UNIVERSITÀ DEGLI STUDI DI PARMA

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e Diagnostica Funzionale e per Immagini del
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Ciclo XVII

Role of Protein Kinase C epsilon in cardiac and skeletal muscle differentiation

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Sommario

La famiglia delle Protein Chinasi C è stata ampiamente studiata durante il differenziamento di diversi tipi cellulari. È noto che l'isoforma ε, appartenente al sottogruppo delle *nuove* PKC, esercita un essenziale ruolo cardioprotettivo e di precondizionamento in seguito a danno da riperfusione. Inoltre, nel muscolo scheletrico adulto, la PKCε fa parte della via segnaletica che regola l'internalizzazione del glucosio in seguito alla contrazione muscolare. L'obiettivo di questa ricerca è stato quello di comprendere il ruolo fisiologico che la PKCε esercita durante il differenziamento muscolare cardiaco e scheletrico e di caratterizzare le vie segnaletiche coinvolte in questi processi.

Come modello di differenziamento cardiaco *in vitro* abbiamo scelto di utilizzare cellule staminali mesenchimali del midollo osseo. I nostri risultati dimostrano che la PKCε regola negativamente l'espressione di due fattori di trascrizione essenziali per il differenziamento cardiaco, Gata4 e Nkx2.5 attraverso l'attivazione delle chinasi ERK1/2.

Abbiamo inoltre studiato il coinvolgimento della PKCε nel differenziamento muscolare scheletrico. Esperimenti effettuati *in vitro* dimostrano che la presenza nel nucleo della forma attiva di questa chinasi, fosforilata a livello della serina 729, inibisce la proteina di legame alla cromatina HMGA1 e promuove l'espressione dei marcatori miogenici Miogenina e MRF4. Questa cascata molecolare promuove la fusione dei mioblasti e induce il differenziamento terminale scheletrico. Infine, abbiamo dimostrato che, in seguito a danno muscolare effettuato *in vivo*, la PKCε è espressa nelle miofibre rigeneranti centro-nucleate. L'utilizzo di un inibitore specifico della PKCε nel muscolo danneggiato inibisce l'espressione dei fattori di trascrizione miogenici MyoD e Miogenina, modulando negativamente il processo rigenerativo.

I nostri risultati dimostrano che la PKCε è un importante regolatore di geni essenziali coinvolti nel differenziamento muscolare scheletrico e cardiaco, suggerendo che l'espressione di questa chinasi deve essere finemente modulata in questi sistemi biologici.

Abstract

Protein kinase C has been studied in the differentiation process of several cellular types. It is well-known that a novel isoform of this family, PKCε, exerts an essential cardio-protective role and mediates the preconditioning in ischemia-reperfusion injury. Furthermore, in adult skeletal muscle, PKCε is involved in the signaling pathway that regulates glucose uptake after muscle contraction. The goal of this research was to elucidate the physiological role that PKCε plays during cardiac and skeletal muscle differentiation and to determine the molecular pathways involved in these processes.

We used rat bone marrow mesenchymal stem cells (BMMSCs) as a model of *in vitro* cardiomyogenic differentiation. Our results show the ability of PKCε to negatively regulate the expression of two essential cardiac transcription factors, Gata4 and Nkx2.5, via activation of ERK1/2.

We also studied the PKCε involvement in skeletal muscle differentiation. *In vitro* experiments reveal that the accumulation of phospho Ser729-PKCε in the nucleus inhibits of the chromatin binding protein HMGA1 and promotes the expression of the myogenic markers Myogenin and MRF4. This molecular cascade promotes *in vitro* myoblast fusion and myogenic terminal differentiation. We also found that, after *in vivo* muscle injury, PKCε accumulates in regenerating, centrally-nucleated myofibers. In damaged muscle, PKCε specific inhibition dramatically impairs the expression of the myogenic transcription factors, MyoD and Myogenin, affecting the regenerative process.

Our findings demonstrate that PKCε is a critical regulator of essential genes involved in cardiac and skeletal muscle differentiation, suggesting that the expression of this kinase has to be finely tuned in these biological systems.

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INTRODUCTION

1.1 The heart

1.1.1 Cardiogenesis

During gastrulation in mammalian organisms, cardiac precursor cells are in the splanchnic mesoderm, exactly in the lateral plate mesoderm. These cells, are responsible for the formation of the two heart-forming fields (Tam et al. 1997). The signaling pathway that regulates this process has been well studied. Eomesodermin, a T-box transcription factor, activates mesoderm posterior 1 (Mesp1) and induces the specification of splanchnic mesoderm into cardiac progenitor cells (Costello et al. 2011). Mesp1 regulates cardiac specification, up-regulating cardiac genes such as GATA4, Nkx2.5 and Mef2c (reviewed in Bondue and Blanpain 2010). External signals are also important in cardiac specification. The balance between positive regulators (Wnt and HegHog ligands, FGF and BMP) secreted by endoderm and negative regulators (Wnt signaling) derived mainly from the neural plate, allows for a correct heart formation.

There are two populations of cardiac progenitor cells that act to generate the primitive heart. The first group of cardiac progenitor cells that differentiate in the embryo are called primary heart field and are responsible for the formation of the atria and left ventricle. These precursor cells express Mesp1, Is11, Flk1, Mef2c and the transcription factors Nkx2.5 and GATA4 (Moses et al. 2001; Yoon et al. 2006). After formation of the heart tube, derived from the folding of the lateral region of the anterior mesoderm toward the ventral midline, a second population of cells, called the secondary heart field, migrate to the growing heart. These cells give rise to the right ventricle, which include portions of the atria and the outflow tracks (Verzi et al. 2005; Kelly et al. 2001). However, these cells express both Flk1 and Nkx2.5 but not Isl1 (Cai et al. 2003).

After a process of growth and remodeling, the heart tube loops to assume a structure that allows for the proper development and position of the future cardiac chambers.

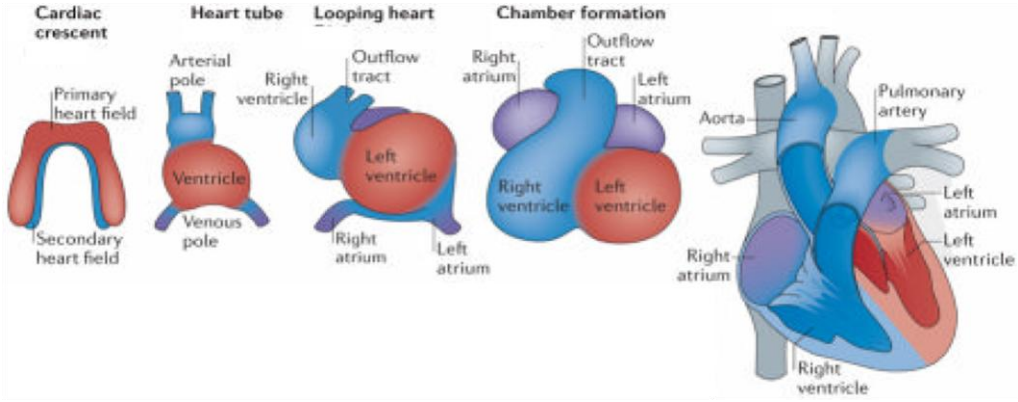

Figure 1.1 Developmental stages of Cardiogenesis (modified from Xei et al. (2013) Nat Rev Mol Cell Biol 14(8): 529-541)

1.1.2 Cardiac regeneration

After birth, cardiomyocytes are mainly binucleated and contain a fully differentiated sarcomeric cytoskeleton. The terminal differentiation of cardiomyocytes is preceded by cell cycle exit. In addition, the down-modulation of cell cycle effectors and the simultaneous up-regulation of cell cycle inhibitors such as p21 and p27 was shown (Tane et al. 2014).

Growing evidence demonstrates that cardiomyocytes are also able to slowly self-renew, thanks to a small pool of cardiac multipotent stem cells described for the first time in 1998 by Anversa and Kajstura (Anversa and Kajstura 1998) and better characterized by Beltrami et al. in 2003 (Beltrami et al. 2003). These cells express the hematopoietic marker c-kit, and are isolable and expandable ex vivo (D'Amario et al. 2011; Rota et al. 2008). Another important evidence about the heart's ability of self-renewal was given by Bergmann and coworkers. Thanks to the analysis of C^{14} in human hearts, they showed that at least 50% of cardiomyocytes are newly produced after birth, demonstrating that new cardiomyocytes are formed throughout adult life (Bergmann et al. 2009).

To understand the contribution of proliferating cardiomyocytes and progenitor cells in the regeneration of injured heart, Malliaras and collegues used an engineered mouse line in which cardiomyocytes express GFP. After induction of myocardial infarction, the percentage of GFP^- cardiomyocytes increased, suggesting a big contribution of progenitor cells in cardiac regeneration (Malliaras et al. 2013). Furthermore, other studies show that a pool of preexisting cardiomyocytes reenter the cell cycle after injury, suggesting a role in the regeneration process (Senyo et al. 2013). The ability for cardiomyocytes to reenter the cell cycle seems to be related with morphological features such as the presence of a sarcomeric cytoskeleton or ploidy. Several groups demonstrated in different animal models and in the human that mononucleated cardiomyocytes are more prone to reenter the cell cycle than binucleated cells, the major population present in an adult heart (Mollova et al. 2013; Bersell et al. 2009).

Bone marrow-derived mesenchymal stem cells (BMMSCs) are one of the most non-cardiac adult stem cell type studied in cardiac regeneration, thanks to their ability to differentiate into cardiomyocyte-like cells (Makino et al. 1999).

TABLE 1. THE STUDIES ADDRESSING THE TRANSDIFFERENTIATION OF BONE MARROW CELLS INTO CARDIAC CELLS

Type of bone marrow cells	*Injection method*	*Identification of donor cells*	*Methods to identify cardiac cells*	*Differentiation into cardiac cells*			*Functional improvement*
				Cardiomyocytes	*Endothelial cells*	*Smooth muscle cells*	
Side population cells (mouse)	BMT[a]	Rosa26_β-galactosidase	IHC[b] stain	0.02%	3.30%	—	Yes
Lin⁻c-kit⁺ cells (mouse)	Intramyocardial injection	EGFP[c] and Y chromosome		53 ± 9%	44 ± 6%	49 ± 7%	Yes
	Mobilization of bone marrow cells	None	—	—	—	—	Yes
Lin⁻c-kit⁺ or LSK[d] cells (mouse)	Intramyocardial injection	Cardiac-specific α-myosin heavy chain promoter_EGFP[c] Cardiac-specific α-myosin heavy chain promoter_β-galactosidase β-actin-EGFP[c]	Expression of cardiomyocyte-specific promoter, IHC[b] staining	0	—	—	—
LSK[d] cells, Thy1.1l[low]LSK[d] cells (mouse)	Intramyocardial injection, parabiotic mice	β-Actin-EGFP[c] (C57BL//Ka-Thy-1.1)	IHC[b] staining	0	0	0	Yes
Whole bone marrow cells, LSK[d] CD45⁻ cells	Intramyocardial injection, BMT[a]	β-Actin-EGFP[c]	IHC[b] staining	Intramyocardial injection: 0	—	—	—
		α-Actin-EGFP[c]		Cytokine-induced bone marrow cell mobilization: Yes but rare (0.75% of all EGFP⁺ cells and 0.0065% of all cardiomyocytes)			
Rat bone marrow cells cultured in the presence of 5-azacytidine	Intramyocardial injection	BrdU labeling during culture (autologous)	IHC[b] staining	Yes, but not quantified	Yes, but not quantified	—	Yes
Human MSCs[e]	Intraventricular injection	Adenovirus carrying β-galactosidase transfection	IHC[b] staining	Yes, but not quantified	—	—	—
Autologous swine MSCs[e]	Intramyocardial injection	DiI labeling	IHC[b] staining	Yes, but not quantified	—	—	Yes
Autologous rat MSCs[e]	Intramyocardial injection	DAPI labeling	IHC[b] staining	No	Yes, but not quantified	Yes, but not quantified	Yes
Human BMSCs[f]	Intramyocardial injection	DiI labeling	IHC[b] staining	Yes, but not quantified	Yes, but not quantified	Yes, but not quantified	Yes
Murine MAPCs[g,h]	Intramyocardial injection	ROSA26_β-galactosidase	IHC[b] staining	No	No	No	Yes

[a]BMT, bone marrow transplantation, [b]IHC, immunohistochemical, [c]EGFP, enhanced green fluorescent protein, [d]LSK, Lin⁻Sca-1⁺c-Kit⁺, [e]MSCs, mesenchymal stem cells, [f]BMSCs, bone marrow-derived multipotent stem cells, [g]MAPCs, multipotent adult progenitor cells.

