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pPKCδ activates SC35 splicing factor during H9c2 myoblastic differentiation

Susi Zara[1], Mirella Falconi[2], Monica Rapino[3], Michela Zago[2], Giovanna Orsini[4], Giovanni Mazzotti[5], Amelia Cataldi[6] and Gabriella Teti[5]

[1]Department of Drug Sciences, Section of Human Anatomy, Faculty of Pharmacy, University "G. d' Annunzio", Chieti-Pescara, [2]Department of Anatomic Sciences, Scientific Pole of Rimini, Alma Mater Studiorum, University of Bologna, [3]Institute of Molecular Genetics CNR, Unit of Chieti, [4]Department of Restorative Dentistry, Institute of Stomatology, University of Marche, Ancona, [5]Department of Anatomic Sciences, Alma Mater Studiorum, University of Bologna and [6]Department Medicine and Ageing Sciences, Section of Human Anatomy, Faculty of Pharmacy, University "G. d' Annunzio", Chieti-Pescara, Italy

Summary. Although Protein Kinase C (PKC) isoforms' role in the neonatal and adult cardiac tissue development and ageing has been widely described "in vivo", the interaction of such enzymes with specific nuclear substrates needs to be investigated.

The aim of our research has been the study of the expression, localization and interaction with the splicing factor SC35 of PKC isoforms (α, δ, ε, ζ) and their potential role in modulating the transcription machinery.

H9c2 cells induced to myoblast differentiation in the presence of 1% Horse Serum (HS) have represented our experimental model. The expression of PKC isoforms, their distribution and interaction with SC35 have been evaluated by western blotting, co-immunoprecipitation and double gold immunolabeling for transmission and scanning electron microscopy.

Our results show PKCδ as the most expressed isoform in differentiated cells. Surprisingly, the distribution of PKCδ and SC35 does not show any significant modification between 10%FBS and 1%HS treated samples and no co-localization is observed. Moreover the interaction between the phosphorylated form of PKCδ (pPKCδ) and SC35 increases, is distributed and co-localizes within the nucleus of differentiated H9c2.

These data represent reasonable evidence of pPKCδ mediated SC35 splicing factor activation, suggesting its direct effect on transcription via interaction with the transcription machinery. Furthermore, this co-localization represents a crucial event resulting in downstream changes in transcription of components which determine the morphological modifications related to cardiomyoblast differentiated phenotype.

Key words: PKCδ, pPKCδ, SC35, Myoblast differentiation

Offprint requests to: Susi Zara, Section of Human Anatomy, Faculty of Pharmacy, University "G. d' Annunzio", Chieti-Pescara, Italy. e-mail: s.zara@unich.it

Introduction

Cardiomyoblast differentiation is a multistep process which implies both molecular and morphological modifications (Sabourin and Rudnicki, 2000). Various cell models are available to investigate the events involved in this process (Brunskill et al., 2001; van der Heyden and Defize 2003; Puceat, 2005). In particular, H9c2 cells are rat embryonic myoblasts with skeletal muscle properties which terminally differentiate by fusing and forming multinucleated myotubes (Brandt and Kimes, 1976). These cells, showing electro-physiological and biochemical properties of cardiac and skeletal muscle, represent an "in vitro" model of both tissues and are ideal to investigate late differentiating events. H9c2 have been used as a cardiac muscle cell model in several biochemical and pathophysiological studies, such as cardiac hypertrophy (Merten and Jiang, 2007), ischemia/reperfusion induced injury (Khaw et al., 1995), response to oxidative stress (Wu et al., 1996), and differentiation (Hong et al., 2001; Pagano and Naviglio 2004).

In the presence of 10% Foetal Bovine Serum (FBS), H9c2 cell line maintains a proliferative state. However, by changing serum from bovine to horse one (HS) and reducing its concentration (1%), proliferation is lowered

while cardiac differentiation is triggered (Ménard et al., 1999).

Cell differentiation is induced by cytoplasmic signalling molecules. In the context of cell membrane receptors and cytoplasmic signalling to the nucleus, pathways are described as cascades of reactions involving kinases, phosphatases, and adaptor proteins controlling, in turn, the functioning of nuclear complexes (Bollen and Beullens, 2002). Among the molecules involved in the phosphorylation processes a role has been assigned to Protein Kinases C (PKC) family (Steinberg, 2008), a multigenic family of protein kinases, composed of three subclasses, classified on the basis of their structure and mode of activation: conventional or classical, novel and atypical PKC (Kazanietz, 2005). Classical (α1, ß1 and γ2) respond to the activation by phorbol esters, phorbol 12,13-dibutyrate or phorbol-12-myristate-13-acetate, phosphatidilserine (PS), diacylglycerol, Ca^{2+}; novel (δ, ε, θ, μ) are PS and diacylglycerol dependent, atypical (ζ, λ, ι) are PS or phosphatidylinositol 3,4,5 triposphate (PI3,4,5P3) dependent (Nishizuka, 1992; Meier et al., 2009).

In cardiac tissue PKCs appear to modulate not only cell differentiation, but also ion transport, contractility, cell proliferation, hypertrophy, and apoptosis (Puceat and Vassort, 1996; Sabri and Steinberg, 2003). In particular, in the rat heart the expression of different PKC isoforms, such as PKCα, PKCδ, PKCε, PKCζ depending on the age of the animals (Cataldi et al., 2009) and during postnatal development (Hamplova et al., 2009; Zara et al., 2009), has been demonstrated. Activation of PKC is associated with the translocation of each isoform from the soluble compartment, where PKC is inactive, to the particulate compartment, where it becomes active (Battaini and Pascale, 2005) and activates specific nuclear domains (Kanashiro and Khalil, 1998) which contain splicing factors.

Splicing Factors are nuclear proteins involved in removing introns (non- coding sequences in the genes) in order to form mature RNA which will be transported to the cytoplasm. Nuclear transcription factor SC35 is associated with multiple active genes and their transcripts (Smith et al., 1999; Moen et al., 2004) and, as elsewhere reported (Bregman et al., 1995), required for the first step of splicing and for the assembly of the earliest detectable ATP- dependent pre-spliceosome complex.

Since molecular modifications, involving the phosphorylated form of Protein Kinase C (pPKC)/SC35 interaction has been demonstrated "in vivo" in the transition from embryonal/fetal to adult cardiomyocytes (Zara et al., 2009) and during ageing (Cataldi et al., 2009), attention has been payed to the possible interactions occurring between different Protein Kinase C (PKC) isoforms (α, δ, ε, ζ) and SC35 nuclear transcription factor during "in vitro" H9c2 differentiation.

