Histochem Cell Biol (2013) 139:35–46
DOI 10.1007/s00418-012-1022-4

ORIGINAL PAPER

The role of PKCε-dependent signaling for cardiac differentiation

D. Galli · G. Gobbi · C. Carrubbi · D. Di Marcantonio · L. Benedetti · M. G. C. De Angelis · T. Meschi · M. Vaccarezza · M. Sampaolesi · P. Mirandola · M. Vitale

Accepted: 13 August 2012 / Published online: 31 August 2012

Abstract Protein kinase Cepsilon (PKCε) exerts a well-known cardio-protective activity in ischemia–reperfusion injury and plays a pivotal role in stem cell proliferation and differentiation. Although many studies have been performed on physiological and morphological effects of PKCε mis-expression in cardiomyocytes, molecular information on the role of PKCε on early cardiac gene expression are still lacking. We addressed the molecular role of PKCε in cardiac cells using mouse cardiomyocytes and rat bone marrow mesenchymal stem cells. We show that PKCε is modulated in cardiac differentiation producing an opposite regulation of the cardiac genes *NK2 transcription factor related, locus 5* (*nkx2.5)* and *GATA binding protein 4* (*gata4)* both in vivo and in vitro. Phospho-extracellular regulated mitogen-activated protein kinase 1/2 (p-ERK1/2) levels increase in PKCε over-expressing cells, while *pkcε* siRNAs produce a decrease in p-ERK1/2. Indeed, pharmacological inhibition of ERK1/2 rescues the expression levels of both *nkx2.5* and *gata4*, suggesting that a reinforced (mitogen-activated protein kinase) MAPK signaling is at the basis of the observed inhibition of cardiac gene expression in the PKCε over-expressing hearts. We demonstrate that PKCε is critical for cardiac cell early gene expression evidencing that this protein is a regulator that has to be fine tuned in precursor cardiac cells.

Keywords Heart · nkx2.5 · gata4 · Kinases

Electronic supplementary material The online version of this article (doi:10.1007/s00418-012-1022-4) contains supplementary material, which is available to authorized users.

D. Galli · G. Gobbi · C. Carrubbi · D. Di Marcantonio · P. Mirandola · M. Vitale (✉)
Department of Biomedical, Biotechnological and Translational Sciences (S.Bi.Bi.T.), University of Parma, Via Gramsci, 14, 43126 Parma, Italy
e-mail: marco.vitale@unipr.it

G. Gobbi · P. Mirandola · M. Vitale
Center for Morphology, Biometry and Body Composition (CMBC), University of Parma, Via Gramsci, 14, 43126 Parma, Italy

L. Benedetti · M. G. C. De Angelis · M. Sampaolesi
Department of Experimental Medicine, Human Anatomy Section, University of Pavia, Via Forlanini, 8, 27100 Pavia, Italy

T. Meschi
Department of Clinical and Experimental Medicine, University of Parma, Via Gramsci, 14, 43126 Parma, Italy

M. Vaccarezza
Department of Health and Sport Science, University of Cassino, Via Sant'Angelo località Folcara, 03043 Cassino, FR, Italy

M. Sampaolesi
University Hospital Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium

Introduction

The protein kinase C (PKC) family, composed by 12 different isoforms, plays a pivotal role in many biological contexts as cell differentiation, proliferation and survival. Several cellular functions have shown to be dependent on PKCε expression levels in different models (Ivaska et al. 2005; Saurin et al. 2008).

In the hematopoietic field, the role of PKCε has been demonstrated in the regulation of erythropoiesis and megakaryocytopoiesis (Mirandola et al. 2006; Gobbi et al. 2007; Redig and Platanias 2007). More recently, PKCε has been found implicated in colorectal cancer cell differentiation (Gobbi et al. 2012). PKCε has been demonstrated to

be relevant for cardio-protection as well as in ischemia–reperfusion injury (Meyer et al. 2009; Budas et al. 2010).

Transgenic mice over-expressing a constitutively active PKCε show increased anterior and posterior cardiac wall thickness that determines concentric hypertrophy (Takeishi et al. 2000) and slowly develop dilated cardiomyopathy with age (Goldspink et al. 2004). The same results were obtained also with a PKCε-specific activator (Mochly-Rosen et al. 2000), confirming the negative effects of a permanently active PKCε in cardiac cells.

In the *pkcε* null mice, there is an up-regulation of the PKCδ isoform and activation of p38 mitogen-activated protein kinase (MAPK) and c-jun N-term kinase (JNK) that leads to interstitial fibrosis and impaired diastolic function after transverse aortic constriction (TAC) (Klein et al. 2005). All these results suggest that PKCε normal expression is required for cardiac morphology and function.

Although the effects of PKCε over-expression have been analyzed both from the physiological and morphological points of view (Takeishi et al. 2000; Goldspink et al. 2004), molecular studies of its consequences on early cardiac marker gene expression are still lacking.

The potential of progenitor stem cells to differentiate into cardiac muscle has been studied by many groups, and different types of stem cells have been identified (rev. in Joggerst and Hatzopoulos 2009), namely bone marrow hematopoietic stem cells (BMHSCs), bone marrow mesenchymal stem cells (BMMSCs), cardiac stem cells and embryonic stem cells. Bone marrow has represented a preferred source of stem cells in the recent years, due to the co-existence of three classes of progenitor cells in this anatomical district: hematopoietic, mesenchymal and endothelial. While hematopoietic c-Kit negative cells and endothelial progenitors do not express cardiac markers once transplanted in infarcted hearts, c-Kit positive progenitors and mesenchymal cells show cardiac differentiation both in vitro and in vivo (Wakitani et al. 1995; Gussoni et al. 1999; Orlic et al. 2003; Huang et al. 2010; Gao et al. 2010). The first report on cardiac potential of BMMSCs induced by 5-azacytidine (5-AZ) from Caplan's group in 1995 (Wakitani et al.). In vivo transplantation of BMMSCs evidenced their capability to differentiate into cardiac-like cells and to form intercalated disks with host cardiomyocytes, although with low efficiency (Huang et al. 2010).

We, therefore, addressed the role of in vivo PKCε over-expression on *nkx2.5* and *gata4* transcriptional regulation. These two genes are early cardiac genes and are considered crucial for cardiac reprogramming (Mauritz et al. 2008; Sperling 2011). The first results that we obtained in vivo suggested a negative role of PKCε on the transcriptional regulation of these two genes, leading us to subsequently study the effects in ex vivo rat BMMSCs, highlighting a novel role for PKCε, mediated by ERK1/2, in cardiomyocyte differentiation.

Materials and methods

Mice

All the experiments, described in this study, regarding the use of laboratory animals were carried out conforming to the "Guide for the Care and Use of Laboratory Animals" (Directive 2010/63/EU of the European Parliament). All the experiments were approved by the Animal Research Ethics Committee of the University of Pavia.

Cell cultures and labeling

BMMSCs were isolated as previously described (Gao et al. 2010) with some modifications. Wistar rats were euthanized with overdoses (≥150 mg/kg) of pentobarbital. Death was checked before femurs and tibia were aseptically harvested. The adherent muscles and soft tissues were removed. The proximal and distal ends of the bone were excised just at the beginning of the marrow cavity. Whole marrow plugs were obtained by flushing the bone marrow cavity with a 18-gauge needle connected to a syringe filled with culture medium composed of low glucose Dulbecco's modified eagle's medium (DMEM, Sigma, Milan, Italy) supplemented with 10 % fetal bovine serum (Euroclone, Milan, Italy). Mononuclear cells were collected at the Percoll interface, rinsed twice in low glucose DMEM and plated at a density of $2 \times 10^5/cm^2$ in low glucose DMEM and incubated at 37 °C in a humidified atmosphere of 95 % air 5 % CO_2. Non-adherent cells were removed after 24 h and culture media were replaced every 2 or 3 days. When these primary BMMSCs reached 90 % confluence, they were trypsinized (trypsin–0.02 % EDTA, Euroclone), counted and passaged at a density of $6 \times 10^5/cm^2$ and the cells of passage 2 were used for study.