Table 1.1 The studies addressing the transdifferentiation of Bone Marrow cells into cardiac cells (modified from Antioxid Redox Signal. 2009 Kim et al. (8):1897-911)

Several protocols and different stimuli that induce MSCs cardiomyogenic differentiation are known. A characterized medium containing insulin, transferrin, dexamethasone, ascorbate phosphate, linoleic acid, and sodium selenite is able to induce cardiomyocytic differentiation in MSCs cultured *in vitro*. These cardiomyocyte-like cells express several cardiac markers like TnI, connexin-43, and β MHC and are negative for specific skeletal markers such as MyoD (Shim et al. 2004).

5-azacytidine is a cytosine analog, able to induce DNA demethylation and *in vitro* cardiac differentiation of MSCs. The upregulation of cardiomyocyte genes after 5-azacytine induction is due in part to demethylation of the glycogen synthase kinase (GSK)-3 promoter and its transcription activation (Yang et al. 2009).

The co-culture of MSCs with neonatal cardiomyocytes induce the stem cells differentiation in cardiomyocyte-like cells that are able to beat synchronously. Evidences *in vivo* show that exogenous MSCs and endogenous cardiomyocytes cooperate during regeneration after myocardial infarction **(**Hatzistergos et al. 2010**).** Another group also demonstrated the regenerative effects of human MSCs and c-kit^{+} cardiomyocytes on the anatomical and functional characteristics of the infarcted heart (Williams et al. 2013).

1.1.3 GATA Family

The mammalian GATA family consists in six isoforms of zinc finger transcription factors that have an important role in the regulation of cell differentiation in several tissues and cell types. All GATA isoforms share a common structure, in which transcriptional activation domains are localized in the N-terminal region and two zinc fingers allow the interaction with DNA. These proteins have also a nuclear localization signal sequence (NLS) that guide their nuclear translocation. The DNA binding domains recognize the consensus sequence (A/T)GATA(A/G) (Morrisey et al. 1997a) and regulate the transcriptional control of target genes.

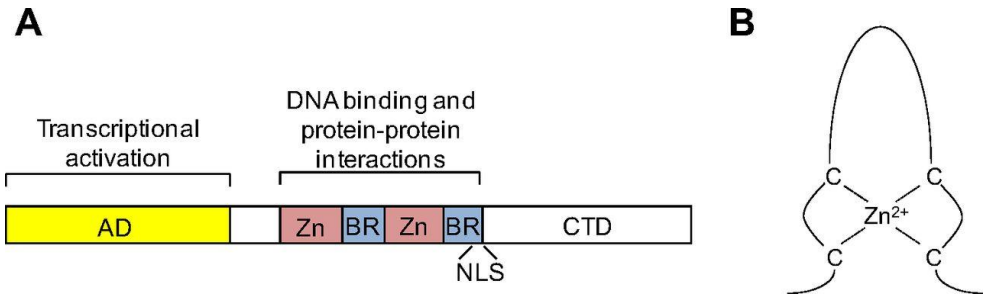

Figure 1.2 **Panel A:** General structure of mammalian GATA family. (AD) Activation domain; (ZN) Zinc fingers domains; (BR) Basic regions; (NLS) Nuclear Localization Signal; (CTD) C-terminal domain. **Panel B:** Schematic structure of a GATA zinc finger (Boaz et al., American Journal of Physiology - Gastrointestinal and Liver Physiology (2014) 306(6), G474-G490

Thanks to the analysis of sequence homology and function, these proteins have been classified in two subgroups. The first, formed by GATA1, GATA2 and GATA3, is expressed mainly in the hematopoietic system, which play important roles in cell specification and development (reviewed by Orkin, 1992 and Weiss, 1995). GATA4, GATA5 and GATA6, belonging to the second subgroup, are well known for its implication in endoderm development during embryogenesis. They are mainly expressed in the heart, liver, pancreas, lung, gonad, and gut (reviewed in Molkentin, 2000a), which control the expression of specific gene subsets.

During heart development and cardiomyocyte differentiation, GATA transcription factors are highly expressed and show an important role in cardiogenesis.

Two independent groups have studied GATA4 null mice demonstrating that this transcription factor is required during heart development to form the primitive heart tube (Kuo et al. 1997; Molkentin et al. 1997). The mechanism proposed is that $GATA4^{-/-}$ mice develop a splanchnic mesoderm in which there are primitive cardiomyocytes, but these cells are not able to migrate in the ventral midline and form the heart tube. These studies suggest that GATA4 expression is required for migration of procardiomyocytes in the embryo and for correct morphogenesis of the primitive heart, but is not essential for cardiac-cell specification. This theory is supported by other evidence that show the importance of GATA4 in cardiac precursor cell survival, but not for cardiac commitment of these cells (Grépin et al. 1997).

During mouse embryogenesis, GATA5 is expressed in the developing heart, first in the precardiac mesoderm and then in the atrial and ventricular chambers but is not detectable in late fetal and post natal heart development (Morrisey et al. 1997b). However, the role of this gene in heart development is not well explained in GATA5$^{-/-}$ mice, in which there are no evident abnormalities in heart formation, suggesting a redundant effect of other GATA factors in this system (Molkentin et al., 2000b).

Finally, GATA6 knock out is lethal in the early stage of embryonic development, before heart formation. Further studies on Xenopus and Zebrafish embryos using a RNA interfering approach demonstrated the role of this transcription factor in maturation and maintenance of cardiac progenitor cells by over-expression of Bone Morphogenetic Protein 4 (BMP-4) and Nkx2 family members (Peterkin et al. 2003).

1.1.4 Nkx 2.5 transcription factor

The NK family members are four homeobox transcription factors classified into two homeodomain protein subgroups (NK1 and NK2- NK4).

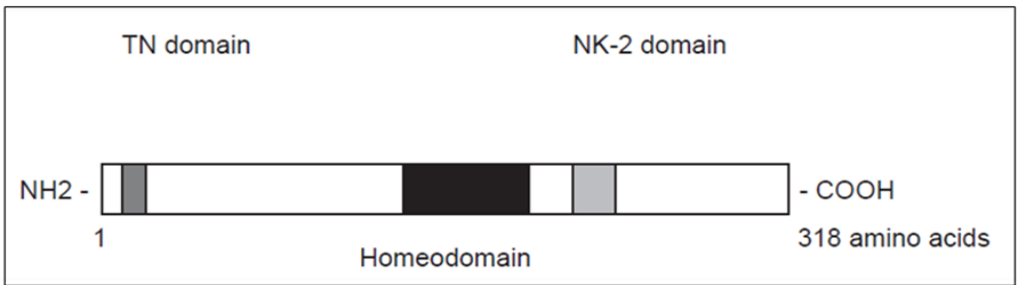

Fig. 1.3. General structure of vertebrate NK2 proteins. (Akazawa and Komuro. (2005) Pharmacology & Therapeutics 107 252 – 268)

Nkx 2.5 is a cardiac transcription factor involved in heart development and post-natal cardiomyocyte gene regulation. In human, mutations of this gene were found in patients affected by congenital heart diseases (Schott et al. 1998) or congenital bicuspid aortic valve (Yuan et al. 2015).

Nkx 2.5 (the fifth gene identified in the NK2 subgroup) is formed by a TN domain, a NK2-SD domain and a homeobox domain that interacts with DNA through a helix-turn-helix DNA-binding motif and recognize the DNA sequence 5'T(C/T)AAGTG3' (Chen and Schwartz, 1995).

During embryogenesis, Nkx2.5 is expressed in both heart fields, suggesting an important role of this transcription factor in the cardiac transcription program during cardiogenesis. At least three different Nkx2.5-deficient mice models were generated and all showed defects on heart tube morphogenesis that are incompatible with life (Lyons et al., 1995; Tanaka et al., 1999a; Biben et al.,2000). Other transgenic mice, in which Nkx2.5 mutation is inducible and restricted only in ventricular cardiomyocytes, have permitted to study the involvement of this gene in ventricular cardiomyocyte specification (Pashmforoush et al. 2004). These mice display a normal morphogenesis of heart structure but are subject to heart failure due to chamber dilatation and hypertrabeculation.

Nkx2.5 activity is also important for the specification and proliferation control of the conduction system in a dose-dependent matter. Indeed, an elegant experiment of Jay and colleagues demonstrate that the Nkx 2.5 mutant lacks the formation of a functional conduction system, but Nkx 2.5 haploinsufficient mice display half of the normal number of functional Purkinje cells (Jay et al. 2004).

The transcriptional activation of the Nkx2.5 gene is hard to completely understand because of the complexity of its upstream regulatory region. The most studied complex that regulates Nkx2.5 expression during heart cardiogenesis is the Smad1/4-GATA4/6 complex. This proteins bind a ~200bp DNA sequence upstream of the Nkx2.5 gene in which are present several binding sites for GATA and SMAD are present. SMAD and GATA act in this system like mutually interacting cofactors that enhance the recruitment and the binding of the other proteins to their sites. (Brown III et al, 2004). Interestingly, Smad proteins are trasducers of bone morphogenetic protein (BMP) signaling, which is known to activated Nkx2.5

transcription and cardiac differentiation in P19CL6 murine embryonal carcinoma cells (Monzen et al., 1999).

Finally, Nkx2.5 drives the expression of essential structural proteins and transcription factors during cardiac differentiation such as ANP, cardiac α-actin, A1 adenosine receptor, connexin 40, calreticulin, myocardin, MEF2-C and other, reviewed by Akazawa and Komuro, (2005).

1.2 Skeletal muscle differentiation

1.2.1 Embryonic development of skeletal muscle

The embryonic development of skeletal muscle is spatially and temporally regulated and allows for the formation of differentiated and functional muscles. In vertebrates, skeletal muscle cells arise from the mesoderm, in the middle layer of the embryo. Trunk and head muscles derive from cells located in different positions. The trunk and limb muscles derive from somites, cells that are located in the segmented paraxial mesoderm. These cells form the dermomyotome in the dorsal part of the neural tube, the sclerotome in the ventral part and the myotome, a product of delamination of $Myf5^{+}$ cells underneath the dermomyotome. In the myotome take place the first event of myogenesis, followed by a second event of differentiation driven by fetal myoblasts that are derived from all four lips of the dermomyotome (Duxon et al. 1989).

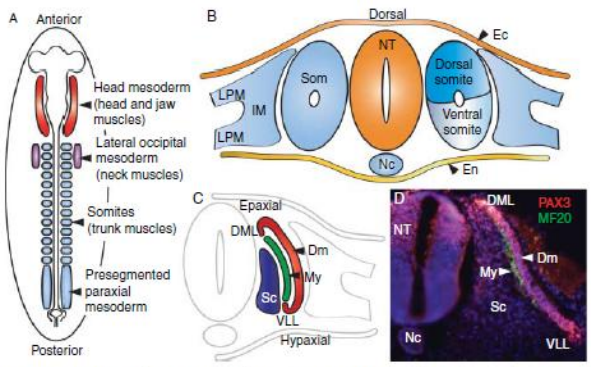

Figure 1.4 Panel A: Schematic representation of a 13 somite amniote embryo (~24 days stage in human, E8.5 in mouse) and the location of myogenic regions. **Panel B:** Illustration of the transverse section of an embryo. The mesodermal derivates are in blue, the ectodermal derivates are in orange and the endoderm is in yellow. **Panel C:** Spatial organization of somites, the dermomiotome is in red, the myotome is in green and the sclerotome is represented in blue. Panel D: Immunostaining of Pax 3 and MF20 (sarcomeric myosin) in a trasverse section of a chicken embryo. (Mok and Sweetman, Reproduction (2011) 141 301–312)

The head muscles have a completely different origin. Head muscles derive from the cranial paraxial mesoderm and the lateral splanchnic mesoderm. The muscle formation

process starts with the progressive differentiation of pluripotent cells, thanks to a complex interplay of soluble factors and transcription factors that result in the formation of functionally specialized cells.

It is known that the differentiation of pluripotent stem cells to form the specialized skeletal muscle tissue is driven by a network of transcription factors that mainly comprise myogenic regulatory factors (MRFs) and other factors like PAX3 and PAX7. Pax3 and Pax7 are transcription factors expressed in the cells of dermomyotome. Pax3 is known to be essential during embryonic myogenesis (Bober et al. 1994) and Pax7 is mostly required during postnatal myogenesis (Oustanina et al. 2004).

The myogenic regulatory factors (Myf5, MyoD, Mrf4 and MyoG) are basic helix–loop–helix transcription factors that bind to the E-box sequence CANNTG and regulate the differentiation of skeletal muscle cells. The myogenic regulatory factors (MRFs) initiate the transcriptional cascade that sustainS the skeletal muscle terminal differentiation during embryronic development and in postnatal life. Myf5 is a determination factor; embrionic cells with the double knockout $Myf5^{-/-}$:$MyoD^{-/-}$ fail to develop skeletal muscle (Rudnickiet al. 1993), suggesting that MyoD and Myf5 are determination factors that are hierarchically upstream of myogenin and MRF4.