Materials and methods

Cell culture and differentiation

H9c2 cells, rat DB1X heart myoblasts, derived from embryonic ventricle (ECACC, Porton Down, Salisbury, UK) were chosen because they represent an "in vitro" model of cardiac and skeletal muscle cells. H9c2 were seeded at a density of $6x10^5$ in a 75 cm^2 flask and cultured at 37°C in a 5% CO_2 humified atmosphere in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FBS. When, after 3-4 days, 70-80% of the cells reached a subconfluence state, they were seeded and cultured for six days in DMEM supplemented with 1% HS, as elsewhere reported (Chun et al., 2000), for the differentiation experiments and photographed in phase contrast. Each parameter was assessed by three different consistent experiments.

Western blotting analysis and Immunoprecipitation

For immunoprecipitation anti phospho Protein Kinase Cδ (pPKCδ) goat polyclonal primary antibody (Santa Cruz, Santa Cruz Biotechnology, CA, USA), was incubated in the presence of 50 μl of the suspended IP matrix (Exacta CRUZ, Santa Cruz Biotechnology Inc, Santa Cruz, California, USA) for 5h at 4°C in 500 μl of PBS. Antibody-IP matrix were pelleted for 30 sec at 4°C and antibody-IP matrix complex was incubated with whole lysates overnight at 4°C on a rotator. After incubation the obtained complex was pelleted for 30 sec at 4°C and washed in RIPA buffer (1X PBS, 1% Igepal CA-630, 0.5% sodium deoxycolate, 0.1% SDS) three times. Samples were stored at -80°C.

Total cell lysates (20 μg) were obtained after centrifugation of the cell suspension at 1200xg and two washes in ice-cold PBS. Pellets were resuspended in RIPA buffer with freshly added inhibitors (PMSF 100 μg/ml, Aprotinin 10 μg/ml, Leupeptin 50 μg/ml and Sodium Ortovanadate 1 mM) and incubated on ice for 30 minutes. The extraction mixture was sonicated three times for 20 sec with 40% amplitude by using a sonicator, incubated with PMSF 100 μg/ml for 30 minutes at 4°C and then centrifuged at 10000xg for 15 minutes at 4°C. Total cell lysates or immunoprecipitates were subjected to electrophoresis on a 10% (SDS)-polyacrylamide gel and transferred to nitrocellulose membrane. Nitrocellulose membranes, blocked in 5% non-fat milk, 10 mmol/l Tris-HCl pH 7.5, 100 mmol/l NaCl, 0.1% (v/v) Tween-20, were probed with mouse monoclonal myogenin, PKCε and PKCα, goat polyclonal PKCζ and pPKCδ (Thr-507), rabbit polyclonal cyclin A and PKCδ antibodies (Santa Cruz, Santa Cruz Biotechnology, CA, USA), and mouse monoclonal SC35 antibody and then incubated in the presence of specific enzyme conjugated IgG horseradish

peroxidase. Samples were normalized by incubating the membranes in the presence of mouseß tubulin monoclonal antibody. Immuno-reactive bands were detected by Super Signal West Dura Extended Duration Substrate (Thermo Scientific Rockford, IL, USA). Densitometric values, expressed as Integrated Optical Intensity (IOI) were estimated in a CHEMIDOC XRS System with the QuantityOne I-D analysis software (Biorad Laboratories Inc., Hercules, CA, USA).

Statistics

Data were analysed using the two-tailed, two-sample t-test (Minitab, statistical analysis software, State College, PA). Results were expressed as mean ±SD. Values of $p<0.05$ were considered significant.

Transmission Electron Microscopy (TEM)

H9C2 cells were seeded in monolayer in a 6 multi well-plate and they were induced to myoblastic differentiation as previously described. To analyse cell morphology samples were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.6 for 1 h at 4°C. After washing in the same buffer, samples were post-fixed in 1% OsO_4 in phosphate buffer for 1h at 4°C, acetone dehydrated and Epon embedded followed by polymerization for three days at 60°C.

To analyse SC35, PKCδ, pPKCδ and actin expression and localization, cell pellets were fixed in 4% paraformaldehyde/0.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.6 for 1h at 4°C, dehydrated in alcohol (progressively higher concentrations), and embedded in London White resin followed by polymerization for three days at 40°C, according to standard procedures. Ultrathin sections were cut and mounted on 300 mesh nickel grids.

In order to detect SC35 and PKCδ or pPKCδ, double gold immunolabelings on opposite sides of ultrathin sections were performed. Briefly, the grids were blocked 20% Normal Goat Serum (NGS) or normal rabbit serum (NRS) for the localization of pPKCδ diluted in Tris buffered saline (TBS) (NaCl 0.15 mol/L, Tris-Base 0.05 mol/L), pH 7.6, to block non-specific binding sites for 30 minutes. Then, the samples were incubated overnight in presence of anti-SC35 and anti-actin mouse monoclonal antibodies (antibodies concentration 1:5) (Sigma, USA), anti-PKCδ rabbit polyclonal antibody (Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted 1:25, and goat pPKCδ polyclonal antibody (antibody concentration 1:10) (Santa Cruz, Santa Cruz Biotechnology, CA, USA). After several washes, the grids were incubated with the specific gold conjugated secondary antibody (secondary antibody concentration 1:10) (Biocell, Cardiff, UK): 15 nm colloidal gold particles for the goat anti SC35, 30 nm colloidal gold particles for rabbit anti pPKCδ, 30 nm gold particles for PKCδ, and 20 nm colloidal gold particles for goat anti actin. Uranyl acetate and lead citrate counterstained grids were observed using transmission electron microscopy Philips CM10 (FEI company, the Netherlands).

Field Emission in-lens-Scanning Electron Microscopy (FEI-SEM) gold immunolabeling

H9c2 cells were grown and differentiated as previously described. Control and differentiated samples were collected after trypsin treatment and centrifuged at 1000 rpm for 10 minutes. Pellets were fixed in 4% paraformaldehyde/0,5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.6 for 1h at 4°C, briefly washed in 0.1 M phosphate buffer, cryopreserved with 2.3 M sucrose in 0.1 M phosphate buffer for 3 hr at 4°C, and finally mounted on a cryoultramicrotome specimen holder and frozen in liquid nitrogen. Frozen cell pellets were cryosectioned in a Reichert JUNG FC 4/E (Leica; Wien, Austria) cryoultramicrotome and sections of 100-120 nm were mounted on silicon chips that were utilized as a specimen support.