Mice cardiomyocytes were isolated as previously described (De Luca et al. 2000). Briefly, mice were euthanized by decapitation after carbon dioxide sedation. Efficacy of the anesthesia was confirmed by the absence of pedal withdrawal reflex. Then, hearts were removed. Great vessels and atria were separated from the ventricles of each heart. Ventricles were minced and digested with collagenase type II (108 U/ml, Roche, Italy) and pancreatin (0.6 mg/ml, Sigma, Italy) until there were no more large tissue fragments left. Cells from each digestion were collected by centrifugation, resuspended in newborn calf serum (Gibco, Italy) and loaded on a discontinuous Percoll (density 1.13 g/ml) (Sigma, Italy) gradient. The band at the

interface (containing myocytes) was removed and washed with PBS.

Cardiac differentiation in BMMSCs was induced by treatment with 10 μM 5-azacytidine (Sigma-Aldrich, Milan, Italy) for 24 h and then cultured in DMEM low glucose (Sigma), 2 % horse serum (Euroclone) for up to 30 days. Osteogenic and adipogenic differentiation was induced with specific media from Stem Cell Technologies (Vancouver, Canada) following manufacturer's instructions and verified by Alizarin red and Oil red Oil staining, as previously described (Galli et al. 2011). For MEK1/2 inhibition we pre-treated with 10 μM U0126 (Cell Signaling, Boston, USA) for 30 min before cell transfection.

FACS analysis

To assess phenotype of rat BMMSCs, 0.3×10^6 cells for each experimental point were labeled with FITC anti-CD34, anti CD-44, anti CD105 and RPE anti-CD45, anti CD90, anti CD14 (all from BD Pharmigen, Franklin Lakes, NJ, USA) for 30 min at room temperature. Dilutions were previously optimized by serial dilution tests. Cells were analyzed by Beckman Coulter Cytomics FC 500 (Beckman Coulter, Fullerton, CA, USA) equipped with a CXP analysis software.

Cell transfections

PKCε expression levels were down-regulated by transfection of double-stranded small interfering RNAs (siRNAs) designed to target sequences corresponding to nucleotides 218–238 (5′-AAGATCAAAATCTGCGAGGCC-3′) and 425–446 (5′-AAGATCGAGCTGGCTGTCTTTC-3′) (Mirandola et al. 2006; Gobbi et al. 2007). The respective sense and antisense RNA sequences were synthesized by Silencer siRNA Construction Kit (Ambion, Austin, TX, USA). Non-specific siRNA duplexes containing the same nucleotides, but in irregular (scrambled) sequence (control siRNA), were used as controls. The GFP–PKCε plasmids (PKCε and PKCε K522M) were kindly provided by Professor Peter Parker (Cancer Research UK, London Research Institute, UK). Transfections were performed using Superfect Reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions, with 1 μg of each plasmid or 100 nM of each siRNA.

RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's protocols. About 1 μg of RNA was treated with DNase-free RNase (Fermentas, MD, USA), precipitated with ethanol and 0.3 M sodium acetate and resuspended in RNase free-water. Equal quantities of RNA for each sample were retro-transcribed with malone murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) according to manufacturer's instructions. 2 μl of 1:5 cDNA dilution were used to perform real-time PCR with SYBR Premix Ex *Taq* (Perfect Real Time) (Takara, Otsu, Japan), 200 nM of each primer in Smart Cycler II system, using SYBR Premix Ex *Taq* standard protocol. In each reaction the number of cycles was 40. Primer sequences were: **rnkx2.5 rt fw**: 5′-TATGAGCTGGAGCGGCGCTT-3′ **rnkx2.5 rt rev**: 5′-TGGAACCAGATCTTGACCTG-3′ **rgata4 fw**: 5′-AGGGTGCTGGGTTTCTTCAA-3′, **rgata4 rev**: 5′-GACAGTGTCTTGAAGCCTCG-3′ **rpkcε rt fw**: 5′-caagcagaagaccaacagtc-3′, **rpkcε rt rev**: 5′-cgaactggatggtgcagttg-3′, **rpgk rt fw**: 5′-tgtgggctcagaagtagaga-3′, **rpgk rt rev**: 5′-tagctggctcagctttaacc-3′. Oligonucleotides sequences for mouse *nkx2.5*, *gata4, mhc* and *gapdh* were derived from Snyder et al. (2010).

Immunofluorescence on cells

Anti-myosin heavy chain antibody (MF-20) was from Developmental Study Hybridoma Bank, while rabbit anti-CONNEXIN43 (CX43) was from Santa Cruz Biotechnology, USA. Cells were fixed with 4 % paraformaldehyde, permeabilized with 1 % BSA, 0.2 % Triton X-100 and blocked in 10 % donkey serum. After 2 h of incubation at room temperature with MF-20 or anti-CX43 diluted 1:200 in 1 % donkey serum, cells were washed and incubated with Alexa Fluor 546 fluorescent anti-mouse or anti-rabbit IgG for 1 h at room temperature, washed again and analyzed by Nikon (Tokyo, Japan) fluorescent microscope. As controls we used BMMSCs treated as described above except for the incubation with primary antibody (i.e., MF-20 or anti-CX43). These controls were used to set microscope parameters (gain, exposition time) before any picture acquisition.

Immunofluorescence on sections

Mouse monoclonal anti-myosin heavy chain antibody (MF-20) was from Developmental Study Hybridoma Bank, while rabbit anti-GFP was from Molecular Probes (Life Technologies, Milan, Italy). Sections were permeabilized with 1 % BSA, 0.2 % Triton X-100 for 5 min and blocked in 10 % donkey serum for 1 h. After 2 h of incubation with MF-20 and GFP diluted 1:200 in 1 % donkey serum, sections were washed three times with PBS and incubated with Alexa Fluor 488 Donkey anti-rabbit IgG and Alexa Fluor 594 Donkey anti-mouse IgG, washed again and visualized with Nikon (Tokyo, Japan) fluorescent microscope. As controls we used serial cardiac sections treated as

described above but not incubated with primary antibody (i.e., MF-20 and GFP). These controls were used to set microscope parameters (gain, exposition time) before any picture acquisition.

Western blot

About 1×10^6 cardiomyocytes or BMMSCs were resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4; 1 % NP-40; 0.25 % sodium deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride; 1 mM Na_3VO_4; 1 mM NaF) and homogenized. Twenty micrograms of each protein sample were loaded on a 10 % SDS gel and blotted onto nitrocellulose. Blotted filters were blocked and incubated with primary antibodies according to manufacturer's instructions. Anti-Phospho-ERK1/2 (Cell Signaling, USA), anti-β-ACTIN (Sigma, Italy) and anti-TUBULIN (Sigma, Italy) were diluted 1:1,000; anti-NKX2.5 (abCam, UK) anti-GATA4 (abCam, UK) and anti-CONNEXIN43 (CX43) (sc-9059) were diluted 1:100. After three washes, filters were incubated for 1.5 h at room temperature with peroxidase-conjugated secondary antibodies diluted 1:5,000. Bands were revealed with ECL SuperSignal West pico Chemiluminescent Substrate Detection System (Thermo Scientific, Rockford, IL, USA).