The Myogenin knockout shows perinatal death due to a total absence of functional skeletal muscle. (Hasty et al. 1993; Nabeshima et al, 1993) These studies suggest that MyoG is a regulator of late myogenesis.

Skeletal muscle development ends during postnatal life, when satellite cells differentiate and fuse with growing myotubes. Few cells remain in a quiescent state and establish the pool of resident stem cells in the adult muscle.

1.2.2 Satellite cells and skeletal muscle regeneration

Although skeletal muscle regeneration appears to be related with different muscle-derived populations, it is mainly sustained by resident stem cells called satellite cells

(Mauro,1961). These mononuclear cells are localized underneath the basal lamina of muscle fibers and represent ~ 2-6% of all nuclei in healthy mammalian muscle fiber.

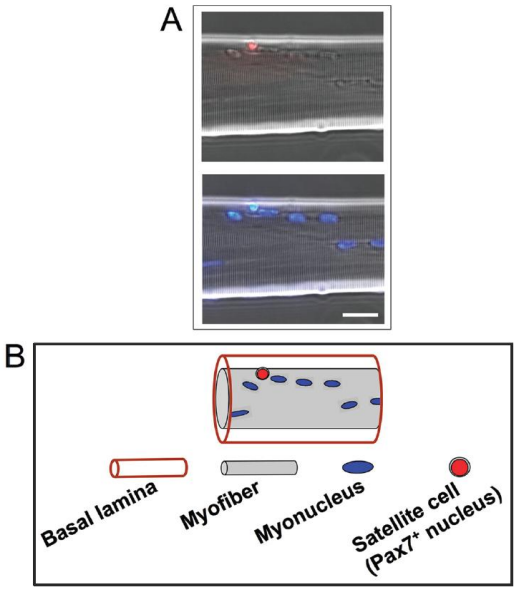

Figure 1.5 Panel A: Adult mouse myofiber stained with anti Pax7 antibodies (Red) and 4′,6-diamidino-2-phenylindole (Blue). **Panel B:** Schematic illustration of the picture showed in Panel A. (Yablonka-Reuveni et al. J ANIM SCI 2008, 86:E207-E216.)

After stimulation by specific factors, satellite cells start to proliferate and differentiate to form new myofibers. At the same time, a subset return in a quiescent status and replenish the satellite pool of dormant stem cells in the muscle (Abou-Khalil et al 2010). The major signaling pathways implicated in the quiescence of satellite cells are the Ang1 /Tie2 signaling pathway(Abou-Khalil et al. 2009), the P38/MAPK pathway (Jones NC et al 2005) and Myostatin via regulation of Pax7 expression (McFarlane et al. 2008).

The staminality of satellite cells was proved by different groups. Collins et al., in 2005, described an engrafting procedure that allowed to transplant a single myofiber in which the satellite cells were tracked with a nuclear Myf5-lacZ reporter. This experiment definitely proved stem cell activity of satellite cells and their ability of self-renewal. (Collins et al. 2005)

More recently, another group showed that satellite cells are able to conserve their stem cell ability after more than seven rounds of serial transplantation (Rocheteau et al. 2012.)

Another fundamental characteristic of stem cells is the ability to undergo asymmetric cell division, giving rise to two different cells, one able to proliferate and the second that remains in the stem cell pool in a quiescent status. Studies on satellite cells reveal that protein like Numb, the Notch inhibitory protein, and MyoD are asymmetrically segregated in daughter cells during cell division. (Conboy et al. 2005; Zammit et al. 2004)

The Paired Box 7 (Pax7) transcription factor is a common marker of quiescent satellite cells. On the other hand, the expression of Pax3 is a characteristic of few specific muscles such as the diaphragm (Relaix F et al.2006). Quiescent cells also express Myf5 but not MyoD. After activation, satellite cells lack the expression of Pax7 and produce Myf5 and MyoD, followed by Myogenin and MRF4.

To study the regenerative property of satellite cells, several different models such as crush, freeze, or chemical injuries have been proposed. One of the most popular and reproducible methods used to induce the activation of the myogenic regenerative program after injury is the intramuscular injection of cardiotoxin or other chemical agents. After injury, the ordinate muscular structure appears disrupted and an interstitial neutrophillic infiltration is detectable. The presence of inflammatory cells in this phase allow for the phagocytosis of necrotic fibers. The activation and proliferation of satellite cells is followed by differentiation in myotube and fusion with preexisting myofibers or toghether to form new growing centronucleated myofibers (Goetsch et al. 2003).

Under pathological conditions, such as dystrophies or aging, satellite cells fail to complete the regenerative process, leading to fibrosis and fatty infiltration in the damaged muscle.

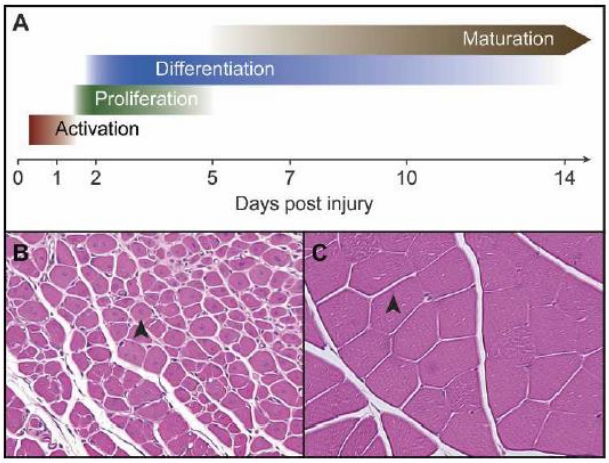

Figure 1.6 Muscle regeneration after injury. **Panel A:** Schematic representation of satellite cell activity during regeneration. **Panel B:** Hematoxylin - eosin staining of a disrupted muscle after cardiotoxin - induced injury. The arrow indicates a centronucleated growing myofiber. **Panel C:** Hematoxylin - eosin staining after 2 weeks of injury. The morphological structure of the muscle is restored. The arrow indicates a mature myofiber in which the nucleus is in a pheripheral position. (Shi and Garry, Genes Dev. 2006 20: 1692-1708)

1.2.3 HMGA family

Chromatin is the structure in which DNA is organized into the nucleus of eukaryotic cells and its structural and functional units are nucleosomes. To allow for gene transcription, chromatin must interact and bind to transcription factors and other DNA binding proteins. The organization of chromatin structure is one of the most important functions of non-histone proteins and the most numerous group is represented by the High Mobility Group (HMG) family.

These proteins are "architectural factors" grouped in three different families based on their different DNA binding domain. Although these three groups have similar functions, each family maintains a typical way to interact and modulate chromatin structure.

The HMGA group is characterized for the presence of an AT-hook DNA-binding domain. This palindromic motif binds preferentially to the minor groove of DNA in A/T rich sequences (Reeves and Nissen, 1990).

HMGB proteins contain the HMG- boxes structure, two tandem DNA-binding regions that bind the minor groove of the DNA with low sequence specificity followed by an unstructured acidic tail. Finally, the six HMGN proteins (HMGN1, HMGN2, HMGN3a, HMGN3b, HMGN4 and HMGN5) contain a nucleosomal binding domain, and an acidic tail called the chromatin-unfolding domain (Bustin, 2001).

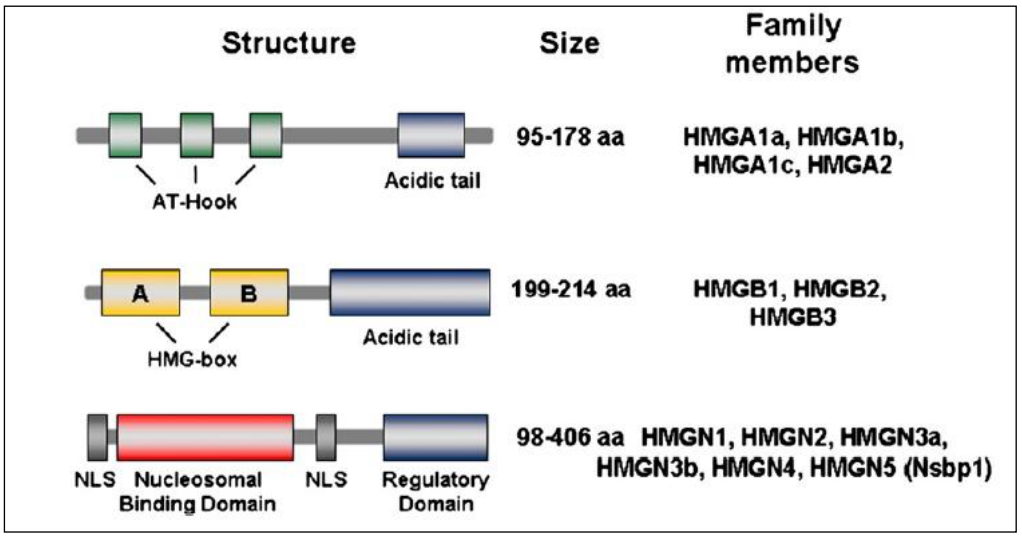

Figure 1.7. Structure of HMG family members. (Katex and Hock Biochimica et Biophysica Acta 1799 (2010) 15–27)

The major role of HMG proteins is to modulate chromatin structure allowing for the binding of other proteins to the DNA and the transcription of specific genes. (Reeves, 2010).
In mammals there are four components of the HMGA subfamily (HMGA1a, HMGA1b, HMGA1c, and HMGA2), encoded by two distinct genes, Hmga1 and Hmga2. HMGA1a, HMGA1b and HMGA1c derive by alternative splicing from the Hmga1 gene and Hmga2 is encoded by its own gene. These proteins, with the rare exception of HMGA1 contain three AT-hook DNA binding motifs.

HMGA proteins are able to modify chromatin condensation affecting the nucleosome structure near the target genes, or changing the conformation of more domains at the same time. The discovery of specific HMGA binding sites in the chromatin structure of metaphase chromosomes (Disney et al., 1989) suggests that these proteins are involved in the

chromosomal changes that occur during the cell cycle. For example, during the G2/M transition, HMGA1 proteins are phosphorylated by cdc2 kinase, decreasing their ability to link DNA (Reeves et al., 1991). HMGA proteins are also able to compete with Histone 1 (H1) for binding to Scaffold Attachment Regions (SARs), which are A/T-rich sequences constitutive of metaphase chromosomes. The competitive binding with SARs of H1 or HMGA proteins is able to modulate chromatin condensation and structure. (Zhao et al., 1993).

HMGA proteins coordinates the formation of the enhanceosomes, multi-subunit complexes that link to A/T-rich promoter regions of specific genes, thus enhancing their transcription. (Merika and Thanos, 2001). One of the most characterized mechanisms in which HMGA1 can promote transcription via the induction of enhanceosome formation is the production of IFN-β after viral infection. HMGA1 coordinates the assembly of the enhanceosome on an A/T-rich sequence near the IFN-β promoter, inducing the transcription of this important gene involved in the innate immune response (Dragan et al. 2008).

Another well characterized mechanism is explained by the study of IL-2 and CRYAB gene transcription. In activated T lymphocytes, HMGA1 is involved in the transcription process of IL-2 and IL-2 α (Himes et al. 1996; John et al. 1995), allowing the formation of the enhanceosome and the transcription initiation of both of these genes (John et al. 1996).

HMGA also activates the alpha-B- crystallin (CRYAB) gene transcription, allowing for the production of the CRYAB heatshock protein. The mechanism comprises the binding to HMGA1 on a response element located near an inhibitory nucleosome and the further recruitment of the transcriptional factors BRG-1 and AP-1 on the gene promoter (Duncan and Zhao, 2007). In both of these cases, HMGA1 allows for the destruction of inhibitory nucleosomes, showing regulatory DNA elements essentials for the binding of key transcriptional factors.

1.3 Protein Kinase C family

The PKC superfamily, belonging to the AGC family of kinases, consists of at least 11 serine/threonine kinase isoforms. They are mainly regulated by calcium (Ca^{2+}) and diacilglicerol (DAG), but also by lipids like phosphatidylserine (PS) and sphingolipids. PKCs are subdivided into three classes, grouped according to their structure and modality of activation. The classic or conventional PKCs (cPKCs) (PKCα, PKCβI, PKCβII and PKCγ) are activated by Ca^{2+}, DAG and PS. However, the novel PKCs (nPKCs) (PKCδ, PKCθ, PKCε,PKCμ and PKCη) are Ca^{2+} independent and regulated by DAG and lipids, whereas the atypical PKCs (PKCζ and PKCί/λ) are both Ca^{2+} and DAG independent (Reviewed by Rosse et al. 2010).