All samples were briefly washed in phosphate buffer and then in TBS 0.05 M, ph 7.6 for 5 minutes. They were subsequently blocked in Normal Goat Serum diluted 1:20 in TBS (blocking buffer) for 30 minutes and incubated over night with mouse splicing factor SC35 antibody, diluted 1:5 in blocking buffer (Sigma-Aldrich, St. Louis, Missouri, USA), and rabbit PKCδ polyclonal antibody (Santa Cruz, Santa Cruz Biotechnology, CA, USA), 1:10 diluted in blocking buffer. After several washes in TBS buffer the samples were incubated respectively with 15 nm gold anti mouse IgG conjugated antibody and 30 nm gold anti rabbit IgG conjugated antibody for 1h and 30 minutes at room temperature (British Biocell, Cardiff, UK). After several washes, samples were post fixed with a solution of 1% glutaraldehyde in 0.1 M phosphate buffer for 30 minutes followed by a fixation in 1% osmium tetroxide in 0.1 M phosphate buffer for 30 min. After being washed in PBS, they were dehydrated in an increasing ethanol series and critical point dried (Critical point dryer CPD 030, Bal-Tec AG, Lichtenstein). The analysis was carried out on uncoated samples with a FEI-SEM Jeol JSM 890 (Jeol, Tokyo, Japan) at 7 kV accelerating voltage and $1x10^{-11}$ A probe current.

siRNA transfection and immunofluorescence

All transfections of H9c2 cells were performed with a Nucleofector device and corresponding kit (Amaxa. Inc., Cologne, Germany) according to the manufacturer's instructions. First, we optimized the transfection protocol for H9c2 cells and PKCδ silencing by assaying different siRNA concentrations. In brief, cells were transfected through a Nucleofector program (C-020) and two different siRNA concentrations (1 and 2 μg); the condition that resulted in the highest transfection efficiency (up to 51.1% of viable cells) with the lowest cytotoxicity (up to the 40% of the cells) was

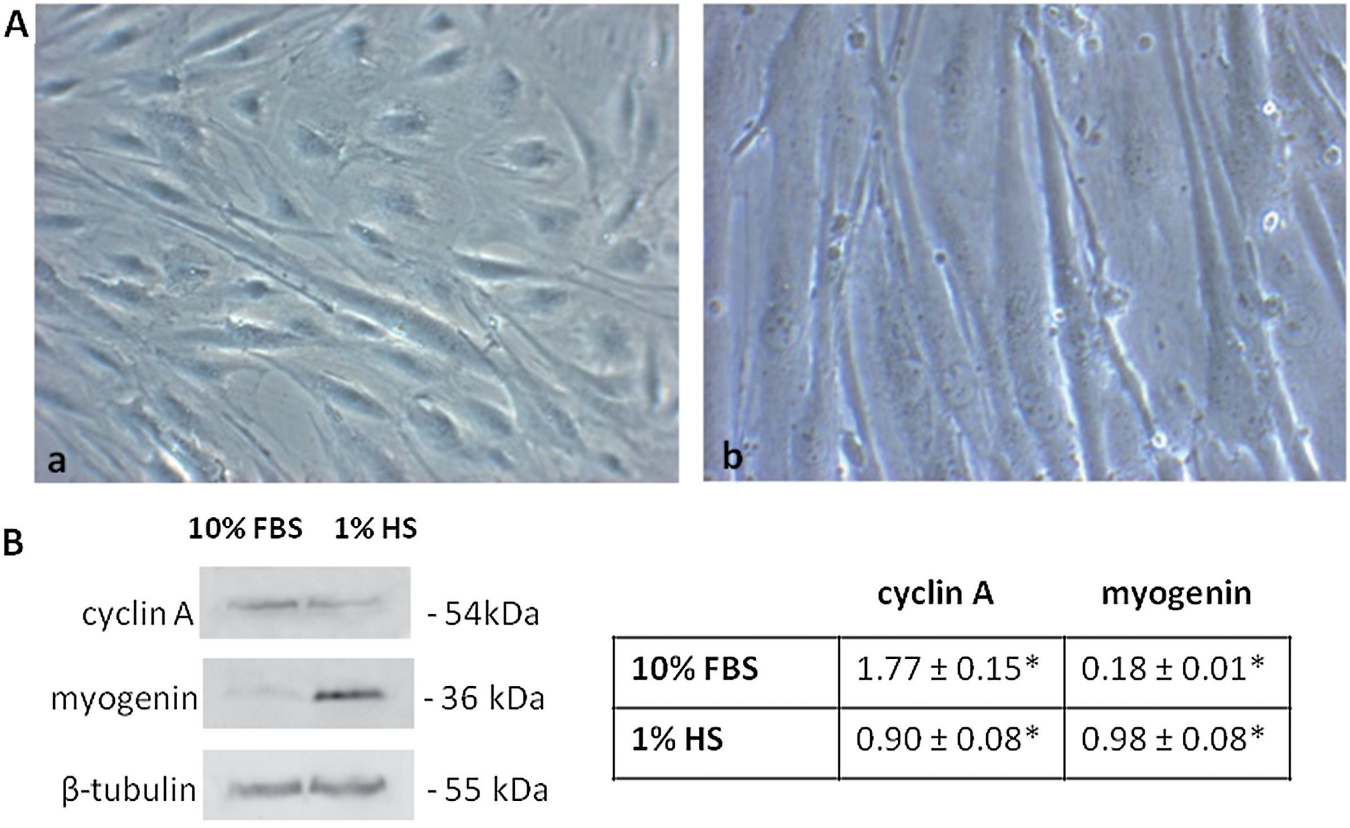
selected. Cells ($4x10^5$) were harvested, transfected with Nucleofector solution L and 2 μg of PKC-δ siRNA (Dharmafect, Lafayette, CO) and reseeded on a glass in a multiwell plate.

Control cells were harvested and fixed when reached sub-confluency (72 hr after transfection), while at this time interval pools of control and PKCδ silenced cells were seeded in 1% HS to reach differentiation within 6 days. In parallel, control cells were transfected with a GFP expression vector (pmax GFP, Amaxa, Inc., Cologne, Germany) and collected 24 hr later to quantify transfection efficiency.