In vivo PKCε over-expression

CD1 outbred mice (3 weeks old) were maintained using standard protocols recognized from University of Pavia and animals were treated according to the EU guidelines. Mice were warmed with a warm box for few minutes to induce vein dilation, anesthetized (pentobarbital 50 mg/kg IP) and kept warmed during all the experiment. The adequacy of anesthesia was determined by the loss of a pedal withdrawal reflex. Forty micrograms of plasmid were diluted in glucose solution (5 %) together with in vivo-jetpei reagent (Polyplus Transfection, Illkirch, France) and injected in the lateral tail vein following the Institutional Animal Care and Use Committee (IACUC) protocol. Final injected volume was 200 µl. 0.9 % NaCl solution (200 µl) was injected as control. DNA and reagents have been prepared following these steps:

1. 40 µg of DNA (resuspended in water for injections) have been diluted into 50 µl of 10 % glucose (supplied by the company); sterile water has been added to reach 100 µl, then vortexed gently and spin down,
2. 6.4 µl of in vivo-jetPEI® have been diluted into 50 µl of 10 % glucose (supplied by the company); sterile water has been added to reach 100 µl, then vortexed gently and spin down,
3. the diluted in vivo-jetPEI® has been added to the diluted DNA at once, vortexed briefly, spin down and incubated for 15 min at room temperature,
4. complexes equilibrated at room temperature have then been injected.

In the hours following injections, mice were monitored and then returned to standard housing. After 36 h, mice were sedated with carbon dioxide and euthanized with cervical dislocation. Hearts were removed and processed to obtain cardiomyocytes as described in "Cell cultures and labeling". RNA and protein extraction were performed on the cardiomyocytes at the interface of the Percoll gradient. Transfection efficiency was calculated by counting the average number of GFP-positive cells relative to the total number of cardiomyocytes in four microscopic fields for each sample (Supplementary Fig. 1). For each plasmid or control, 2 mice were used and each experiment was repeated three times.

Statistical analysis

Three independent experiments were performed for each assay and statistically analyzed using ANOVA test. Statistical significance was set at $p < 0.05$.

Results

In vivo over-expression of PKCε down-regulates *nkx2.5* and *gata4* gene expression

To study the effect of PKCε over-expression in vivo, we injected in the tail vein of CD1 mice the plasmid carrying the wt isoform (*pkcε*) or the plasmid carrying a mutated isoform in its catalytic site (*pkcε* K522M). After 72 h, we performed another injection. *Pkcε* expression (by qRT-PCR) was analyzed on cardiomyocyte fraction of control and transfected hearts after 24 h.

A significant increase in *pkcε* mRNA was observed in the cardiomyocytes of transfected animals (Fig. 1a). We therefore decided to analyze the expression of *nkx2.5* and *gata4*. These two genes were significantly down-regulated when wt PKCε was over-expressed (Fig. 1b), suggesting a negative effect of PKCε over-expression on *nkx2.5* and *gata4*. Interestingly, *myosin heavy chain* (*mhc*) mRNA did not seem to be affected by over-expression of PKCε. Transfection was evaluated in cardiomyocytes in vivo by double immunofluorescence for GFP and myosin heavy chain (Fig. 2). Notably, we found GFP expression in the vessels and cardiomyocytes surrounding vessels. This is probably due to the technique of injection. A quantitative analysis of GFP-positive cells and pictures of GFP cells

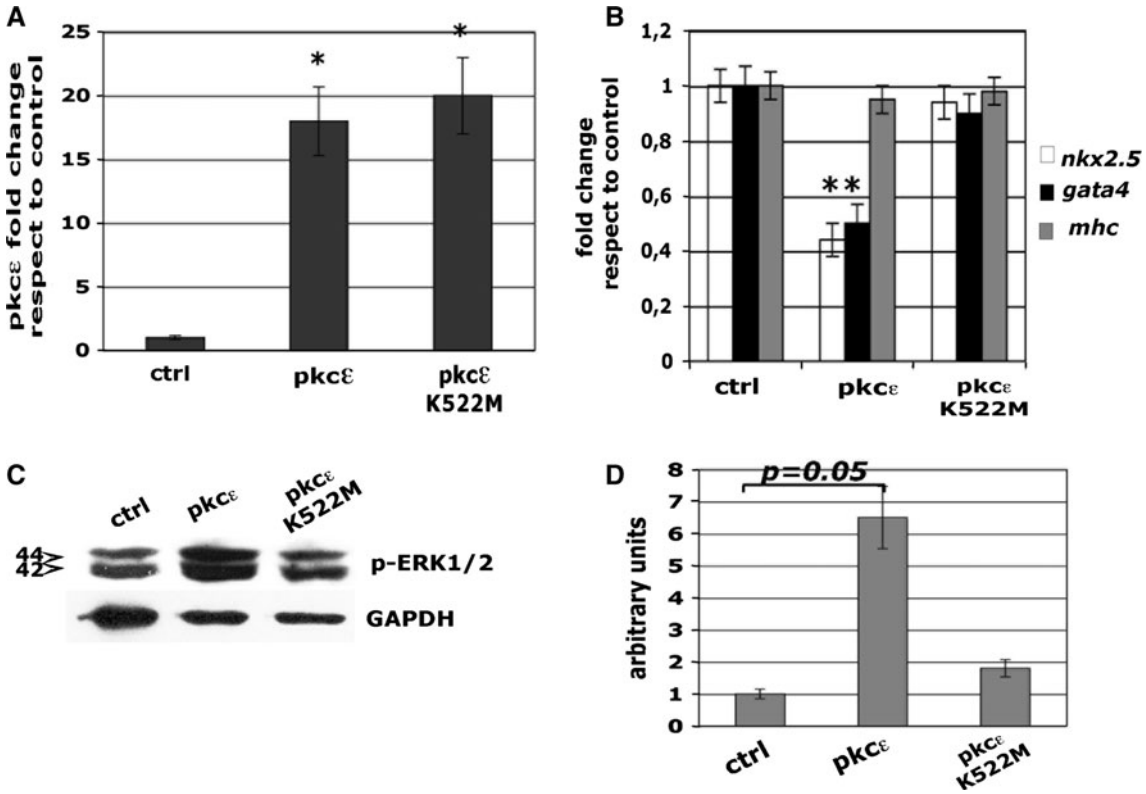


Fig. 1 In vivo analysis of *nkx2.5* and *gata4* expression in hearts transfected with *pkcε* or *pkcε* K522M. **a** qRT-PCR for *pkcε* in cardiomyocytes of mice injected with *pkcε* or *pkcε* K522M compared with 0.9 % NaCl injected mice (*ctrl*). $*p = 0.01$. **b** qRT-PCR for *nkx2.5* (*empty bar*), *gata4* (*black bar*) and *myosin heavy chain* (*gray bar*) in the same samples as in **a**. In all qRT-PCR, housekeeping *gapdh* was used as reference gene. $**p = 0.04$. **c** Western blot for phospho-ERK-1/2; GAPDH for loading control. **d** Quantitative analysis of the bands in **c** expressed as arbitrary units respect to the intensity of the corresponding bands of control after normalization with the GAPDH of the same sample

just after Percoll gradient are reported in Supplementary Fig. 1.

We next looked for possible PKCε-related mechanisms of cardiac genes regulation. Starting from the notion that: (i) PKCε stimulates cell proliferation through ERK1/2 (Basu and Sivaprasad 2007) and, (ii) ERK1/2 has a negative effect on calcium channel expression in cardiomyocytes through *nkx2.5* (Marni et al. 2009), we decided to focus on ERKs as candidate molecular intermediates between PKCε and *nkx2.5*/*gata4*. Western blot analysis showed an increase of phospho-ERK1/2 in PKCε over-expressing cells (*pkcε*) (Fig. 1c, d), suggesting that a reinforced MAPK signaling could be at the basis of the observed inhibition of early cardiac gene expression in the PKCε over-expressing hearts.

PKCε expression is modulated during cardiac differentiation of BMMSCs

Following the results obtained in vivo, we moved to analyze *pkcε* expression in adult BMMSCs induced with 5-azacytidine. In fact, BMMSCs represent also a useful tool for in vitro cardiac trans-differentiation, providing an inducible (5-azacytidine) system, thus permitting to observe early phenomena in cardiac differentiation.