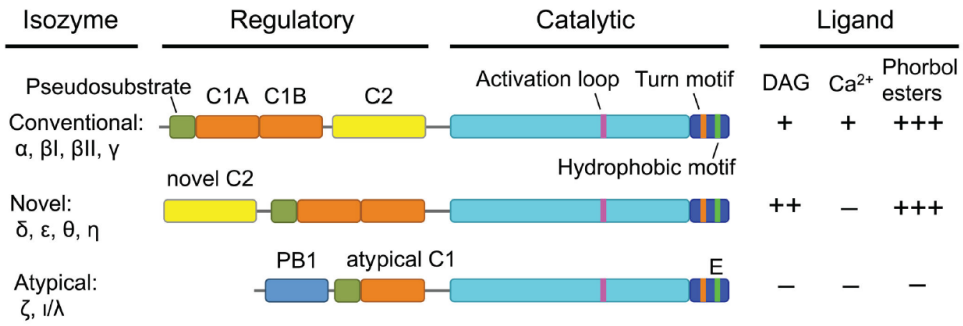

Figure 1.8. Structure of PKC family members (Wu-Zang and Newton, Biochem J. (2013) 452(2): 195–209.)

Most of these kinases are widely expressed in mammalian tissues, with the exception of PKCγ that is typical of the nervous system (Hughes et al. 2008) and the PKCη that is found predominantly in epithelia (Suzuki et al. 2009).

The common structure of PKCs includes a N-terminal regulatory domain and a highly conserved C-terminal catalytic domain separated by a hinge region.

- cPKCs possess two tandem membrane-targeting domains: C1A and C1B bind DAG and phorbol esters in membranes and the C2 domain binds membranes in the presence of the second messenger Ca^{2+}.

- nPKCs contain two tandem C1 domains with a 100-fold higher affinity for DAG than the C1B domain of cPKCs (Giorgione et al. 2006). They also possess a novel C2 domain that does not bind the second messenger Ca^{2+} .
- aPKCs possess a C1B domain that allows them to bind anionic phospholipids and a PB1 domain that mediates protein-protein interactions.

All these isoenzymes have a short autoinhibitory pseudosubstrate sequence in the regulatory domain. When this sequence occupies the substrate-binding pocket, it maintains PKC in an inactive conformation. Binding of second messengers in the regulatory domain induces a conformational modification that allows for the pseudosubstrate release and activation of the active site (Dutil and Newton 2000)

The catalytic domain contains an ATP-binding site and a substrate binding site. To be catalytically competent, PKCs need to be phosphorylated in three different sites in the catalytic domain. These sites are in the activation loop, in the turn motif and in the hydrophobic motif.

Targets of PKCs show a phosporylation site (Serine or Threonine) surrounded by a basic amino acid at N-terminal -2 or -3 position and a hydrophobic amino acid at C-teminal +1 position. Studying these characteristics, many consensus phosphorylation sites for PKCs are known. The most common are (R/K)X(S/T), (R/K)(R/K)X(S/T), (R/K)XX(S/T), (R/K)X(S/T)(R/K, and (R/K)XX(S/T)XR/K (Nishikawa et al. 1997).

In physiological conditions, PLC - PIP2 - DAG is the major pathway of PKC's activation. The α-adrenergic receptors activate phospholipase C (PLC) via Gq proteins. This pathway involves the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2), generating inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG), the major physiologic activator of PKC.

A mechanism of self-inhibition of PKC activity is the interaction of the pseudosubstrate sequence with the substrate-binding motif of the catalytic domain that leads to the inability to link and phosphorilate substrates (Orr and Newton, 1994). Binding of

activators like DAG and PMA on the regulatory domain causes a conformational change that release the active site from the inhibition of the pseudosubstrate motif and activates PKCs. PKCs are also sensitive to cleavage by proteolytic enzymes like calpain or caspases in the hinge region. The final products are usually constitutively active, even in the absence of second messengers (Kishimoto, 1989).

Regulation of PKCs activity occurs also via interaction with transporters and other proteins. The most characterized are Receptors for Activated C Kinases (RACKs), A-Kinase Anchoring Proteins (AKAPs) and 14-3-3 proteins. RACKs are intracellular PKC receptors that interact with the regulatory domain of PKCc and are responsible for their subcellular localization. Their function is critical for PKCs activation, interaction with substrates, and cellular responses.

Since the PKC family was discovered, it has been a goal to develop specific molecules capable to modulate the function of these kinases in an isoform specific manner. The high sequence homology between the different groups and isoforms has made this goal difficult to achieve. Different approaches were tried, including the development of active site inhibitors, which are small molecules that activate or inhibit PKC mimicking the binding of DAG, the physiological activator of the classical and novel PKC, and peptides that act disrupting the protein-protein interaction.

The active site inhibitors, are small molecules that compete with ATP to bind to the ATP-binding site. These type of inhibitors are efficient in activating PKC but have low specificity, because the ATP binding pocket is a well conserved region of these proteins and it shows high sequence homology not only between different isozymes but also with other serine/threonine kinases. The best characterized is the bisindolylmaleimide family. They are water soluble compounds, isoenzyme-non-specific PKC inhibitors that act on all three classes of PKC isoenzymes *in vitro*, but are more effective against conventional and novel PKCs than atypical isoenzymes. They do not inhibit the closely related PKA or PKD but are highly

effective against other kinases like FLT3, GSK3, GSK3β, PIM1, PIM3 and RSK1–RSK4 (Anastassiadis et al. 2011).

The non-active site activators/inhibitors are molecules that target the regulatory domains of these enzymes. A well-characterized family mimics the binding of DAG - the physiological activator of classical and novel PKCs - to the C1 domain. Examples of activators are the phorbol esters, that cause an irreversible activation of PKCs (Blumberg1980) or diacylglycerol-lactones. Modified diacylgliycerols show higher affinity for the PKC's C1 domain than the natural counterpart. (Marquez et al., 1999).

A new class of PKC inhibitors is composed by small peptides that are able to interfere with PKC interaction with specific transporters, crucial for their translocation and subcellular localization. The peptide inhibitors are competitive antagonists that have the same sequence and structure of the PKC's C2 domain and compete with the native kinase to the binding with RACK. This results in the inhibition of translocation and phosphorylation of the substrate. Instead, the peptide shows sequence homology with the PKC pseudo-RACK site and binds PKC, thus stabilizing the active conformation of the protein. Interestingly, RACK has a higher affinity than the activator and it is able to bind the activated PKC and mediate the translocation. (Churchill et al. 2009)

1.3.1 Protein Kinase C ε (PKCε)

PKCε is a novel isoform characterized by wide expression in many tissues and organs and with well known activity in the cardiac (Budas and Mochly-Rosen, 2007), nervous (Shirai 2008) and immune system (Aksoy, 2004) as well as in cancer development.

Commonly with the other classical and novel PKC isoforms, three major sites of phosphorylation were identified in the C-terminus of PKCε. In the Activation-loop, phosphorylation of Thr566 is necessary for catalysis because it induces conformational modifications that stabilize the active conformation of this kinase. The most characterized

kinase that catalyses this phosphorilation is the Phosphoinositide- Dependent Protein Kinase-1 (PDK1).

Studies *in vitro* have revealed that the over-expression of PDK1 increases PKCε ^{Thr566}, Interestingly, this first phosphorilation event triggers autophosphorilation of the Ser729, located in the hydrophobic motif (Cenni et al. 2002). Also PDK1 down-modulation has important effects on PKCε phosphorilation and activity. Balendran *et al.* shown that murine PDK1$^{-/-}$ embryonic stem cells have low levels of PKCε including other novel and conventional PKCs, suggesting that phosphorilation in the activation loop could also have a role in the stabilization of this protein. (Balendran et al. 2000). However, more recent studies on other related PKCs suggest that PDK1 is not the only kinase that can phosphorilate this site. Ser729 is a target of the mTORC1 complex and the treatment with rapamycin, a mTORC1 inhibitor, can affect the PKCε phosphorilation in this site (Parekh D,1999). Other possible sites of phosphorilation are Ser-234, Ser-316, and Ser-368. Little is known about the functional effects of this phosphorilation, but they are probably targets of conventional PKC or auto-phosphorilation sites (Durganet al. 2008).

After activation, PKCε translocates to membranes or other subcellular compartments, thanks to the anchoring proteins Receptor for Activated C-Kinase1 and 2 (RACK1 and RACK2). Specifically, RACK2 allows the active phospho - Ser^{729} kinase to translocate to the Golgi membrane.

The role and function of PKCε in several tissues has been investigated. In the nervous system, PKCε is the most abundant PKC and has various effects in this system. Interestingly, several studies conducted in murine animal models show that this kinase is able to modulate the sensibility of GABAA receptors and up-regulate the expression of N-type channels inducing alcohol dependency (Besheer et al. 2006). Moreover, activation of PKCε led to an improvement of the functionality of neuronal cells in Alzheimer's disease that correlates with a reduction of β-amyloid protein levels. (Nelson et al. 2009). In the colon, the down-modulation of PKCε is required for TRAIL and butyrate induction of colonic epithelial

cell differentiation (Gobbi G et al. 2012). In the hematopoietic system, PKCε is needed to protect erythrocytes and acute myeloid leukemia against apoptosis (Gobbi G et al. 2009; Mirandola P et al. 2006). PKCε's role and its fine regulation in megakaryocytic differentiation is also well documented (Gobbi G et al. 2007; Gobbi G et al. 2013).

In skeletal muscle, high levels of PKCε are able to modulate the expression and sensitivity of the Insulin Receptor (IR) causing insulin resistance (Dey et al. 2007). During muscle contraction, PKCε promotes glucose uptake through the modulation of GLUT4 traffic (Niu et al., 2011). Less is known about the involvement of PKCs in muscle differentiation. PKCθ isoform principally regulates the fusion process, modulating the expression of caveolin-3 and β1D integrin (Madaro L et al., 2011). The same group has published conflicting data, demostrating that the deletion of PKCθ in an animal model of muscular dystrophy improves muscle regeneration. The possible explanation for this phenotype is that PKCθ is a potent inflammatory promoter and in its absence the exaggerated inflammatory response in damaged and pathologic muscle is reduced (Madaro et al. 2012). Finally, PKCε mRNA and protein expression increases during insulin-induced myogenic differentiation of the C_2C_{12} cell line (Gaboardi GC et al., 2010).

In the heart, PKCε has well known cardioprotective effects and mediates the preconditioning in ischemia-reperfusion injury. Studies performed *in vivo* with peptic activators show that pretreatment with the activator peptide before heart ischemia results in a strong cardioprotective effect (Inagaki et al. 2005). One of the mechanisms proposed is that activation of PKCε after short-term periods of ischemia leads to a positive regulation of mitochondrial Aldehyde Dehydrogenase 2 (ALD2) and a consequent decrease of damage in the heart. (Dorn et al. 1999; Chen et al. 2008). PKCε is also able to increase sarcK_{ATP} channel activity after preconditioning, leading to ATP preservation and a reduction of Ca^{2+} entry. (Aizawa et al. 2004). Finally connexin43, a well known component of cardiomyocyte Gap junctions, is a direct target of PKCε in human and rat cardiomyocytes (Doble et al., 2000; Bowling et al., 2001).

AIMS

AIMS

Although the role of the PKCε pathway has been extensively studied in cardiac preconditioning and in the adult heart (Inagaki et al. 2005: Dorn et al. 1999; Chen et al. 2008), little is known about its implication in cardiac differentiation.

The role of PKCε in skeletal muscle differentiation is also less clear. Only a precedent study suggests that PKCε is up-regulated during insulin-induced myogenic differentiation of the C_2C_{12} cell line (Gaboardi et al., 2010).

The goal of the present study was to evaluate the PKCε pathway during cardiac and skeletal muscle differentiation. In particular my interest has been focused on the BMMSCs cardiac differentiation induced by 5-azacytine treatment and murine C_2C_{12} myoblast and primary satellite cells *ex vivo*.

- The first aim was to understand a possible connection between PKCε and the cardimyocyte transcription factors Nkx2.5 and GATA4, two well known markers of early cardiac differentiation.

- The second aim was to understand the PKCε pathway in skeletal muscle differentiation *in vitro* and *ex vivo* and explain the possible interconnection between PKCε and the chromatin binding protein HMGA1 signaling.

MATERIALS AND METHODS

3.1 Mice

All animal experiments described in this thesis were approved by the Local Animal Research Ethics Committee. In addition, the experimental procedures were conducted according to the "Guide for the Care and Use of Laboratory Animals" (Directive 2010/63/EU of the European Parliament).