Cells cultured on coverslips were fixed in 4% paraformaldehyde for 10 min, and washed in Phosphate Buffer Saline (PBS). For myogenin and PKCδ immune labeling, cells were incubated in 5% donkey serum in PBS for 20 min at room temperature followed by a 45 min incubation in the presence of 5 μg/ml mouse monoclonal myogenin or rabbit polyclonal PKCδ antibodies (Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted in PBS, 5% Tween-20, 2% BSA (Bovine Serum Albumin) for 1 hr at 37°C. Slides were washed in PBS and reacted for 45 min with FITC (fluorescein - isothiocyanate) -conjugated anti-mouse or anti-rabbit IgG (immunoglobulin) antibodies (Boheringer Mannheim, Germany) diluted 1:50 in PBS, 5% Tween-20, 2% BSA for 45 min at 37°C. After several washes in PBS, slides were mounted in glycerol-DABCO (1-4-Diazabicyclo[2-2-2]octane) containing 5 μg/ml DAPI (4-6,diamidino-2-phenyl-indol) to counterstain nuclei. Internal controls, performed omitting the primary antibody, did not disclose any FITC staining. The labelled slides were examined with light microscope Leica DM 4000 (Leica Cambridge Ltd, Cambridge, UK) equipped with a Leica DFC 320 videocamera (Leica Cambridge Ltd, Cambridge, UK) to acquire computerized images.

Computerized morphometry measurements and image analysis

After digitizing the images deriving from immunofluorecence, Leica Qwin 3.5 Plus Software System (Leica Cambridge Ltd, Cambridge, UK) was



	cyclin A	myogenin
10% FBS	1.77 ± 0.15*	0.18 ± 0.01*
1% HS	0.90 ± 0.08*	0.98 ± 0.08*

Fig. 1. Morphological and molecular modifications in 10% FBS cells and 1%HS cells. **A.** Phase contrast microscopy analysis of H9c2 cells; note thin and elongated 1% HS cells **(b)**, compared to round 10% FBS shaped cells **(a)**. x 40 **B.** Western blotting analysis of cyclin A and myogenin expression. Each membrane has been probed with anti ß-tubulin antibody to verify loading evenness. The most representative out of three separate experiments is shown. Data in the table are the densitometric measurement of protein bands expressed as mean ± SD of three separate experiments; * 1% HS vs 10% FBS cyclin $p<0,05$; 1% HS vs 10% FBS myogenin $p<0,05$.

used to evaluate myogenin and PKCδ expression. Image analysis of protein expression was performed through the quantification of the number of positive nuclei per field of light microscope observation.

Leica Qwin assessments were logged to Microsoft Excel and processed for Percentage, Standard Deviations and Histograms.

Results

H9c2 cells are rat embryonic myoblasts with skeletal muscle properties which reach differentiation when cultured for 6 days in HS low concentration (1%). Their terminal differentiation consists of fusing and forming multinucleated myotubes (Andrés and Walsh, 1996) (Fig. 1A). The modified synthesis of specific proteins accompanies such morphological events: in particular, the expression of cyclin A, required cell cycle regulator for the onset of DNA replication, decreases, and the expression of myogenin, marker of skeletal muscle differentiation, increases (Fig. 1B).

At ultrastructural level the organization of the cells

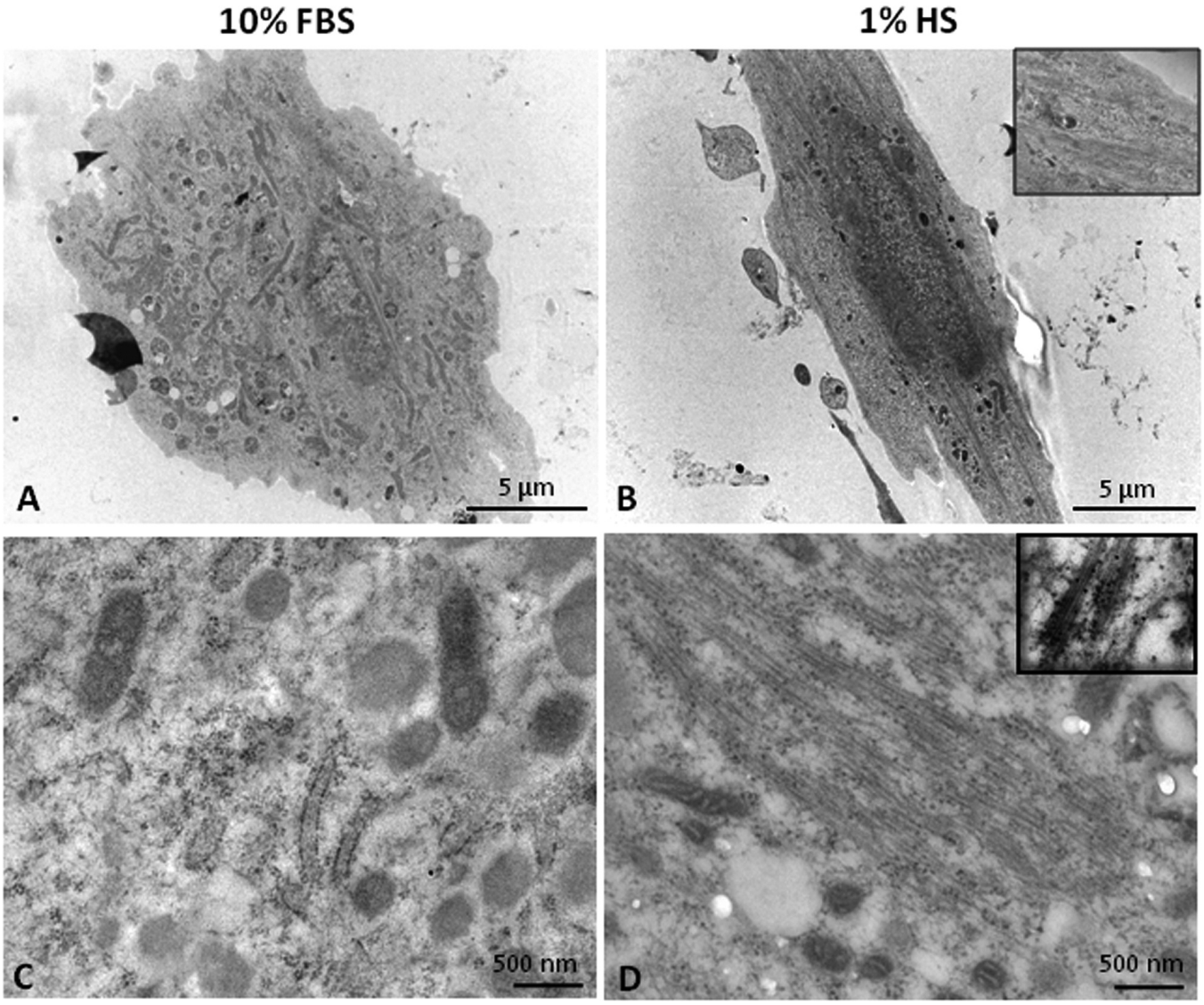


Fig. 2. A-B. Ultramorphological analysis of 10% FBS and 1% HS H9c2 cells respectively. Differentiated cells show a thin and elongated shape compared to the round shape of control cells. Inset in figure B shows a large number of actin filaments demonstrating a terminal cardiomyoblast differentiation. **C-D.** TEM immunogold labeling of actin in 10% FBS cells and 1% HS respectively. Inset shows gold granules on actin filaments in 1% HS cells **(D)**. No gold granules labeling actin filaments are observed in 10%FBS H9c2 cells **(C)**. Inset B, x 25000; Inset D, x 34000