We performed a time course of *pkcε*, *nkx2.5* and *gata4* mRNAs by real-time RT-PCR in 5-AZ-treated BMMSCs (Fig. 3a). Interestingly, while *pkcε* was maximal at day 2 (2d) and minimal at day 7 (7d) (arrow heads), we found expression kinetics opposite to that of *pkcε*, i.e., maximal at 7d and minimal at 2d for the two cardiac transcription factors. PKCε protein kinetic was confirmed by Western blot (Fig. 3b) and relative densitometric analysis (Fig. 3c).

On the other side, we characterized BMMSCs both phenotypically and functionally. They were positive for CD44 (90 ± 3 %), CD90 (87 ± 2.7 %) and CD105 (92 ± 1 %) and negative for CD34, CD45, CD14 (not shown), a phenotypic profile characteristic of rat BMMSCs (Gao et al. 2010).

To assay their general differentiation potential, we first tested BMMSCs for osteogenic and adipogenic

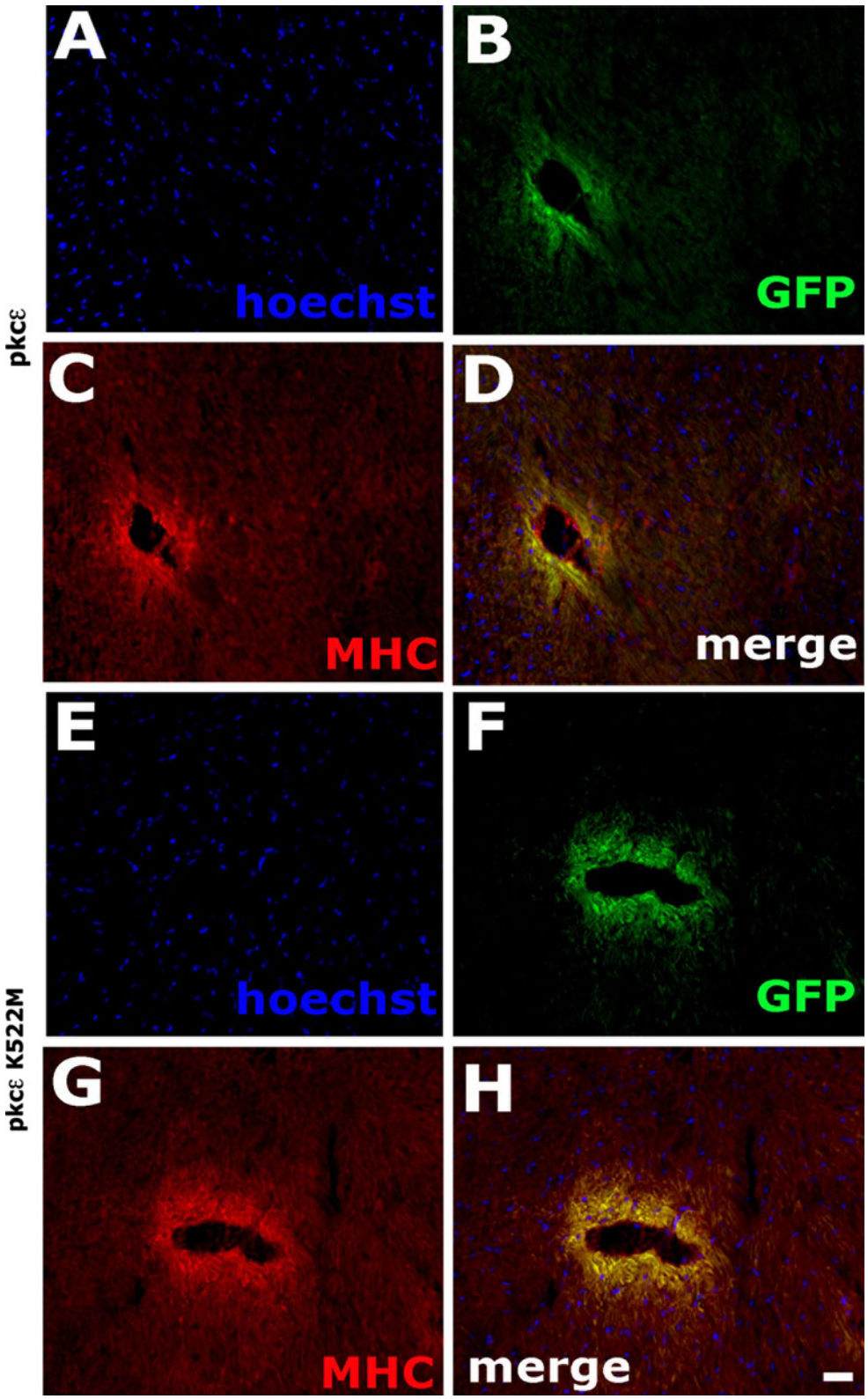


Fig. 2 In vivo analysis of PKCε over-expression by anti-GFP immunolocalization. **a–d** Immunofluorescence on sections of hearts injected with plasmid encoding wt PKCε–GFP. **a** Hoechst staining; **b** GFP immunofluorescence; **c** myosin heavy chain immunofluorescence; **d** merge of **a**, **b** and **c**. **e–h** Immunofluorescence on sections of hearts injected with plasmid encoding K522M PKCε–GFP; **e** Hoechst staining; **f** GFP immunofluorescence; **g** myosin heavy chain immunofluorescence; **h** merge of **e**, **f** and **g**. *Scale bar* in **h** is the same for all the panels and corresponds to 30 μm

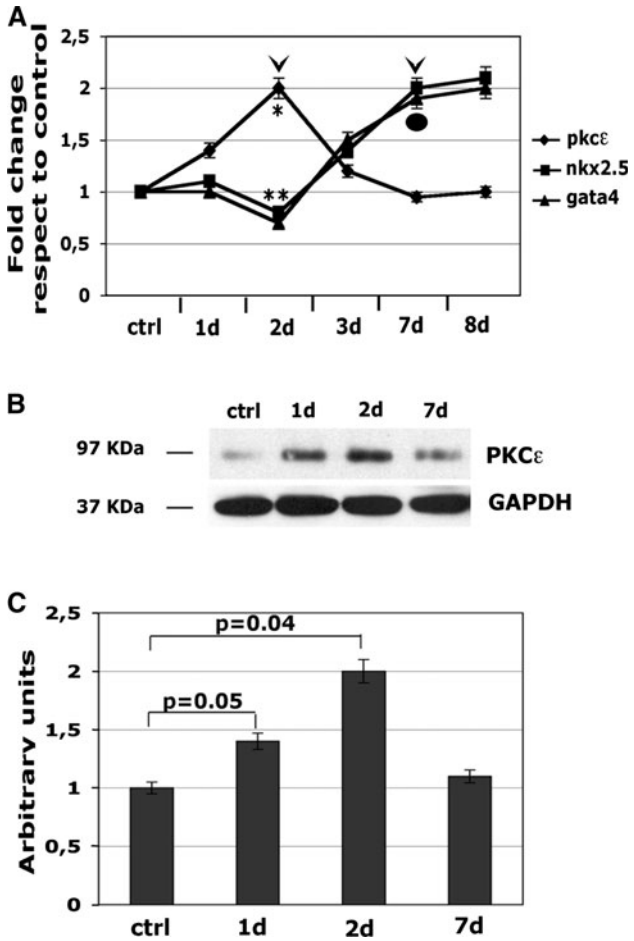


Fig. 3 Quantitative analysis of *pkcε* mRNA during cardiac differentiation of rBMMSCs. **a** qRT-PCR of *pkcε*, *nkx2.5* and *gata4* mRNA 1 day (1d), 2 days (2d), 3 days (3d), 7 days (7d), 8 days (8d) after treatment with 5-AZ and expressed as fold increase respect to control (untreated cells, *ctrl*). Housekeeping *phosphoglycerate kinase 1* (*pgk*) gene was used as reference. *Arrowheads* highlight samples 2d and 7d (*$p = 0.01$, **$p = 0.009$, *black ball* $p = 0.01$). **b** Western blot for PKCε at 1 day (1d), 2 days (2d) and 7 days (7d) after treatment with 5-AZ, *ctrl* corresponds to the untreated sample. GAPDH was used as loading control. **c** Quantitative analysis of band intensities for PKCε expressed as arbitrary units with respect to control (*ctrl*) after normalization with the GAPDH of the same sample

differentiation under appropriate culture conditions. Osteogenic potential was demonstrated with Alizarin Red S that forms red precipitates on calcium crystals formed by osteocytes (Fig. 4a). Adipogenic differentiation was indeed shown by the formation of Oil Red Oil stained lipid vacuoles (Fig. 4b). Specific in vitro cardiac potential of this mesenchymal population was confirmed by anti-myosin heavy chain (Fig. 4c–h) antibody immunofluorescence staining 30 days after the addition of 5-AZ. We also performed immunofluorescence (Fig. 4i–k) and Western blot analysis (Fig. 4l) for Connexin43 expression to be convinced of the cardiogenic read-out of BMMSCs.