3.2 Cell cultures

Bone Marrow Mesenchymal Stem Cells (BMMSCs) were isolated from Wistar rats' bone marrow after euthanization with overdoses of pentobarbital. Tibia and femurs were collected in aseptic conditions and cleaned from muscles and other soft adherent tissues. After excision of the proximal and distal ends, the marrow plugs were flushed from the bone marrow cavity and collected in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS). To isolate BMMSCs, Percoll media (density 1.13 g/ml) was used to isolate mononuclear cells by density centrifugation. The mononuclear fraction was grown in low glucose DMEM with 10% FBS in a humidified 5% CO_2 atmosphere at 37°C and non-adherent cells were removed after 24 h. BMMSCs were then induced to differentiate in different cell types:

- Cardiac differentiation was induced by treatment with 10 μM 5-azacytidine (Sigma-Aldrich, Milan, Italy) for 24 h. Cells were then cultured in a differentiation media (DMEM low glucose, 2 % horse serum) for up to 30 days. In order to inhibit the ERK pathway, cardiomyocytes-like cells were pre-treated with 10 μM of the MEK1/2 inhibitor U0126 (Cell Signaling, Boston, USA) for 30 min before cell transfection.
- Osteogenic and adipogenic differentiation were induced by treatment with specific media from Stem Cell Technologies (Vancouver, Canada) and verified by Alizarin red and Oil red Oil staining, respectively.

Satellite cells (SCs) were isolated from hindlimb muscles of neonatal (2 days old) CD1 mice. Muscles were minced and then incubated with a collagenase/dispase solution (Roche, Basel, Switzerland) for a total of 4 digestions. Cell suspension was filtered with 40 µm nylon cell strainer and stained with the Feeder Removal Microbeads kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and immunomagnetic separated following the manufacturer's instructions. Fibroblast negative fraction was seeded at a density of 1.25 x $10^5/cm^2$ in collagen-coated culture dishes. Non-adherent cells were removed after 24 h and satellite cells were grown in a fibroblast-conditioned medium obtained by mixing (1:1 ratio) Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Growth Medium, GM) with filtered supernatant of primary cultures of mouse fibroblasts grown in GM. Mouse myoblast C_2C_{12} cell line and primary SC were cultured in a humidified 5% CO_2 atmosphere at 37°C. To induce myogenic differentiation, when the cell cultures reached 80% confluence the GM was substituted with DMEM supplemented with 2% horse serum (Differentiation Medium, DM).

3.3 RNA extraction and quantitative RT-PCR

Total RNA was extracted using Trizol reagent or the RNeasy mini kit (Qiagen) according to the manufacturer's instructions. 1 µg of total RNA was reverse transcribed using ImProm-II™ Reverse Transcription System (Promega, Fitchburg, WI) in a final volume of 20 µl. Quantitative real-time PCR assay was performed on 2µl of the 1:5 dilution of cDNA using Syber Green method. Polymerase chain reactions were made by StepOne Real-Time PCR System (Applied Biosystems) and GoTaq ® qPCR Master Mix (Promega). For each well, the 20 µl reaction medium contained: 10 µl of 2X GoTaq ® qPCR Master Mix (with SYBR Green), 100 nM each forward and reverse primer, 7,6 µl of RNase-free water and 2 µl cDNA template 1:5. The cycling conditions were: 95°C for 20s followed by 40 cycles of 95°C for 3s and 60°C for 30s. Real-Time RT-PCR products were confirmed by the analysis of melting

curves. The amount of the target transcript was related to that of the reference *gusb* gene by the method of Comparative C_T.

The sequence of primers used in this study is summarized in Table 3.1.

GENE	*SEQUENCE*
Rat nkx2.5	fw: 5'-TATGAGCTGGAGCGGCGCTT-3' rev: 5'-TGGAACCAGATCTTGACCTG-3'
Rat gata4	fw: 5'-AGGGTGCTGGGTTTCTTCAA-3' rev: 5'-GACAGTGTCTTGAAGCCTCG-3'
Rat pkcε	fw: 5'-CAAGCAGAAGACCAACAGTC-3' rev: 5'-CGAACTGGATGGTGCAGTTG-3'
Rat pgk	fw: 5'-TGTGGGCTCAGAAGTAGAGA-3' rev: 5'-TAGCTGGCTCAGCTTTAACC-3'
Mouse myf5	fw 5'- TGAGGGAACAGGTGGAGAAC -3' rev 5'-AGCTGGACACGGAGCTTTTA -3'
Mouse mrf4	fw 5'-GAGATTCTGCGGAGTGCCAT -3' rev 5'-TTCTTGCTTGGGTTTGTAGC-3'
Mouse pkcε	fw 5'- ATGTGTGCAATGGGCGCAAG -3' rev 5'-CGAGAGATCGATGATCACGT -3'
Mouse hmga1	fw 5'-CAAGCAGCCTCCGGTGAG -3' rev 5'- TGTGGTGACTTTCCGGGTCTTG -3'
Mouse gusb	fw 5'-CCGCTGAGAGTAATCGGAAAC- 3' rev 5'- TCTCGCAAAATAAAGGCCG -3'

Table 3.1 Primer sequences.

3.4 Immunofluorescence

BMMSCs 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 anti-myosin heavy chain antibody (clone MF-20; Developmental Study Hybridoma Bank) or anti-connexin43 (CX43) (Santa Cruz Biotechnology, USA) 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. Nuclei were counterstained with DAPI.

C_2C_{12} were fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized 3 times with 1% BSA, 0.2% Triton X-100 in PBS for 5 minutes at room temperature and

incubated in 10% goat serum in PBS for 1 hour at room temperature to saturate non-specific binding sites. Samples were incubated for 1.5 hours with primary antibody diluted 1:200 in 1% goat serum in PBS. PKCε and myosin were detected by anti-PKCε rabbit serum (Novus Biologicals, Littleton, CO NBP1-30126) and anti-myosin heavy chain antibody, respectively. Cells were washed in PBS and then incubated with secondary antibody (Alexa Fluor 488 Donkey anti-mouse IgG and Alexa Fluor 594 anti-rabbit Donkey IgG) 1:1000 for 1 hour at room temperature. Nuclei were counterstained with DAPI.

Fluorescence was viewed with a Nikon Eclipse 80i (Tokyo, Japan) fluorescent microscope equipped with Nikon Plan color 20X/0.50, Ph1 DLL, ∞/0.17, WD 2.1 and Nikon Plan color 40X/0.75, Ph2 DLL, ∞/0.17, WD 0,72 objectives and a camera (Nikon Camera DS-JMC). Image acquisition were performed using Nis element F2.30 (Nikon, Japan).

3.5 Cellular fractions separation and Western Blot analysis

In cellular fractions separation experiments, $5x10^6$ cells were treated with NE-PER Nuclear and Cyotplasmic Extraction Reagents (Pierce), used according to manufacturer's protocol. For Western Blot analysis, samples 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 Na3VO4; 1 mM NaF). 30 μg of total proteins were loaded on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose. Blots were incubated with the specific primary antibody (dilutions and buffers were as indicated by manufacturer) anti-Phospho-ERK1/2 (Cell Signaling, USA), anti-b-ACTIN (Sigma, Italy), anti-NKX2.5 (abCam, UK) anti-GATA4 (abCam, UK) and anti-CONNEXIN43 (CX43) (sc-9059), anti-PKCε (Merck Millipore, Darmstadt, Germany 06-991), anti-HSP70 (Sigma-Aldrich, St. Louis, MO, H5147), anti-insulin receptor β chain (IRβ, (Cell Signaling, Danvers, MA)#3025), anti-myogenin (Santa Cruz, Dallas, TE sc-12732), anti-myoD (Santa Cruz sc-32758), anti

GAPDH (Merk Millipore MAB374) anti-HMGA1 (Abcam, Cambridge, UK ab4078), washed and incubated with 1:5000 peroxidase-conjugated anti-rabbit or with 1:2000 peroxidase conjugated anti-mouse IgG (Pierce). Signals were revealed by ECL Supersignal West Pico Chemiluminescent Substrate detection system (Pierce).

3.6 Cell transfection

PKCε expression levels were up-regulated by the transfection of murine GFP-PKCε plasmid and GFP-K522M mutated PKCε control plasmid (kindly provided by Prof. Peter Parker, Cancer Research Institute, UK) using the Superfect Transfection reagent (Qiagen, Hilden, Germany). Small interfering RNA (siRNA) silencing was obtained by transfection of 400 nM specific siRNAs or control siRNA (Ambion, Austin, TX). PKCε activity was also pharmacologically modulated by the εV1-2 (CEAVSLKPT) and ψεRACK (CHDAPIGYD) peptides, conjugated to TAT_{47-57} (CYGRKKRRQRRR) by a cysteine disulfide bound. Briefly, εV1-2 is a specific PKCε inhibitor that acts as a binding competitor between PKCε and its anchoring protein εRACK. Instead, ψεRACK is a PKCε allosteric activator, implicated in auto inhibitory intramolecular interactions. Peptides are highly specific for PKCε and they don't interact with other PKC isozymes. C_2C_{12} cells and SC were incubated with DM and treated with 1μM of peptides every 24 hours for 48 or 72 hours.

3.7 Short hairpin RNA (shRNA) cell infection

PKCε expression was also down-modulated by shRNA gene silencing using a pLKO.1 lentiviral vector encoding shRNA against mouse *Pkcε* (Open-Biosystem, Thermo Scientific,Waltham, MA) and the MISSION pLKO.1-puro Non- Target shRNA Control Plasmid (Sigma-Aldrich, St. Louis, MO). The shRNA expressing viruses were produced in 293TL cells according to standard protocols. Mouse proliferating C_2C_{12} cell line was infected

with *Pkcε* shRNA or CTRL shRNA and then cultured in the presence of puromycin (2 μg/ml) to select infected, puromycin-resistant cells.

3.8 Cardiotoxin injury and immunohistochemistry

Acute skeletal muscle injury was induced by intramuscular injection of Cardiotoxin (10 μM) in the tibialis muscle of CD1 adult mice. In some exeriments, εV1-2 or ψεRACK (100 nM) were directly added to the cardiotoxin mix. To study the regenerative process, mice were euthanised for histological analysis 3 and 7 days after injury. Muscle samples were fixed with 4% paraformaldehyde and embedded in paraffin. Sections (4 μm) were blocked with goat serum and incubated with primary anti PKCε antibody (Novus Biological NBP1-30126). Detection was performed using Vectastain elite ABC kit (Vector Laboratories) and nuclei were counterstained with haematoxylin.

3.9 Statistical analysis

All Panels show the mean values and Standard Deviations (SDs). p-values were calculated using the Anova - Dunnett test.

RESULTS

4.1 Role of PKCε in BMMSCs cardiac differentiation

4.1.1 Characterization of BMMSCs and 5-azacytidine induction of cardiac differentiation

Bone marrow-derived mesenchymal stem cells (BMMSCs) are adult stem cells known to be able to differentiate in cardiomyocyte-like cells after 5-azacytidine treatment (Makino et al. 1999). In order to phenotypically and functionally characterize the BMMSCs used in these experiments, cells were isolated as extensively described in the Materials and Methods section. Cytofluorimetric analysis of surface markers CD14, CD34, CD44, CD45, CD90 and CD105 revealed a phenotypic profile consistent with that previously characterized in rat BMMSCs by Gao and colleagues (Gao et al. 2010).

CD	%	CD	%
CD14	-	CD44	90±3
CD34	-	CD90	87±2.7
CD45	-	CD95	92±1

Table 4.1 Cytofluorimetric analysis of BMMSCs surface markers

To verify their ability to undergo osteogenic or adipogenic differentiation *in vitro*, BMMSCs were cultured with specific pro differentiation media. Cells stained with Alizarin Red show evident red precipitates formed by the reaction of this reagent with calcium crystals typically present in osteocytes (Fig. 4.1 a). Adipogenic differentiation was evaluated using the Oil Red Oil staining, that reacts with lipid vacuoles, a typical structure of adipocytes (Fig. 4.1 b).

Finally, we proved the BMMSCs cardiac potential *in vitro* after 5-azacytidine treatment. The immunofluorescence analysis reveal the expression of cardiac markers like myosin heavy chain and Connexin43, an essential component of cardiomyocytes gap junction. (Fig. 4.1 c-h). The expression of Connexin43 was also confirmed by Western Blot analysis.

