three alpha-muscle actin isoforms are sequentially expressed during in vivo cardiac development. alpha-Smooth muscle actin is first and transiently expressed, followed by alpha-skeletal and finally alpha-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 alpha-smooth muscle and alpha-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 alpha-smooth muscle actin during a time window preceding spontaneous beating, prevents proper cardiac sarcomyogenesis, whereas alpha-skeletal actin-fusion peptide has no effect. Knockdown of alpha-smooth muscle actin in embryonic stem cells using RNA interference also affects cardiac differentiation. The application [...]
Expression and function of $\alpha$-smooth muscle actin during embryonic-stem-cell-derived cardiomyocyte differentiation

Sophie Clément$^{1,*,*}$, Michael Stouffs$^{1,*}$, Esther Bettiol$^{1,*}$, Sandy Kampf$^{1,*}$, Karl-Heinz Krause$^{1,*}$, Christine Chaponnier$^{2}$ and Marisa Jaconi$^{1,*}$

$^1$Department of Geriatrics, Laboratory of Ageing, Geneva Hospital, Chêne-Bourg, Geneva, Switzerland
$^2$Department of Pathology and Immunology, Faculty of Medicine, CMU, Geneva, Switzerland

*Author for correspondence (e-mail: sophie.clement@medecine.unige.ch)

$^*$Present address: Department of Pathology and Immunology, CMU, 1, Rue Michel-Servet, 1211 Geneva 4, Switzerland

Summary

Three $\alpha$-muscle actin isoforms are sequentially expressed during in vivo cardiac development. $\alpha$-Smooth muscle actin is first and transiently expressed, followed by $\alpha$-skeletal and finally $\alpha$-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 $\alpha$-smooth muscle and $\alpha$-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 $\alpha$-smooth muscle actin during a time window preceding spontaneous beating, prevents proper cardiac sarcomyogenesis, whereas $\alpha$-skeletal actin-fusion peptide has no effect. Knockdown of $\alpha$-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 mammals constitute a family of closely related proteins expressed in a tissue-specific way. $\beta$- and $\gamma$-cytoplasmic actins are ubiquitous, and four muscle actins with very primary sequences [{$\alpha$}-skeletal ({$\alpha$}-SKA), $\alpha$-cardiac ({$\alpha$}-CAA), $\alpha$-smooth muscle ({$\alpha$}-SMA) and $\gamma$-smooth muscle actin ({$\gamma$}-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: $\alpha$-SMA (Skalli et al., 1986), $\beta$-cytoplasmic actin (Gimona et al., 1994), $\alpha$-SKA (Clement et al., 1999) and $\alpha$-CAA (Clement et al., 2003; Franke et al., 1996).

In normal myocardium, $\alpha$-CAA, $\alpha$-SKA and $\alpha$-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, $\alpha$-SMA marks the onset of cardiomyocyte differentiation, and as development proceeds, it is sequentially replaced by $\alpha$-SKA and $\alpha$-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 $\alpha$-muscle actin isoforms, with $\alpha$-CAA being predominantly expressed throughout development (Sassoon et al., 1988). In normal adult myocardium, the two sarcomeric actins, $\alpha$-CAA and $\alpha$-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 $\alpha$-SMA, $\alpha$-SKA, $\beta$-myosin heavy chain ($\beta$-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 $\gamma$-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 $\alpha$-CAA usually die in the neonatal period. However, when $\gamma$-SMA is expressed under the control of the cardiac $\alpha$-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 normally 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 immunohistochemistry 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); 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 mimics 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 previously 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
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...
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).
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 disappearance of $\gamma$H9251-SMA and $\gamma$H9251-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 characteristic 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 $\alpha$-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 $\alpha$-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 $\alpha$-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).

$\alpha$-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 $\alpha$-SMA during cardiac differentiation.
Our results, using both 
SMA-FP and siRNAs underline the
importance of transient \( \alpha \)-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) \( \alpha \)-SMA could constitute a scaffold for contractile protein
organization during myofibrillogenesis. The fact that \( \alpha \)-SMA-FP
specifically impairs the formation of myofibrils is an
argument in favor of this notion, as previously hypothesized
(Clement et al., 2001; Ehler et al., 2004). (2) \( \alpha \)-SMA could
be a major contributor to the production of cellular tension.
The implication of \( \alpha \)-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 \( \alpha \)-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-

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 \( \alpha \)-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 \( \alpha \)-SMA, it is possible that hindrance by cell
contractility in only the \( \alpha \)-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. \( \alpha \)-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 \( \alpha \)-SKA and \( \alpha \)-SMA functions during cardiomyocyte differentiation and on
cell rhythmicity. In addition, this work confirms that the isoactin
N-terminus is functionally crucial, as recently suggested for \( \alpha \)-

---

**Fig. 4.** Design of shRNAs and their effect on \( \alpha \)-SMA expression in lung fibroblasts (A) Schematic representation of shRNA constructs targeting \( \alpha \)-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-iTTM 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 \( \alpha \)-SMA (green). DAPI (blue) was used to stain nuclei. Bar, 20 \( \mu \)m.
Actin isoforms in cardiac differentiation

SMA (Hinz et al., 2002) in myofibroblasts. The results shown here further demonstrate that the N-terminal sequences of \( \alpha \)-SMA and \( \alpha \)-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, \( \beta \)-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.
were incubated with anti-SMA [ 
Electrophoretic and immunoblot analysis

Indirect immunofluorescence, immunohistochemistry and confocal laser scanning microscopy

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 EB. Beating of these regions generated movements detectable by phase-contrast microscopy. Beating-rate variations in the gray level, which were recorded over time with Ultraview software. The resulting frequency plots accurately represent the beating activity. 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’.
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 (siSMA1, bp 1135-1157) or the 3′ UTR sequence (siSMA1, bp 1310-1332 and siSMA2, bp 1306-1328) of mouse α-SMA (accession number BC064800) were converted to short hairpin RNAs (shRNAs) using the BLOCK-i™ RNAi designer (Invitrogen). They were then cloned in the pENTRY™i-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-i™ RNAi vectors (Invitrogen) using the Gateway® LR plus cloning 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 (10^5 cells/well in six-well plates) or mouse lung fibroblasts (10^5 cells/well) were subjected to lentiviral infection, 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 cDNA was amplified using specific primers 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 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

Statistical analysis was performed using the StatView program. The results were presented as mean ± S.E.M. Differences between means were analyzed by Student’s t-test. p<0.05 was considered as a statistically significant difference.

References


Garrits, I., Minty, A. J., Alonso, S., Barton, P. J. and Buckingham, M. E. (1986). A 5′ duplication of the alpha-cardiac actin gene in BALB/c mice is associated with abnormal levels of alpha-cardiac and alpha-skeletal actin mRNAs in adult cardiac tissue. EMBO J. 5, 2559-2567.


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).

Actin isoforms in cardiac differentiation