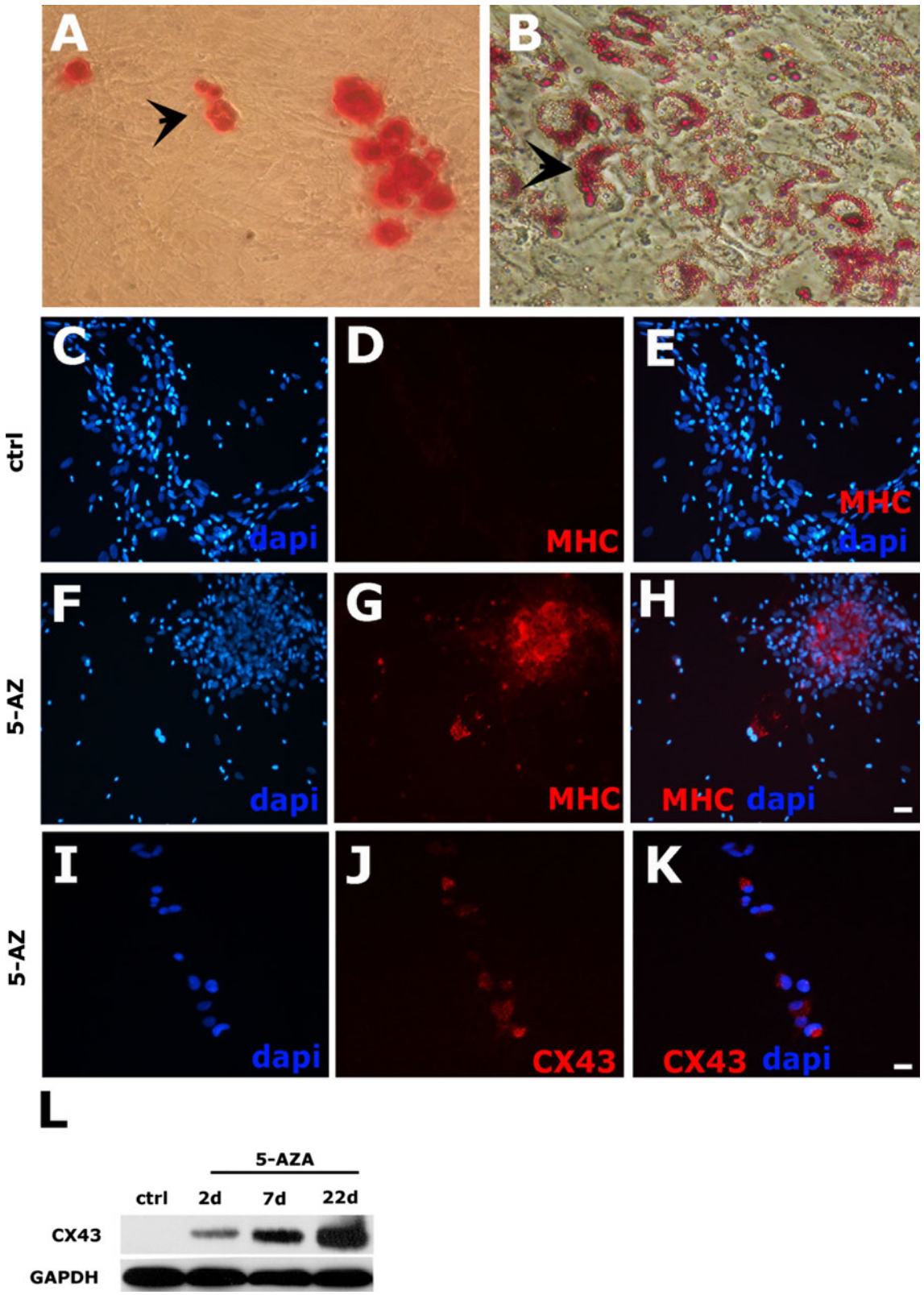
PKCε has a negative role in nkx2.5 and gata4 expression

To test the role of PKCε in *nkx2.5* and *gata4* regulation, we both down-regulated and up-regulated PKCε expression by transfecting BMMSCs 2 days after 5-AZ treatment with siRNAs and plasmids carrying either the wt isoform of PKCε (*pkcε*) or a mutated isoform in its catalytic site (*pkcε* K522M). We chose this time because the PKCε level was maximum. Relative PKCε over-expression and silencing were evaluated by qRT-PCR and normalized on not transfected 5-AZ-treated cells (Fig. 5a, 3d ctrl). We also observed a tenfold higher expression for both plasmids while *pkcε* siRNAs generated a 50 % decrease of *pkcε* mRNA.

Over-expression of PKCε produced a significant decrease of both *nkx2.5* and *gata4* mRNAs (Fig. 5b, arrowhead) and protein (Fig. 6a); on the contrary, BMMSCs over-expressing the mutated PKCε form (Fig. 5b, black ball), showed an increase in the expression of the two genes, suggesting a negative role for PKCε in their regulation. The down-regulation of PKCε expression by *pkcε* siRNAs (Fig. 5b, asterisk), produced similar effects as those obtained with *pkcε* K522M. In Fig. 6b, a quantitative analysis of NKX2.5 and GATA4 Western blot band intensities are reported. Finally, both mRNA and protein patterns of the two cardiac marker genes were similar, thus definitively showing a negative effect of PKCε over-expression on these genes. The same experiments repeated 7 days after 5-AZ treatment did not produce any effect on *nkx2.5* and *gata4* transcription; instead, in these conditions, we observed a strong cell death (data not shown).

PKCε effects on nkx2.5 and gata4 are mediated by ERKs signaling

On the basis of preliminary data obtained in vivo, suggesting ERKs as candidate molecular intermediates between PKCε and *nkx2.5*/*gata4*, we studied in deeper detail the MAPKinase signaling axis in the BMMSCs model system. Western blot analysis showed an increase of phospho-ERK1/2 in PKCε over-expressing cells (Fig. 7a, b, lane 2), while transfection with *pkcε* siRNAs produced a decrease of phospho-ERK1/2 (Fig. 7a, b, lane 4), suggesting that a reinforced MAPK signaling could be at the basis of the observed inhibition of cardiac gene expression in the PKCε over-expressing hearts. Functionally, treatment of BMMSCs over-expressing PKCε with the MEK1/2 inhibitor U0126 rescued the expression levels of both *nkx2.5* and *gata4* (Fig. 7c). Moreover, treatment with



U0126 significantly increased the expression of *nkx2.5* and *gata4* confirming a role for ERK1/2 in the inhibition of cardiac differentiation. Efficacy of U0126 treatment is shown in Supplementary Fig. 2.