undergoes modification in terms of morphological features: 1% HS cells appear thin and elongated (Fig 2B), often fused to form multinucleated syncytia (myotubes) and well organized actin filaments are recognizable and detected by immunogold labeling (Fig. 2D).

Obviously, at the basis of such morphological modifications lie biochemical events, which start at plasma membrane level and are transduced into the nucleus. Since PKC are molecules known to be involved in a cascade of reactions controlling the functioning of nuclear complexes, we have checked the expression and localization of PKC α, δ, ε and ζ.

While PKCδ expression does not undergo modification in all experimental samples, a ten fold greater signal of pPKCδ in 1%HS cells with respect to 10%FBS cells is detected (Fig. 3). No signal of α, δ, ε and ζ PKC isoform expression is evidenced (data not shown).

In order to investigate a potential activation of the splicing factor SC35 by pPKCδ, an immunoprecipitation assay has been performed. When pPKCδ is immunoprecipitated and probed against mouse SC35 monoclonal antibody, pPKCδ-SC35 co-immunoprecipitation is revealed only in differentiated cells, supporting a possible interaction between the two proteins (Fig. 4).

To better understand the relationship between PKCδ or pPKCδ, and SC35 in the nuclear compartment, immunogold co-localization of SC35 factor, either with PKCδ or with pPKCδ, have been carried out by FEI-SEM and TEM (Figs. 5, 6).

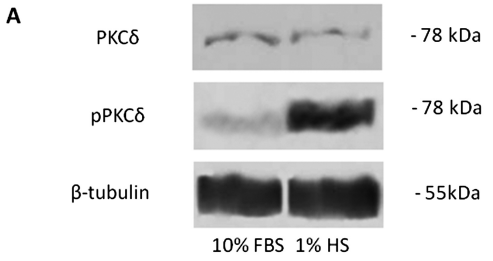
FEI-SEM analysis reveals 10%FBS H9c2 cells with well preserved chromatin structure, in which small areas are delimited by fibers of different diameters, and few gold particles corresponding to PKCδ and SC35 are detected in the chromatin structure (Fig. 5A,B). In 1% HS cells, the chromatin ultrastructure does not show any deep morphological modification. PKCδ 30nm gold particles show a low signal comparable with undifferentiated samples, while SC35 15 nm gold particles demonstrate an increased signal scattered in the chromatin structure (Fig. 5C,D). No significant colocalization between the two proteins is detectable in 1% HS cells.

Since no variation in PKCδ signal between 10% FBS and 1% HS H9c2 cells is observed by FEI-SEM analysis, and a co-immunoprecipitation of the phosphorylated form of PKCδ, pPKCδ, and SC35 is observed in 1% HS cells, we have focused our attention on pPKCδ and its potential involvement in SC35 activation. To compare the different expression and ultrastructure localization of PKCδ and pPKCδ, and their co-localization with the splicing factor SC35, a double immunogold labeling for PKCδ/SC35 and pPKCδ /SC35 has been performed for TEM analysis. 10% FBS and 1% HS H9c2 cells do not disclose any difference in the expression and localization of PKCδ

Table 1. PKCδ, pPKCδ and SC35 expression in nuclear and cytoplasmic compartments.

	10%FBS		1% HS	
	nuclei	cytoplasm	nuclei	cytoplasm
PKCδ	2.82±0.30	2.35±0.20	3.13±0.29	3.27±0.30
pPKCδ	7.20±0.70*	4.00±0.37	18.00±1.77*	8.00±0.77
SC35	8.40±0.87*	—	13.60±1.31*	—

Number of immunogold particles for pPKCδ (30 nm) and SC35 (15 nm) in nuclear and cytoplasmic compartments in H9c2 cells gown in 10% FBS and differentiated in 1%HS, respectively. SC35 splicing factor is exclusively localized at nuclear level while PKCδ and pPKCδ are localized both at nuclear and cytoplasmic level. The number of the gold granules are presented as mean ± SD determination in 100 randomly selected fields for each experimental point. * 1% HS pPKCδ *vs* 10% FBS; 1% HS SC35 *vs* 10% FBS.



B

	PKCδ	pPKCδ	pPKCδ/PKCδ
10% FBS	0.070 ± 0.006	0.090 ± 0.008*	1.28 ± 0.13*
1% HS	0.050 ± 0.005	0.900 ± 0.085*	18.75 ± 1.90*

Fig. 3. A. Western blotting analysis of PKCδ and pPKCδ expression in 10% FBS H9c2 cells and 1% HS cells. Each membrane has been probed with anti β-tubulin antibody to verify loading evenness. The most representative out of three separate experiments is shown. **B.** Data in the table are the densitometric measurement of protein bands expressed as mean ± SD of three separate experiments; *1% HS vs 10% FBS pPKCδ p<0,05; 1% HS vs 10% FBS p<0,05

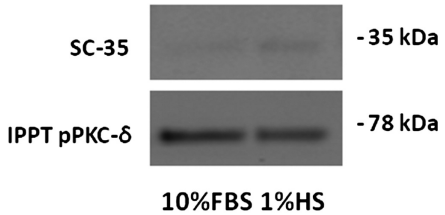


Fig. 4. Co-immunoprecipitation of pPKCδ and SC35. Immunoprecipitated pPKC δ has been probed against mouse SC35 monoclonal antibody, and reprobed against rabbit pPKCδ polyclonal antibody. pPKCδ/SC35 immune complex is observed only in 1% HS cells. The most representative out of three separate experiments is shown

(Fig. 6A, B), as already shown by FEI-SEM analysis.