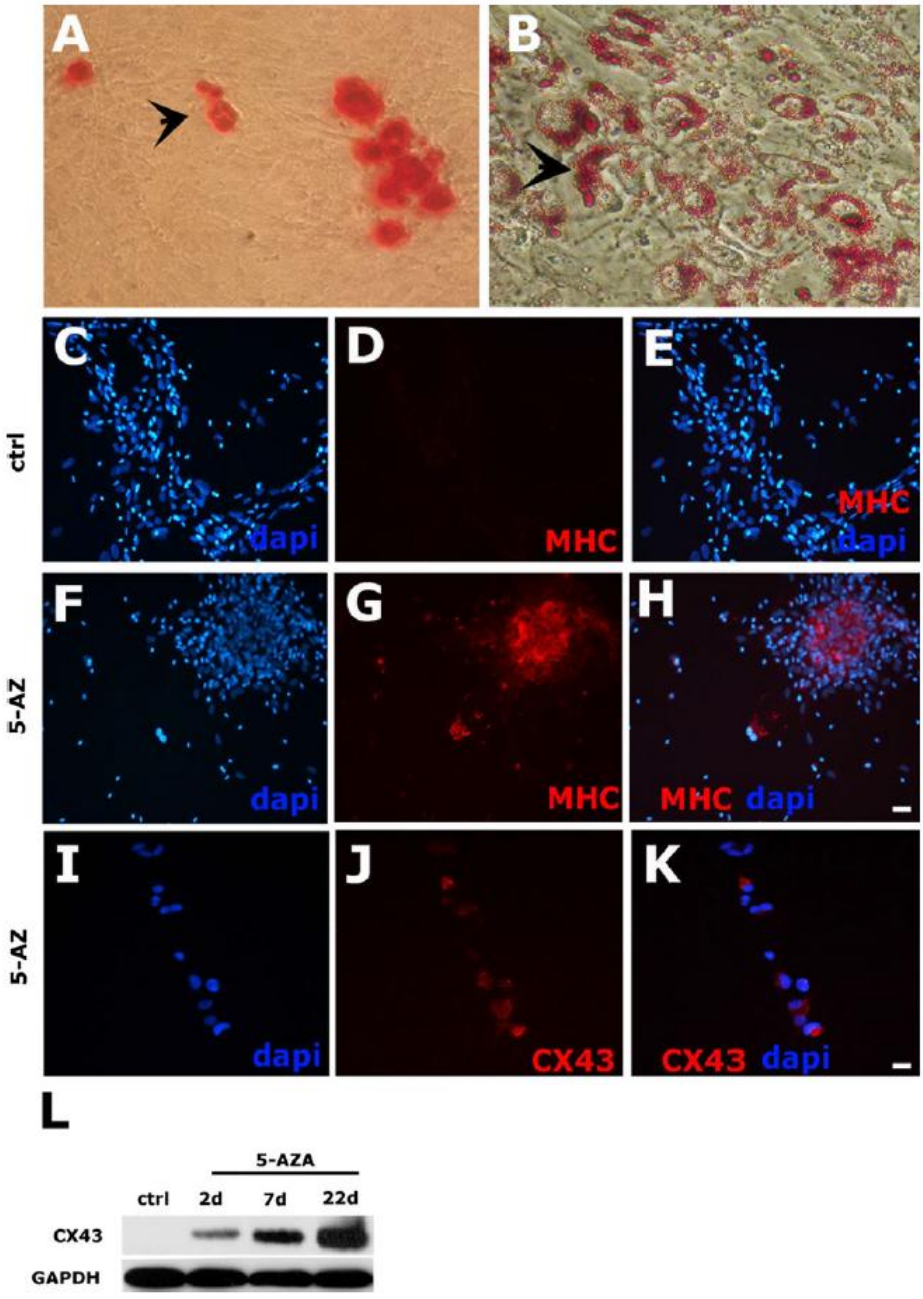

Figure 4.1
Panel A: Alizarin red staining of BMMSCs cultured in osteogenic inductive medium. Arrowhead highlights the red staining of calcium deposits. **Panel B:** Oil Red Oil staining of BMMSCs cultured in adipogenic inductive medium. Arrowhead highlights lipid vacuoles. **Panel C-E:** Myosin (MHC) immunofluorescence in control cells. Panel F-H: Myosin immunofluorescence in 5-Azacytidine treated cells. Panel I-K: Connexin43 (CX43) immunofluorescence in 5-Azacytidine treated cells. Scale bar corresponds to 50µm. Panel L: Western Blot analysis of CX43 expression with (2, 7 and 22 days) or without 5-azacytidine. GAPDH was used as housekeeping protein.

4.1.2 PKCε expression during BMMSCs cardiac differentiation

In order to study the expression of PKCε during 5-azacytidine induced - cardiac differentiation of BMMSC, mRNA levels of pkce, nkx2.5 and gata4 were analyzed by real-time RT-PCR at days 1, 2, 3, 7 and 8 after treatment with 5-azacytidine. (Fig. 4.2 a). The mRNA expression of PKCε is detectable in all samples analyzed but is maximal at day 2 and it's down-modulated up to day 7, in which detection of PKCε was lowest. PKCε protein expression was analyzed by Western blot. Figure 4.2 b-c shows that the protein expression levels are consistent with the results obtained by Western Blot. Interestingly, we founded that the nkx2.5 and gata4 mRNA profiles are opposite to that of pkce (Fig. 4.2 a). Further studies were conducted to evaluate the possible implication of PKCε in nkx2.5 and gata4 expression during cardiac differentiation.

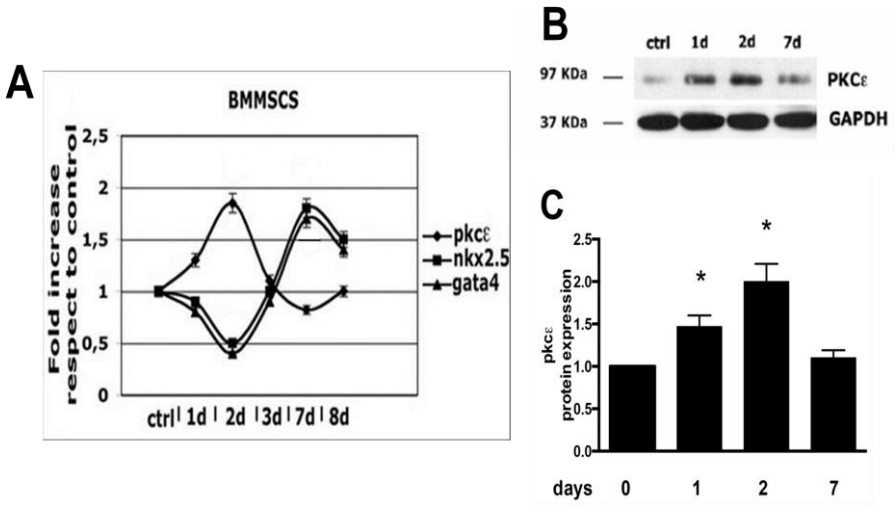

Figure 4.2
Panel A: Quantitative Real Time-PCR for pkce, nkx2.5 and gata4 mRNA expression in BMMSCs at different time points (1 day, 2 days, 3 days, 7 days and 8 days after treatment with 5-azacytidine). Housekeeping phosphoglycerate kinase 1 (pgk) gene was used as reference. **Panel B:** Western blot analysis of PKCε expression at 1, 2 and 7 days after treatment with 5-azacytidine. Day 0 corresponds to the untreated sample. GAPDH was used as housekeeping protein. **Panel C:** densitometric analysis of PKCε protein expression. Values are means of 3 independent experiments ± standard deviation. GAPDH was used for normalization. n=3; *p<0.05 Anova-Dunnet test (vs untreated cells).

4.1.3 PKCε role in nkx2.5 and gata4 expression during BMMSCs cardiac differentiation

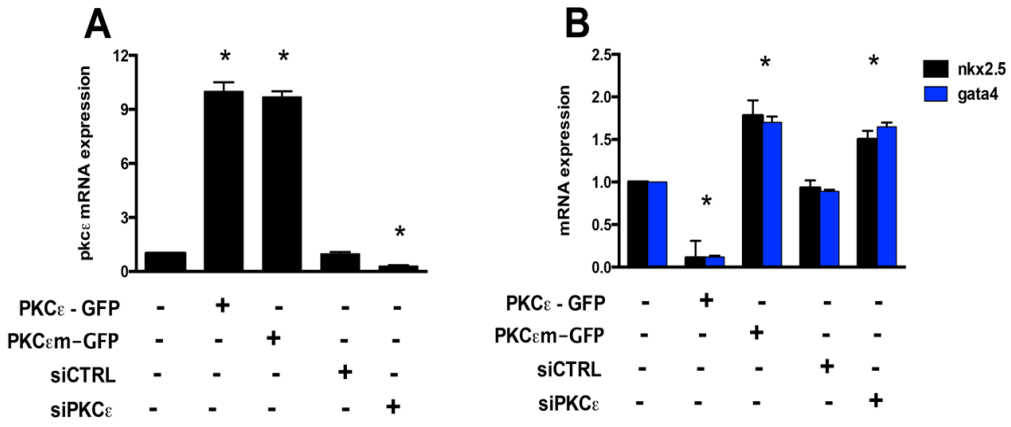

Figure 4.3
Panel A: Quantitative Real Time-PCR for PKCε mRNA expression in BMMSC cultures transfected with wild type pkcε (PKCε-GFP), mutated pkcε (PKCεm-GFP), control siRNA (siCTRL) and specific pkcε siRNA (siPKCε) compared with untrasfected cells (-). n=3; *p<0,05 Anova-Dunnett test (vs untreated cells). **Panel B:** Quantitative Real Time-PCR for nkx2.5 and gata4 mRNAs in the cells transfected as explained in Panel A. Housekeeping pgk was used as reference gene. Values are reported as means of 3 independent experiments ± standard deviation. Cell cultures were transfected 2 day after 5-axacytidine treatment and collected 24h later. n=3; *p<0,05 Anova-Dunnett test (vs untreated cells).

To test the role of PKCε in nkx2.5 and gata4 regulation, we both down-regulated and up-regulated PKCε expression in BMMSCs after 5-azacytidine treatment. Cells were engineered to express either a wild type mouse PKCε-GFP fusion protein (PKCε-GFP) or an inactive PKCε-GFP fusion protein carrying a point mutation in the catalytic core of the enzyme (PKCεm-GFP). The down-modulation was performed by using specific pkcε siRNA or control siRNA that has no known target in the mammalian genome. The analysis of gene expression was performed 2 days after 5-azacytidine treatment, when the PKCε protein level was maximum. mRNA expression of pkcε was analyzed to verify the efficiency of transfection (Fig. 4.3 a). Expression of PKCε-GFP significantly decreased the expression of both nkx2.5 and gata4 mRNAs, while specific pkcε siRNAs and the PKCεm-GFP plasmid induced the expression of these two cardiac markers of differentiation (Fig. 4.3 b). Taking

together, these data suggested that PKCε has a negative role in nkx2.5 and gata4 expression during BMMSCs cardiac differentiation.

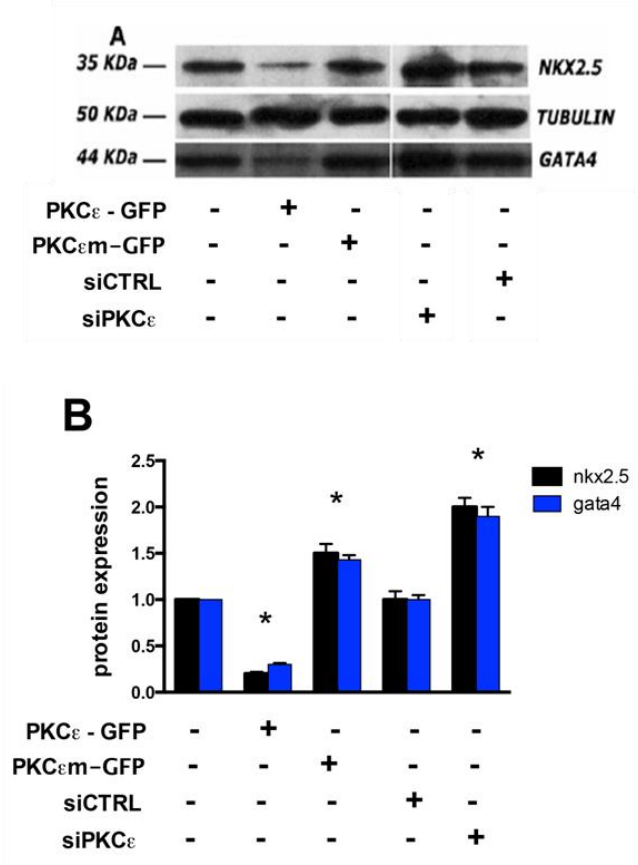

Figure 4.4
Panel A: Western blot analysis of NKX2.5 and GATA4 in BMMSC cultures transfected with wild type pkcε (PKCε-GFP), mutated pkcε (PKCεm-GFP), control siRNA (siCTRL) and specific pkcε siRNA (siPKCε) compared with untrasfected cells (-). TUBULIN was used for normalization. **Panel B:** densitometric analysis of NKX2.5 and GATA4 protein expression. Values are means of 3 independent experiments ± standard deviation. TUBULIN was used for normalization. n=3; *p<0.05 Anova-Dunnet test (vs untreated cells).