Discussion

Although it is well known that, during development, cardiac differentiation is induced by FGFs and BMPs

◀ **Fig. 4** BMMSCs differentiation analysis. **a** Alizarin red staining of BMMSCs cultured in osteogenic inductive medium. *Arrowhead* indicates red calcium deposits. **b** Oil Red Oil staining of BMMSCs cultured in adipogenic inductive medium. *Arrowhead* indicates lipid vacuoles. **c–e** Myosin immunofluorescence in control cells. **f–h** Myosin immunofluorescence in 5-AZ-treated cells. **c**, **f** Dapi counterstaining of nuclei. **d**, **g** Anti-myosin immunolocalization. **e** merge of **c** and **d**. **h** Merge of **f** and **g**. **i–k** Connexin43 immunofluorescence in 5-AZ-treated cells. **i** Dapi counterstaining of nuclei. **j** Anti-CONNEXIN43 (CX43) immunolocalization. **k** Merge of **i** and **j**. *Scale bar* for **c–h** is reported in **h** and corresponds to 50 µm, for **a** and **b** corresponds to 30 µm. *Scale bar* for **i–k** is reported in **k** and corresponds to 20 µm. **l** Western blot analysis with anti-CONNEXIN43 (CX43) antibody on untreated cells (*ctrl*), 2 days (2d), 7 days (7d) and 22 days (22d) after 5-AZ treatment

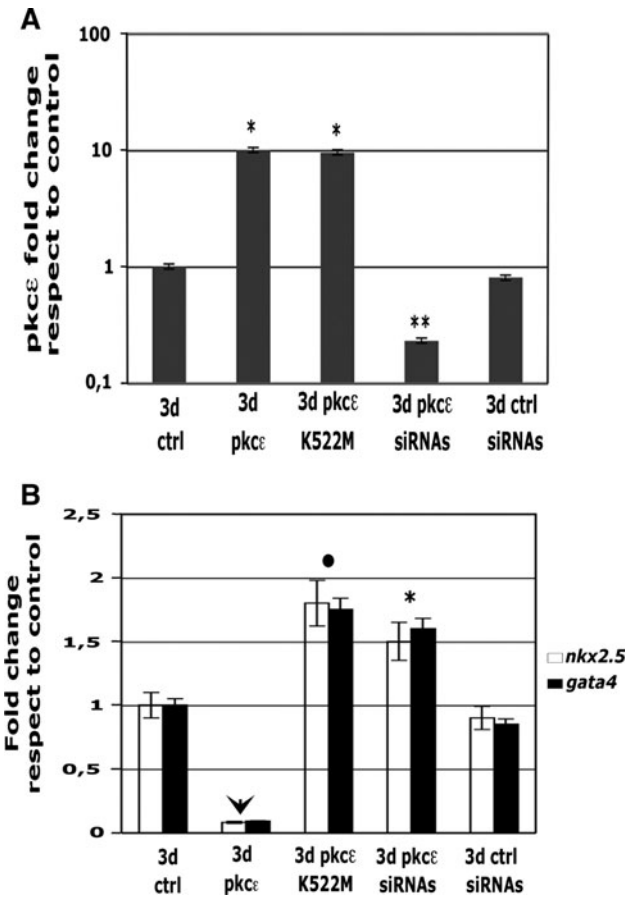


Fig. 5 Effect of PKCε over-expression and silencing on *nkx2.5* and *gata4* gene expression in rBMMSCs. **a** qRT-PCR for *pkcε* in cells treated with 5-AZ and transfected with *pkcε* (*3d pkcε*), *pkcε* K522M (*3d pkcε K522M*), *pkcε* siRNAs (*3d pkcε siRNAs*) and ctrl siRNAs (*3d ctrl siRNAs*); *pkcε* indicates cells transfected with the plasmid containing wt isoform of murine PKCε, while *pkcε* K522M refers to cells transfected with mutated isoform of murine PKCε. $*p = 0.01$, $**p = 0.03$, **b** *nkx2.5* (*empty bar*) and *gata4* (*black bar*) qRT-PCR in rBMMSCs treated with 5-AZ (*3d ctrl*), transfected with PKCε (*3d pkcε*) or PKCε K522M (*3d pkcε K522M*), or *pkcε* siRNAs (*3d pkcε siRNAs*), or control siRNAs (*3d ctrl siRNA*). All transfections were performed 2 days after treatment with 5-AZ and cells were processed after 24 h. *Arrowhead* $p = 0.01$, *black ball* $p = 0.04$, $*p = 0.035$

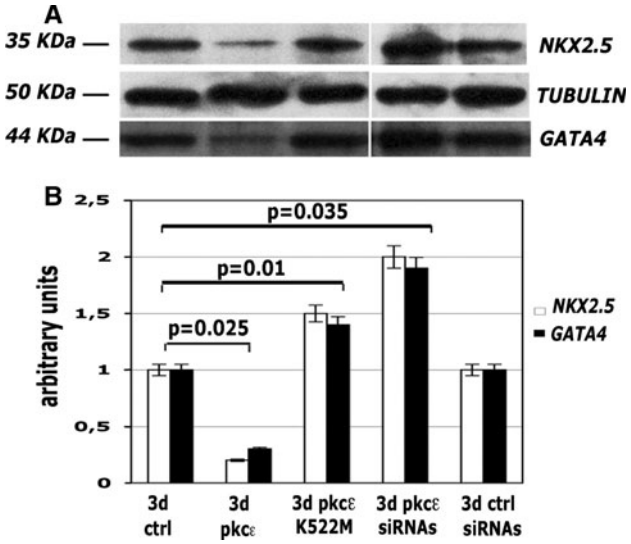


Fig. 6 Effect of PKCε over-expression and silencing on *nkx2.5* and *gata4* protein in rBMMSCs. **a** Western blot for NKX2.5 GATA4 and TUBULIN as housekeeping in in rBMMSCs treated with 5-AZ (*3d ctrl*), transfected with PKCε (*3d pkcε*) or PKCε K522M (*3d pkcε K522M*), or *pkcε* siRNAs (*3d pkcε siRNAs*), or control siRNAs (*3d ctrl siRNA*). **b** Quantitative analysis of band intensities for NKX2.5 and GATA4 expressed as arbitrary units with respect to control (*3d ctrl*) after normalization with the TUBULIN of the same sample

signaling pathways (Lincoln et al. 2006; Lopez-Sanchez et al. 2002), there is still a paucity of information regarding the driving mechanisms of cardiomyogenesis (Adam Young et al. 2011). In particular, the signal transduction pathways involved in the activation of cardiac transcription factors like NKX2.5, GATA4, TBX5 (Bruneau et al. 2001; Snyder et al. 2010) are only partially known.

On the other side, although the importance of PKCε in cardio-protection is well established, its role on cardiac cell differentiation has not yet been unraveled. In this perspective, we decided to study the role of PKCε on early cardiac gene expression using both in vivo and in vitro models.

Firstly, data obtained in vivo pointed at a negative role of PKCε over-expression on cardiac gene regulation. Of note, the wt isoform of PKCε determined a significant decrease in *nkx2.5* and *gata4* expression in hearts together with an increase of phospho-ERK1/2, suggesting that PKCε imbalance could act as a key event of early cardiac gene expression. Interestingly, a late differentiation marker gene like *myosin heavy chain* did not seem to be affected by PKCε over-expression. One hypothesis could be that PKCε acts in different manner on early and late cardiac genes. In fact, in PKCε over-expressing mice, there is hypertrophy and higher content of myosin respect to wt.