In 1% HS cells an increased level of pPKCδ and its accumulation in the nucleus, which represents reasonable evidence of protein activation, along with an increased expression of SC35 is detected. In addition, pPKCδ and SC35 localization in structures corresponding to interchromatin granules and perichromatin fibrils, respectively, suggests a possible colocalization. (Fig. 6C,D, Table 1). In order to check the specificity of PKCδ involvement in the events related to H9c2 differentiation, PKCδ silencing has been performed and both PKCδ and myogenin expression have been detected by immunofluorescence microscopy analysis in the different experimental conditions. Fig. 7 evidences a decrease of myogenin expression in 1% HS PKCδ siRNA- transfected H9c2 cells in parallel to reduced percentage of PKCδ positive cells, compared to both untransfected cells.

Discussion

H9c2 cells are currently used as an "in vitro" model of both skeletal and cardiac muscle, since they show electrophysiological and biochemical properties of both

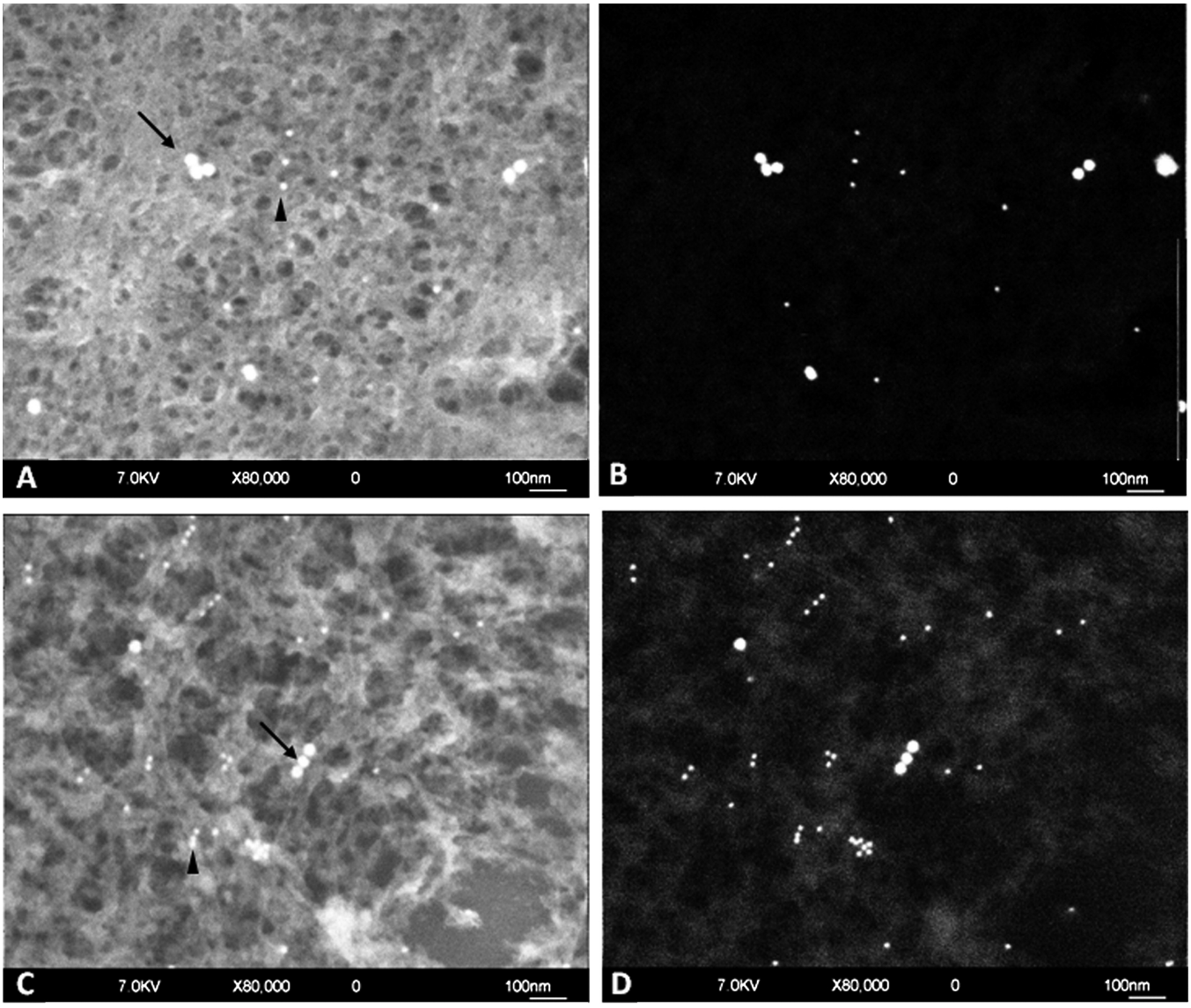


Fig. 5. FEI-SEM double immunogold ultrastructural localization of PKCδ and SC35 in 10% FBS H9c2 and 1% HS cells. Arrow indicates 30 nm gold particles (PKCδ labeling), arrowhead indicates 15 nm gold particles (SC35 labeling). **A.** 10% FBS H9c2 cells. Small areas delimited by euchromatin fibers of different diameter are detectable in the nucleus. **B.** A few gold particles indicating PKCδ and SC35 are scattered in the nucleus. **C.** 1% HS H9c2 cells. **D.** There is no modification in the expression and localization of 30 nm gold particles corresponding to PKCδ compared to the undifferentiated sample, while the signal of 15 nm gold particles corresponding to SC35 is strongly increased.

tissues, such as depolarizing responses to acethylcholine (Kimes and Brandt, 1976), or rapidly activating L-type Ca^{2+} currents (Hescheler et al., 1991; Mejia-Alvarez et al., 1994; Wang et al., 1999).

Furthermore, this cell line, derived from embryonic rat heart (Kimes and Brandt, 1976), represents an intriguing experimental model for studying the progression from proliferative myoblasts to differentiated myocytes.

In this study, we have evaluated the expression of different PKC isoforms and, specifically, the localization and possible interaction between PKCδ or pPKCδ and SC35 splicing factor in H9c2 cells induced to differentiation. The differentiation of H9c2 cells has been triggered by serum reduction and by substitution of FBS with HS, as elsewhere already reported (Langen et al., 2001; Pagano and Naviglio, 2004). By phase contrast light microscopy and TEM ultramorphological analyses, 1% HS H9c2 cells show an elongated cell body and several actin filaments, when compared to the round shape and to the absence of actin fibers in 10% FBS cells. These morphological data are strongly confirmed by the increased expression of the muscle development marker, myogenin, which is involved in the terminal differentiation of myoblasts versus non proliferating myocytes/ myotubes, and by the decreased expression of the proliferative marker, cyclin A (Pagano and Naviglio, 2004).