4.1.4 PKCε modulates nkx2.5 and gata4 expression via ERK1/2 signaling pathway

To understand how PKCε is able to modulate nkx2.5 and gata4 expression during BMMSCs cardiac differentiation, we decided to study mitogen-activated protein kinases (MAPKs). Extracellular signal-regulated kinases 1/2 (ERK1/2) are known to be downstream of PKCε in a complex signaling pathway that regulate cell proliferation in several models (Basu and Sivaprasad 2007). ERK1/2 proteins are also expressed in cardiomyocytes, where

they are implicated in the regulation of calcium channel expression via nkx2.5 (Marni et al. 2009). Western blot analysis of phospho-ERK1/2 , the active kinase form, showed that PKCε over-expression increases the phosphorilation of ERK1/2, while the siRNA - mediated down-modulation has an opposite effect (Fig. 4.5 a-b). Interestingly, treatment of BMMSCs over-expressing PKCε with the MEK1/2 inhibitor U0126 is able to rescue the expression levels of nkx2.5 and gata4 (Fig. 4.5c).

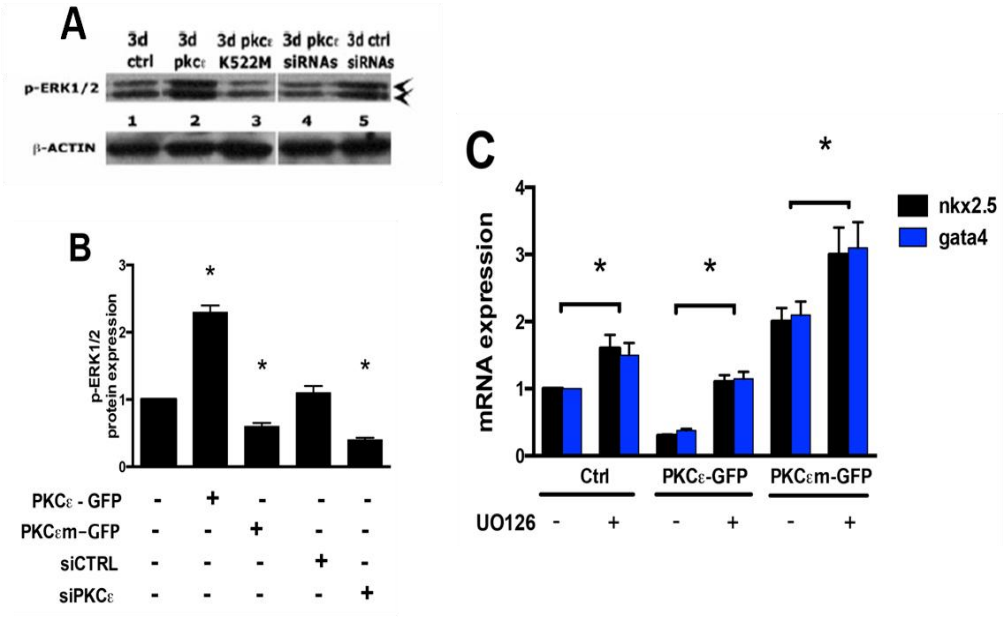

Figure 4.5
Panel A: Western blot analysis of phospho-ERK1/2 (pERK1/2) in BMMSC cultures transfected with wild type pkcε (3d pkcε), mutated pkcε (3d pkcε K522M), control siRNA (3d ctrl siRNAs) and specific pkcε siRNA (3d pkcε siRNAs) compared with untransfected cells (3d ctrl). β-ACTIN was used for normalization. **Panel B:** densitometric analysis of p-ERK1/2 protein expression. Values are means of 3 independent experiments ± standard deviation. β-ACTIN was used for normalization. n=3; *p<0.05 Anova-Dunnet test (vs untreated cells). **Panel C:** Quantitative Real Time-PCR for nkx2.5 and gata4 mRNAs in controls (Ctrl), wild type pkcε (PKCε-GFP) transfected cells and mutated pkcε (PKCεm-GFP) transfected cells, treated with or without U0126. *p<0.05 Anova-Dunnet test (vs U0126 untreated cells).

4.2 Role of PKCε in C_2C_{12} and primary satellite cells skeletal muscle differentiation

4.2.1 PKCε expression is modulated during C_2C_{12} and primary satellite cell differentiation.

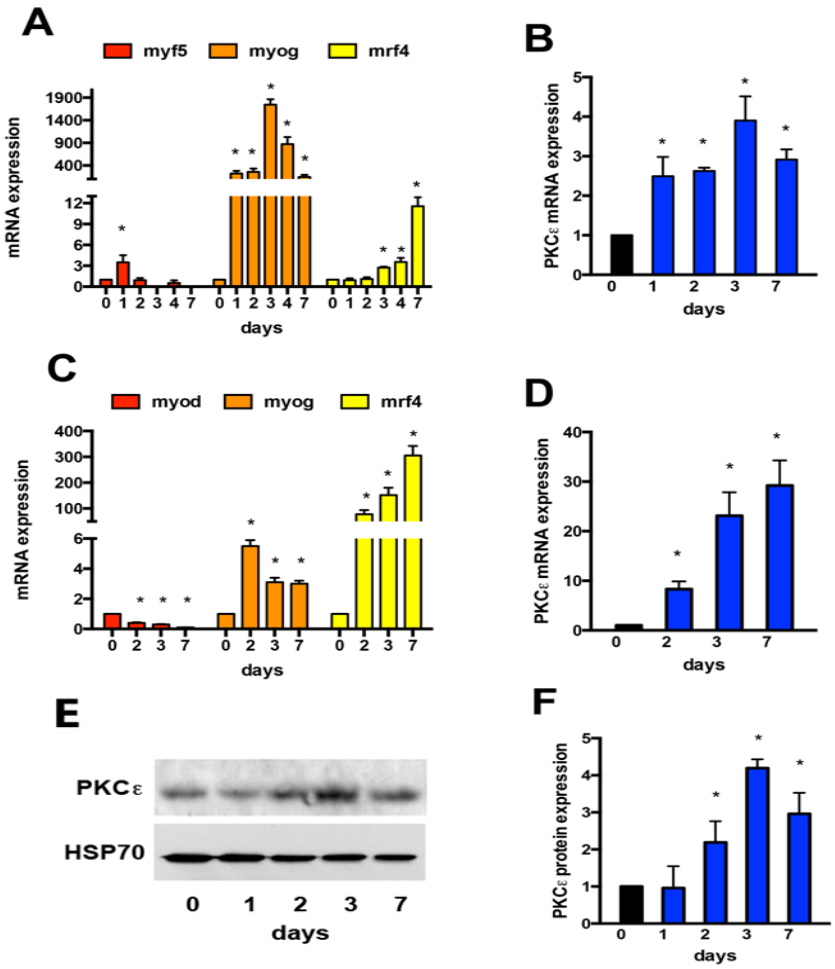

Figure 4.6
Panel A-B: PCR Real Time analysis of myf5, myogenin, mrf4 and pkcε during C_2C_{12} cell differentiation. **Panel C-D:** PCR Real Time analysis of myoD, myogenin, mrf4 and pkcε during primary SC cultures differentiation. **Panel E:** Western Blot analysis of PKCε protein expression levels during C2C12 cell differentiation; HSP70 was used as a housekeeping protein. **Panel F:** densitometric analysis of PKCε protein levels. Results are representative of three independent experiments; values are reported as fold increase of control cell cultures (0 days) ± standard deviation. *p<0.05 Anova-Dunnett test (vs undifferentiated cells).

To evaluate PKCε expression during skeletal myotube formation *in vitro* and *ex vivo*, C_2C_{12} and SC cells, respectively were cultured in Differentiation Medium (DM) for one week.

Quantitative real time PCR and Western Blot analyses of cells collected at several time points during differentiation show that both *Pkcε* mRNA and protein levels were low in proliferating myoblasts but increased significantly during differentiation and subsequent myotube formation (Fig. 4.6 b, d, e, f). We also evaluated the expression of MRFs during differentiation and confirmed that expression of the early myogenic differentiation markers myod and myf5, progressively decreased during the differentiation of primary SC and C_2C_{12} cells, while myog and mrf4 accumulated during myofibers formation (Fig. 4.6 a-c).

4.2.2 Cellular localization of PKCε and phospho-PKCε

To evaluate the subcellular localization of PKCε during the differentiation of C_2C_{12} cell cultures, we used different approaches.

First, immunofluorescence microscopy was applied. In undifferentiated C_2C_{12} cells, PKCε levels were low but significantly increased after the induction of differentiation. PKCε preferentially localized to the nucleus (Fig 4.7a arrow heads) during the first 24 hours of skeletal muscle differentiation, however some cytoplasmic staining was observed at later time points (72 hours) (Fig. 4.7 a). The expression of the late muscle cell differentiation marker myosin was not detected in undifferentiated C_2C_{12} cells, but progressively accumulated in the cytoplasm of forming myotubes.

Second, biochemical fractionation of C_2C_{12} cells revealed that the nuclear content of both total and phosphorylated PKCε protein significantly increased 3 days after the induction of differentiation (Fig.4.7 b-c). Phosphorilation of Ser729 is required for the kinase to achieve the mature conformation and it is a well-known marker of PKCε activation (Xu et al. 2007). We have also observed a concomitant down-regulation of HMGA1, a non-histone nuclear protein involved in the regulation of chromatin condensation and gene transcription and has also been implicated in preventing muscle cell differentiation (Brocher et al.. 2010).

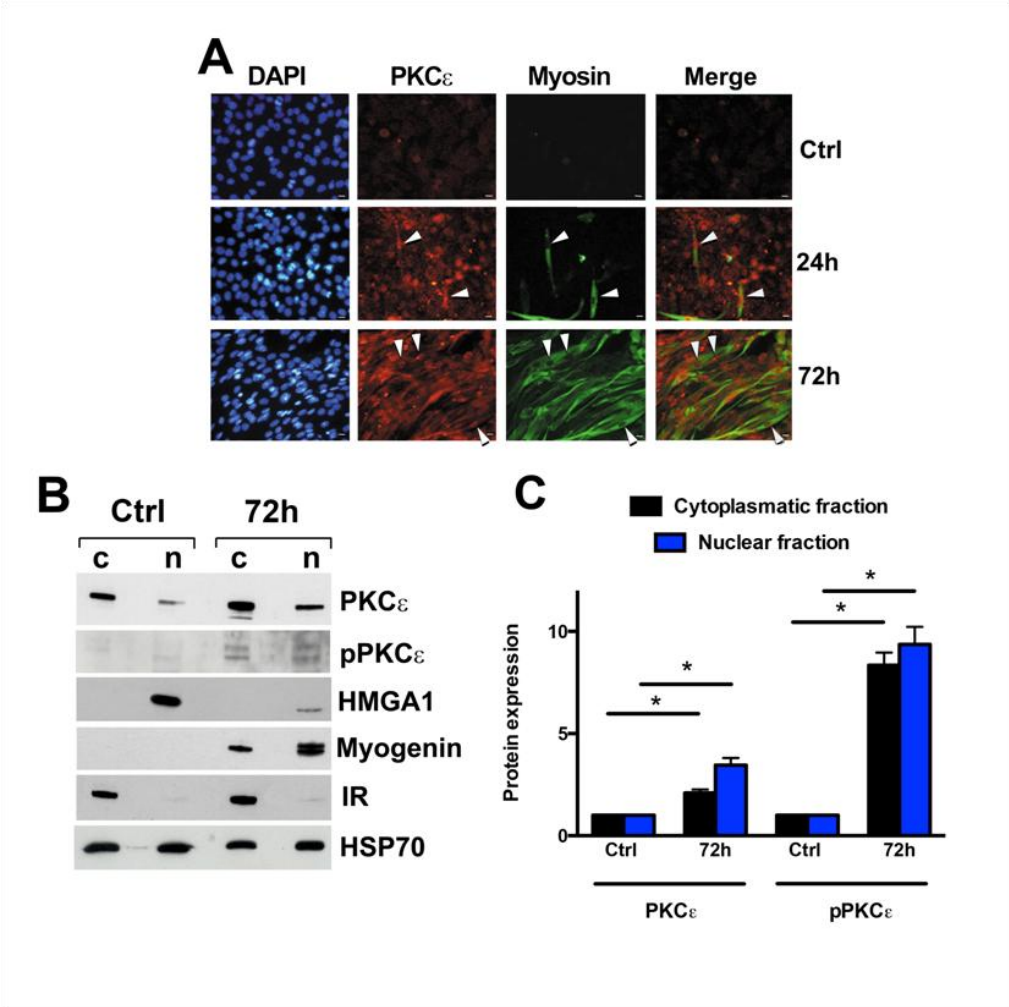

Figure 4.7

The subcellular localization of PKCε was studied by immunofluorescence and western blot analysis of protein expression in nuclear and cytoplasmic fractions of C_2C_{12} undifferentiated (control) and differentiated cell cultures. **Panel A:** DAPI counterstaining of nuclei is shown in blue; PKCε staining shown as red fluorescence; myosin staining shown as green fluorescence. Arrow heads indicate cells with strong PKCε nuclear staining. Scale bar corresponds to 10 μm. **Panel B:** Nuclear (n) and cytoplasmic (c) extracts from undifferentiated (control) and 72h differentiated C2C12 cells (72hs) were resolved by SDS-PAGE; membranes were probed with anti-PKCε, anti phospho-PKCε (pPKCε, Ser-729), anti-HMGA1, anti HSP70, and anti-myogenin antibodies. Anti-IR was used to exclude nuclear contamination by the cytoplasmic fraction. **Panel C:** Densitometric analysis of PKCε and phospho-PKCε expression levels. The values, normalized with respect to HSP70, are the mean of three independent experiments ± standard deviations (n=3). *$p<0.05$ Anova-Dunnett test (vs control cells).