We recapitulated in vitro cardiac cell differentiation using 5-AZ-treated BMMSCs cultures. We found that *pkcε* transcription has a kinetic of expression with a peak at day

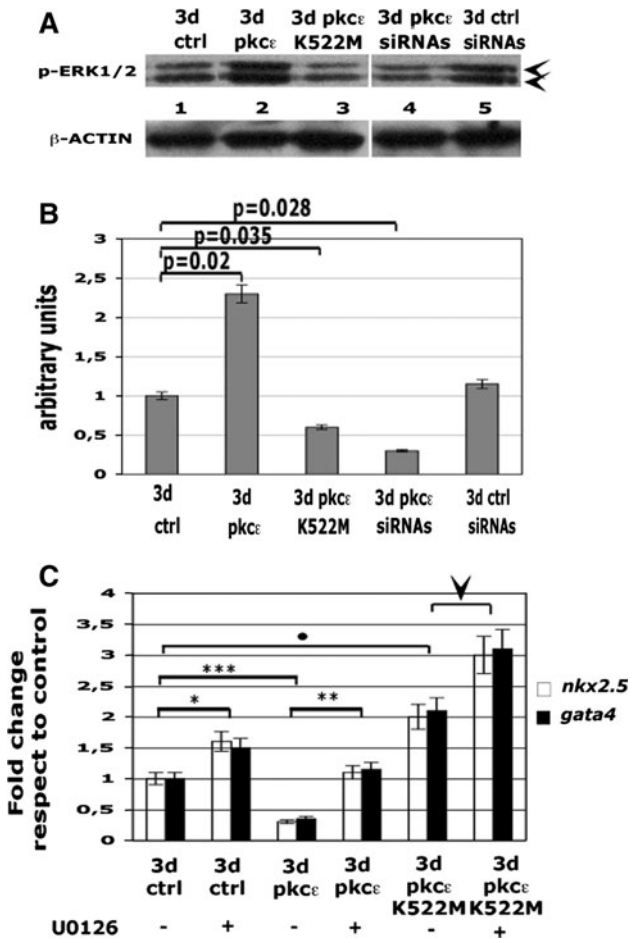


Fig. 7 Phospho-ERK1/2 in BMMSCs over-expressing PKCε. **a** p-ERK1/2 Western blot in control cells (*3d ctrl*, *lane 1*), PKCε over-expressing cells (*3d pkcε*, *lane 2*), mutated PKCε over-expressing cells (*3d pkcε K522M*, *lane 3*), pkcε silenced cells (*3d pkcε siRNAs*, *lane 4*) and cells transfected with control siRNAs (*3d ctrl siRNAs*, *lane 5*). β-ACTIN was used for normalization. **b** densitometric analysis of p-ERK1/2 bands expressed as arbitrary units with respect to control (*3d ctrl*) after normalization with the β-ACTIN of the same sample. **c** *nkx 2.5* (*empty bar*) and *gata4* (*black bar*) expression levels (qRT-PCR) in controls (*3d ctrl*), controls treated with U0126 (*3d ctrl + U0126*), pkcε transfected (*3d pkcε*), pkcε transfected and treated with U0126 (*pkcε + U0126*), pkcε K522M transfected (*pkcε K522M*) and *pkcε* transfected and treated with U0126 (*pkcε K522M + U0126*). $*p = 0.05$, $**p = 0.01$, $***p = 0.03$, *black ball* $p = 0.02$, *arrowhead* $p = 0.04$

2 and a minimum at day 7. Notably, cardiac transcription factors *nkx2.5* and *gata4* show opposite kinetics with respect to *pkcε*, suggesting a negative regulatory role for PKCε on the transcription of these genes.

While over-expression of PKCε in cardiac-induced BMMSCs produced a strong decrease of *nkx2.5* and *gata4* levels, the opposite experiments, designed to knock-out PKCε expression by specific siRNAs induced a significant increase of both *nkx2.5* and *gata4* expression, confirming a negative role of PKCε on these genes. Transfection with a plasmid encoding the K522M mutated isoform of PKCε determined a significant increase in *nkx2.5* and *gata4* mRNA expression. Although dominant negative effects of this mutated isoform have not been described so far, it must be said that other phosphorylation sites in the same region of PKCε are known to generate dominant negative effects when mutated (Garcia-Paramio et al. 1998; Moschella et al. 2007).

It is note worthy that the same transfection experiments done at day 7 after 5-AZ treatment did not show any of the described effects, instead killing the cells, likely due to the irreversible addressing to differentiation.

Both in PKCε over-expressing cells and in PKCε silenced cells, Tbx5 expression was not affected (data not shown) suggesting that this gene could be regulated by other pathways not involving PKCε.

Current notion is that PKCε induces ventricular hypertrophy through stimulation of cell proliferation (Duquesnes et al. 2011), in fact PKCε mainly regulates proliferation (through ERK1/2 pathway) or apoptosis (through indirect caspase activation) (Basu and Sivaprasad 2007). Since ERK1/2 has a negative role on *nkx2.5* expression in cardiomyocytes (Marni et al. 2009), we wondered if PKCε could also act on differentiation option of the cells. Thus, we chose ERK1/2 as reasonable molecular intermediates between *gata4* and *nkx2.5* and PKCε. First, in vivo results suggested that phospho-ERK1/2 levels increased when PKCε was over-expressed. In vitro experiments evidenced that phospho-ERK1/2 was increased when PKCε was over-expressed and decreased when PKCε was silenced. In control and in PKCε over-expressing cells treated with U0126 (that completely abolished ERK1/2 phosphorylation), we observed a significant increase of *nkx2.5* and *gata4*, confirming the negative role of phospho-ERK1/2 in cardiac differentiation. These results may appear in contrast with others (Fukuda 2003; Kim et al. 2007; Qian et al. 2012), which, however, used different cellular models (ES cells) and/or explored completely different time frames. On another side, our results are in line with those obtained in vivo in mouse embryos where inhibition of FGF/ERK signaling is necessary and sufficient for cardiac differentiation of anterior heart field progenitors (Tirosh-Finkel et al. 2010).

Proteomic analysis has shown that PKCε is associated with at least 36 proteins including p38 MAPKs, ERKs, JNKs, AKT (Duquesnes et al. 2011). While ERKs seem to have a role also in differentiation, the others have been demonstrated to act in cardio-protection through regulation of apoptosis and stress resistance. Interestingly, in *pkcε* null mice (Klein et al. 2005), p38 MAPKs, JNKs and AKT activation seem not to be affected in the heart, while ERKs activation was attenuated. We think that these findings can strongly support our results.

As others, we used BMMSCs exclusively for in vitro experiments, since it is known that their cardiac differentiation efficiency is very low in vivo (Chong et al. 2011). However, they represent a good model for in vitro experiments (Orlic et al. 2003; Huang et al. 2010), as they share common properties with pericardial mesenchymal cells, probably originating from a common progenitor that lost its in vivo cardiac potential upon localization in the bone marrow.

Finally, we here evidenced that highly controlled levels of PKCε are important for early cardiac genes paralleling the notion that PKCε likely plays a role in the differentiation fate of most mesoderm-derived stem cells.

Acknowledgments We thank Cristina Micheloni, Luciana Cerasuolo, Vincenzo Palermo and Davide Dallatana for technical help. This work was supported by Programma di ricerca Regione Emilia-Romagna-Università (ER-Università) 2007–2009 *Therapeutic application of stem cells in the treatment of heart failure* and Finanziamento Italiano per la Ricerca di Base (FIRB) RBAP10KCNS_002.

Conflict of interest None.