PKC family plays a pivotal role in the signal transduction of cardiac growth and development. Cardiomyocytes co-express multiple protein kinase C isoforms involved in many adaptative and maladaptative

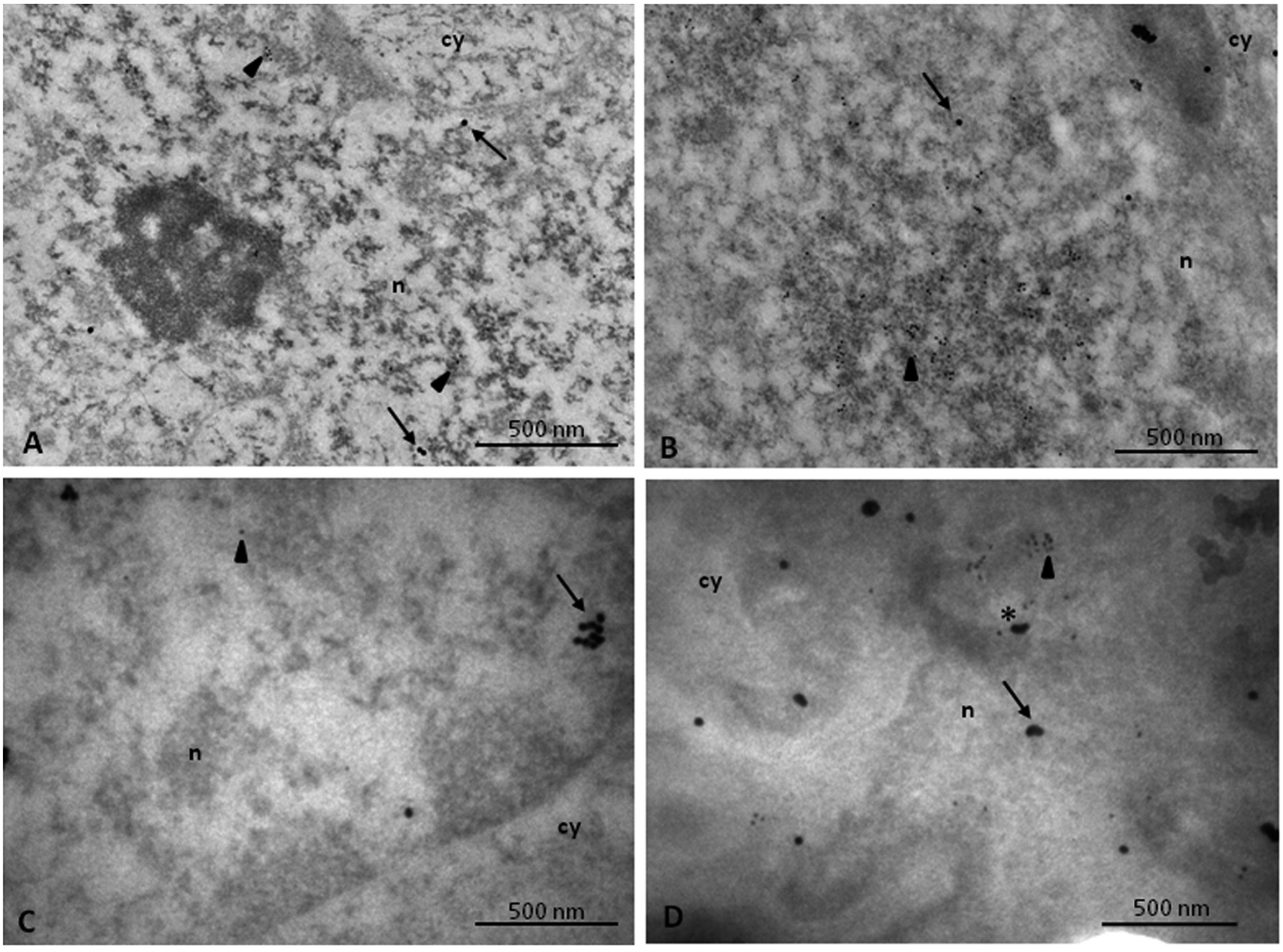


Fig. 6. A,B. TEM double immunogold localization of PKCδ/SC35 in H9c2 grown in 10% FBS and differentiated in 1% HS, respectively. No alteration in PKCδ expression and localization is observed, while the signal of SC35 is increased. **C-D.** TEM double immunogold localization of pPKCδ/SC35 in 10% FBS H9c2 and in 1% HS cells, respectively. A deep increase of both pPKCδ and SC35 levels detectable in 1% HS H9c2. A colocalization between the two proteins is evident (asterisk). n: nucleus; cy: cytoplasm; arrow indicates 30 nm gold particles (PKCδ or pPKCδ labeling), arrowhead indicates 15 nm gold particles (SC35 labeling).

cardiac responses, such as hypertrophy and heart failure (Malhotra et al., 2001; Sabri and Stainberg, 2003). PKC α, δ, ε and ς have been mostly identified in neonatal rat heart and all of them diminished markedly until adulthood (Hamplova et al., 2005). Among the PKC isoforms checked in our experimental model, western blotting analysis reveals PKC δ isoform as the most expressed, while the signal corresponding to the other isoforms is almost absent.

According to our preliminary results, we have decided to focus our attention on PKCδ isoform and on its potential regulative role in H9c2 differentiation process. The specific activity of different PKC is characterized by a dependence upon lipids (Jaken, 1996). More recently, the activation of individual isoforms of PKC has been demonstrated to be modulated by their phosphorylation (Parekh et al., 2000).PKCδ retains a low catalytic activity when it is expressed in the unphosphorylated form. This behaviour contrasts with the classic PKC isoforms whose phosphorylation is essential for activation (Cazaubon et al., 1994). Thus, in order to demonstrate a co-localization and a pPKCδ dependent-functional regulation of SC35, a potential interaction between pPKCδ and SC35 has been proposed. pPKCδ–SC35 co-immunoprecipitation suggests an interaction between the two molecules in 1% HS cells. Phosphorylated PKCδ seems to be the most active form but it holds an acceptable catalytic activity in its unphosphorylated form (Stempka et al., 1997). In light of these findings FEI-SEM and TEM double gold immunolabeling analyses against PKCδ and SC35 have been performed. No significant modification of PKCδ