References

Adam Young D, Dequach JA, Christman KL (2011) Human cardiomyogenesis and the need for systems biology analysis. Wiley Interdiscip Rev Syst Biol Med 3:666–680

Basu A, Sivaprasad U (2007) Protein Kinase Cepsilon makes the life and death decision. Cell Signal 19:1633–1642

Bruneau BG, Nemer G, Schmitt JP, Charron F, Robitaille L, Caron S, Conner DA, Gessler M, Nemer M, Seidman CE, Seidman JG (2001) A murine model of Holt–Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. Cell 106:709–721

Budas GR, Churchill EN, Disatnik MH, Sun L, Mochly-Rosen D (2010) Mitochondrial import of PKCepsilon is mediated by HSP90: a role in cardioprotection from ischaemia and reperfusion injury. Cardiovasc Res 88:83–92

Chong JJ, Chandrakanthan V, Xaymardan M, Asli NS, Li J, Ahmed I, Heffernan C, Menon MK, Scarlett CJ, Rashidianfar A, Biben C, Zoellner H, Colvin EK, Pimanda JE, Biankin AV, Zhou B, Pu WT, Prall OW, Harvey RP (2011) Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell 9:527–540

De Luca A, Sargiacomo M, Puca A, Sgaramella G, De Paolis P, Frati G, Morisco C, Trimarco B, Volpe M, Condorelli G (2000) Characterization of caveolae from rat heart: localization of postreceptor signal transduction molecules and their rearrangement after norepinephrine stimulation. J Cell Biochem 77:529–539

Duquesnes N, Lezoualc'h F, Crozatier B (2011) PKC-delta and PKC-epsilon: foes of the same family or strangers? J Mol Cell Cardiol 51:665–673

Fukuda K (2003) Regeneration of cardiomyocytes from bone marrow: use of mesenchymal stem cell for cardiovascular tissue engineering. Cytotechnology 41:165–175

Galli D, Benedetti L, Bongio M, Maliardi V, Silvani G, Ceccarelli G, Ronzoni F, Conte S, Benazzo F, Graziano A, Papaccio G, Sampaolesi M, De Angelis MG (2011) In vitro osteoblastic differentiation of human mesenchymal stem cells and human dental pulp stem cells on poly-L-lysine-treated titanium-6-aluminium-4-vanadium. J Biomed Mater Res A 97:118–126

Gao LR, Zhang NK, Bai J, Ding QA, Wang ZG, Zhu ZM, Fei YX, Yang Y, Xu RY, Chen Y (2010) The apelin-APJ pathway exists in cardiomyogenic cells derived from mesenchymal stem cells in vitro and in vivo. Cell Transplant 19:949–958

Garcia-Paramio P, Cabrerizo Y, Bornancin F, Parker PJ (1998) The broad specificity of dominant inhibitory protein kinase C mutants infers a common step in phosphorylation. Biochem J 333:631–636

Gobbi G, Mirandola P, Sponzilli I, Micheloni C, Malinverno C, Cocco L, Vitale M (2007) Timing and expression level of protein kinase C epsilon regulate the megakaryocytic differentiation of human CD34 cells. Stem Cells 25:2322–2329

Gobbi G, Marcantonio DD, Micheloni C, Carubbi C, Galli D, Vaccarezza M, Bucci G, Vitale M, Mirandola P (2012) Trail up-regulation must be accompanied by a reciprocal PKCε down-regulation during differentiation of colonic epithelial cell: implications for colorectal cancer cell differentiation. J Cell Physiol 227:630–638

Goldspink PH, Montgomery DE, Walker LA, Urboniene D, McKinney RD, Geenen DL, Solaro RJ, Buttrick PM (2004) Protein kinase Cepsilon overexpression alters myofilament properties and composition during the progression of heart failure. Circ Res 95:424–432

Gussoni E, Soneoka Y, Strickland CD, Buzney EA, Khan MK, Flint AF, Kunkel LM, Mulligan RC (1999) Dystrophin expression in the mdx mouse restored by stem cell transplantation. Nature 401:390–394

Huang XP, Sun Z, Miyagi Y, McDonald Kinkaid H, Zhang L, Weisel RD, Li RK (2010) Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. Circulation 122:2419–2429

Ivaska J, Vuoriluoto K, Huovinen T, Izawa I, Inagaki M, Parker PJ (2005) PKCepsilon-mediated phosphorylation of vimentin controls integrin recycling and motility. EMBO J 24:3834–3845

Joggerst SJ, Hatzopoulos AK (2009) Stem cell therapy for cardiac repair: benefits and barriers. Expert Rev Mol Med 11:e20

Kim HS, Cho JW, Hidaka K, Morisaki T (2007) Activation of MEK-ERK by heregulin-beta1 promotes the development of cardiomyocytes derived from ES cells. Biochem Biophys Res Commun 361:732–738

Klein G, Schaefer A, Hilfiker-Kleiner D, Oppermann D, Shukla P, Quint A, Podewski E, Hilfiker A, Schröder F, Leitges M, Drexler H (2005) Increased collagen deposition and diastolic dysfunction but preserved myocardial hypertrophy after pressure overload in mice lacking PKCepsilon. Circ Res 96:748–755

Lincoln J, Alfieri CM, Yutzey KE (2006) BMP and FGF regulatory pathways control cell lineage diversification of heart valve precursor cells. Dev Biol 292:292–302

Lopez-Sanchez C, Climent V, Schoenwolf GC, Alvarez IS, Garcia-Martinez V (2002) Induction of cardiogenesis by Hensen's node and fibroblast growth factors. Cell Tissue Res 309:237–249

Marni F, Wang Y, Morishima M, Shimaoka T, Uchino T, Zheng M, Kaku T, Ono K (2009) 17β-Estradiol modulates expression of low-voltage-activated $Ca_v3.2$ T-Type calcium channel via extracellularly regulated kinase pathway in cardiomyocytes. Endocrinology 150:879–888

Mauritz C, Schwanke K, Reppel M, Neef S, Katsirntaki K, Maier LS, Nguemo F, Menke S, Haustein M, Hescheler J, Hasenfuss G, Martin U (2008) Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation 118:507–517

Meyer KD, Zhang H, Zhang L (2009) Prenatal cocaine exposure abolished ischemic preconditioning-induced protection in adult

male rat hearts: role of PKCepsilon. Am J Physiol Heart Circ Physiol 296:H1566–H1576

Mirandola P, Gobbi G, Ponti C, Sponzilli I, Cocco L, Vitale M (2006) PKCepsilon controls protection against TRAIL in erythroid progenitors. Blood 107:508–513

Mochly-Rosen D, Wu G, Hahn H, Osinska H, Liron T, Lorenz JN, Yatani A, Robbins J, Dorn GW 2nd (2000) Cardiotrophic effects of protein kinase C epsilon: analysis by in vivo modulation of PKCepsilon translocation. Circ Res 86:1173–1179

Moschella PC, Rao VU, McDermott PJ, Kuppuswamy D (2007) Regulation of mTOR and S6K1 activation by the nPKC isoforms, PKCepsilon and PKCdelta, in adult cardiac muscle cells. J Mol Cell Cardiol 43:754–766

Orlic D, Kajstura J, Chimenti S, Bodine DM, Leri A, Anversa P (2003) Bone marrow stem cells regenerate infarcted myocardium. Pediatr Transplant 7(Suppl 3):86–88

Qian Q, Qian H, Zhang X, Zhu W, Yan Y, Ye S, Peng X, Li W, Xu Z, Sun L, Xu W (2012) 5-Azacytidine induces cardiac differentiation of human umbilical cord-derived mesenchymal stem cells by activating extracellular regulated kinase. Stem Cells Dev 21:67–75

Redig AJ, Platanias LC (2007) The protein kinase C (PKC) family of proteins in cytokine signaling in hematopoiesis. J Interferon Cytokine Res 27:623–636 Review

Saurin AT, Durgan J, Cameron AJ, Faisal A, Marber MS, Parker PJ (2008) The regulated assembly of a PKCepsilon complex controls the completion of cytokinesis. Nat Cell Biol 10:891–901

Snyder M, Huang XY, Zhang JJ (2010) Stat3 directly controls the expression of Tbx5, Nkx2.5, and GATA4 and is essential for cardiomyocyte differentiation of P19CL6 cells. J Biol Chem 285:23639–23646

Sperling SR (2011) Systems biology approaches to heart development and congenital heart disease. Cardiovasc Res 91:269–278

Takeishi Y, Ping P, Bolli R, Kirkpatrick DL, Hoit BD, Walsh RA (2000) Transgenic overexpression of constitutively active protein kinase Cε causes concentric cardiac hypertrophy. Circ Res 86:1218–1223

Tirosh-Finkel L, Zeisel A, Brodt-Ivenshitz M, Shamai A, Yao Z, Seger R, Domany E, Tzahor E (2010) BMP-mediated inhibition of FGF signaling promotes cardiomyocyte differentiation of anterior heart field progenitors. Development 137:2989–3000

Wakitani S, Saito T, Caplan AI (1995) Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve 18:1417–1426