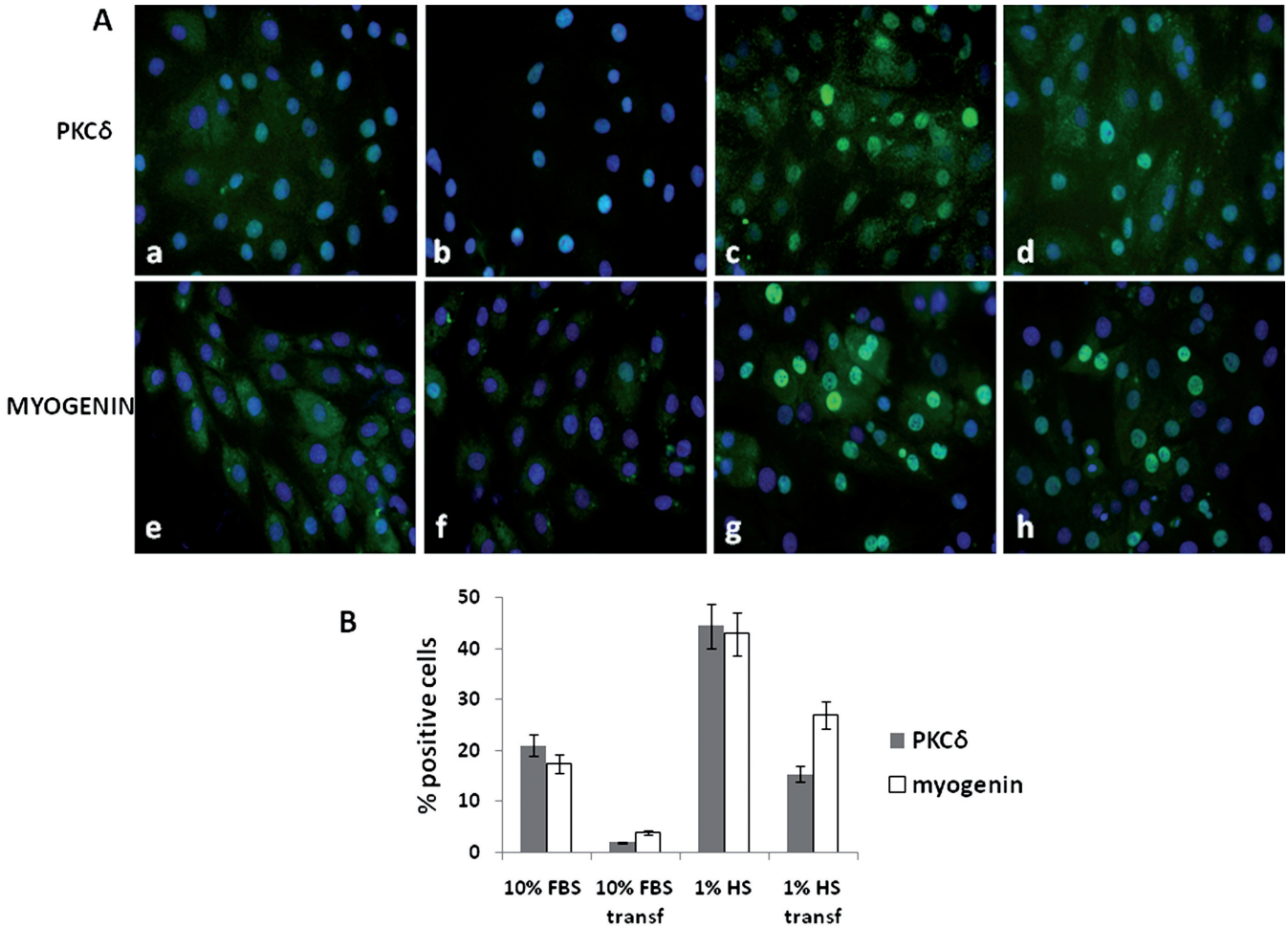


Fig. 7. A. Immunofluorescence analysis of PKCδ (a-d) and myogenin positive cells percentage **(e-h)**, in untransfected and PKCδ siRNA-transfected H9c2 cells. **a, e.** 10% FBS H9c2. **b, f.** 10% FBS PKCδ siRNA-transfected H9c2 cells. **c, g.** 1% HS cells. **d, h.** 1% HS PKCδ siRNA- transfected H9c2 cells. Green fluorescence of FITC (Fluorescein-Isothiocyanate) refers to PKCδ and myogenin labeling; blue fluorescence of DAPI (4-6diamino-2-phenyl-indol)-counterstains nuclei. The most representative out of three separate experiments is shown. **B.** Graphic representation of positive cell percentage. Note that myogenin positive cell percentage decreases in 1% HS PKCς siRNA- transfected H9c2. Results are the mean ± SD of three different consistent experiments. $p<0.05$ is considered statistically significant. A, x 20

level in 1% HS cells compared to 10% FBS ones in parallel to no significant co-localization both in FEI-SEM and in TEM labeling have been detected. Immunogold electron microscopy analysis has been chosen instead of confocal laser scanning microscopy analysis because it requires the permeabilization of the cell, which strongly modifies cell ultrastructure, and has a light microscope resolution.

These findings have led us to focus on the phosphorylated form of PKCδ and its involvement in SC35 activation, since we have previously demonstrated a strict co-localization between SC35 and a specific PKC isoform, namely pPKCα, during neonatal rat heart development, suggesting a main role of pPKCα in modulating the early transcription of components related to cardiac growth and development (Zara et al., 2009). In fact TEM double gold immunolabeling results demonstrate an accumulation of pPKCδ and an deep increase of SC35 expression in the cell nucleus of 1% HS H9c2 cells. In addition, pPKCδ and SC35 localize in structures corresponding to interchromatin granules and perichromatin fibrils, respectively, suggesting their possible colocalization. These data represent a reasonable evidence of pPKCδ mediated SC35 splicing factor activation, suggesting its direct effect on transcription via interaction with the transcription machinery. Furthermore, this co-localization represents a crucial event resulting in downstream changes in transcription of components which determine the morphological modifications related to cardiomyocyte differentiated phenotype.

This evidence is supported by experiments of PKCδ silencing performed in our lab which display the specific role played by PKCδ in the H9c2 differentiative events, since PKCδ silenced cells do not undergo differentiation. PKCδ/SC35 interaction in silenced cells will be investigated in order to confirm our hypothesis.

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