4.2.3 PKCε up-regulation induces skeletal muscle differentiation via Myogenin and Mrf4 modulation

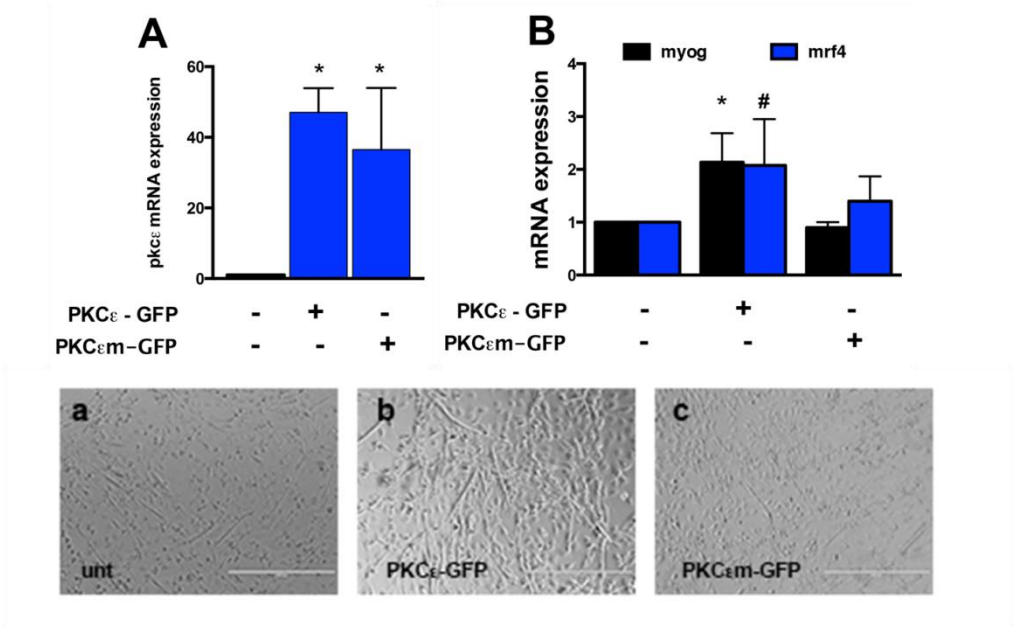

Figure 4.8
Panel A: Quantitative Real Time-PCR for PKCε mRNA expression in C_2C_{12} cell cultures transfected with wild type pkcε (PKCε-GFP) or mutated pkcε (PKCεm-GFP) compared with untrasfected cells (-). n=3; *p<0,05 Anova-Dunnett test (vs untreated cells). Panel B:
Quantitative Real Time-PCR for myogenin and mrf4 mRNA in C_2C_{12} transfected with wild type pkcε (PKCε-GFP) or mutated pkcε (PKCεm-GFP). Housekeeping gusb was used as reference gene. Values are reported as means of 3 independent experiments ± standard deviation. Cell cultures were transfected and differentiated for 2 days. n=3; *p<0,05 Anova-Dunnett test of MRF4 expression (vs untreated cells); #p<0,05 Anova-Dunnett test of Myogenin expression (vs untreated cells). Panel B a, b and c: Cell morphology was analyzed by bright-field observation.

To determine whether PKCε expression was correlated to myoblast differentiation and MRFs induction in the *in vitro* C_2C_{12} cell model, these cells were engineered to express either a wild type mouse PKCε -GFP fusion protein (PKCε -GFP) or an inactive PKCε -GFP fusion protein carrying a point mutation in the catalytic core of the enzyme (PKCεm-GFP). Two days after differentiation induction, cell morphology was analyzed by bright-field observation showing that the myotube numbers increased in PKCε-overexpressed cells, comparing with the inactive PKCε transfected cells (Figure 4.8 a, b , c). At the same time point, cells were collected and analyzed for myog and mrf4 expression by quantitative RT-PCR. Expression of PKCε-GFP, but not the inactive mutated PKCεm-GFP, significantly increased myog and mrf4 mRNA expression (Fig. 4.8 A-B) with respect to untreated cells.

These results were confirmed using a pharmacological approach to modulate PKCε expression. C_2C_{12} cells and primary SC cultures were treated with the ψεRACK PKCε specific activator displaying an increased myog and mrf4 mRNA expression, whereas the εV1-2 PKCε inhibitor yielded the opposite effect (Fig. 4.9).

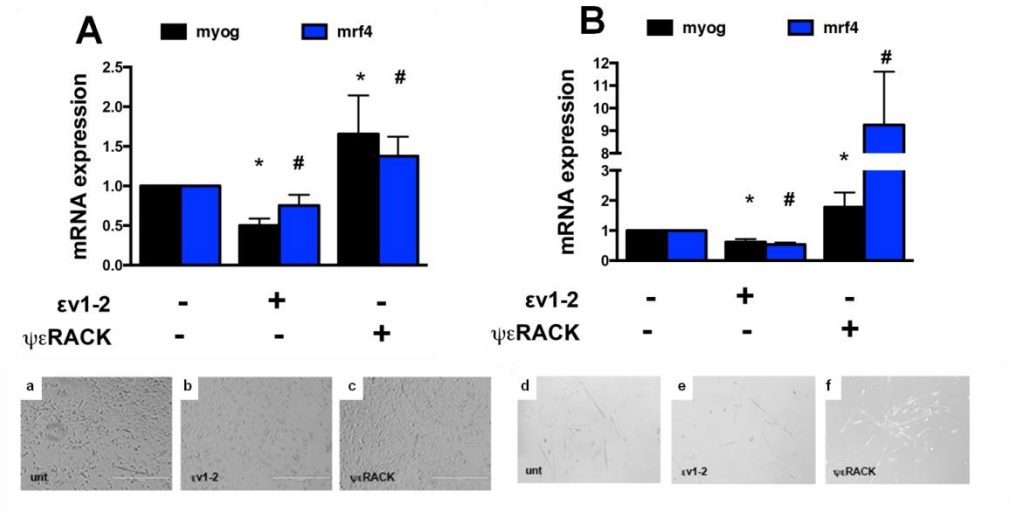

Figure 4.9
Quantitative Real Time-PCR for myogenin and mrf4 mRNA in C_2C_{12} (Panel A) and SC cultures (Panel B) treated with 1 μM of PKCε specific activator and inhibitor (ψεRACK and εV1-2 peptides, respectively). Housekeeping gusb was used as reference gene. Values are reported as means of 3 independent experiments ± standard deviation. Cell cultures were transfected and differentiated for 2 or 3 days (Panel A and B, respectively). n=3; *p<0,05 Anova-Dunnett test of Myogenin expression (vs untreated cells); #p<0,05 Anova-Dunnett test of MRF4 expression (vs untreated cells). Cell morphology was analyzed by bright-field observation (Panel A a, b and c; Panel B d, e and f).

4.2.4 PKCε down-modulates hmga1 during C_2C_{12} cell differentiation.

Looking for a molecular target of PKCε signaling, we then analyzed the expression levels of HMGA1 during myogenic cell differentiation. According to Brocher et al., we found a progressive decrease of HMGA1 expression (Fig. 4.10 a) in C_2C_{12} cell cultures induced to terminal differentiation. To formally demonstrate that PKCε could remove HMGA1 inhibition, allowing myoblasts to start the differentiation program, we then over-expressed PKCε in C_2C_{12} cells growing in complete medium. Figures 4.10 b and c show that the rapid accumulation of PKCε in undifferentiated C_2C_{12} cells promoted a parallel decrease of HMGA1 expression. At the same time Myogenin started to accumulate, notwithstanding the

persistence of mitogenic stimuli (10% of serum). To definitively prove the functional link between PKCε and HMGA1, we further performed double-transfection experiments with PKCε-specific shRNA and HMGA1-specific siRNA. Figure 4.10d shows that the sole down-modulation of hmga1 crucially increases myog and Mrf4 transcription, as expected. On the contrary, pkcε silencing dramatically inhibited myog and mrf4 expression, blocking muscle differentiation. Of note, double silencing of Pkcε and Hmga1 induced the expression of muscle differentiation markers, indicating the functional necessity of Hmga1 down-regulation in the induction of the muscle cell differentiation program (Fig. 4.10 d).

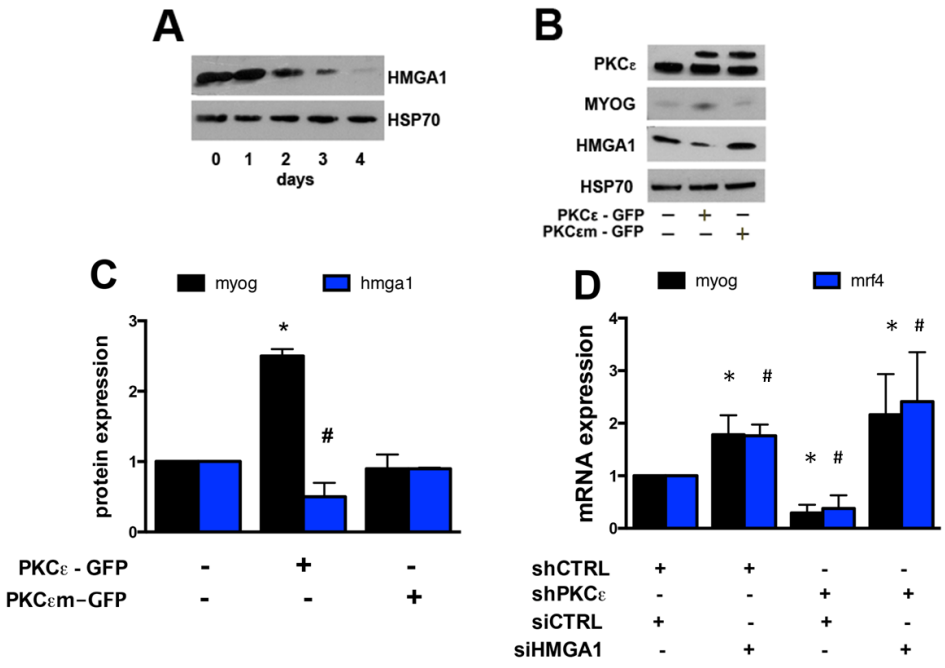

Figure 4.10
Panel A: Western blot analysis of HMGA1 during C_2C_{12} myogenic differentiation for 4 days. HSP70 was used for normalization. **Panel B:** Western blot analysis of HMGA1, Myogenin, PKCε and HSP70 in undifferentiated C_2C_{12} cell cultures treated with vectors expressing wild type PKCε (PKCε-GFP) or mutated PKCε (PKCεm-GFP). **Panel C:** densitometric analysis of HMGA1 and myogenin protein expression in C_2C_{12} cells transfected with wild type or mutated PKCε. Values are means of 3 independent experiments ± standard deviation. HSP70 was used for normalization. n=3; *p<0.05 Anova-Dunnet test (vs untreated cells). **Panel D:** Quantitative Real Time-PCR for myog and mrf4 mRNA expression in C_2C_{12} cell cultures infected with PKCε specific shRNA (shPKCε) or control shRNA (shCTRL). After selection of infected cells with puromycin (2μg/ml), cells were transfected with HMGA1 specific siRNAs (siHMGA1) or control siRNA (siCTRL) and then induced to muscle differentiation. Sample was collected at 2 days of differentiation. n=3 *p<0,05 Anova-Dunnett test of mrf4 expression (vs control cell cultures); #p<0,05 Anova-Dunnett test of Myogenin expression (vs control cell cultures).

4.2.5 In vivo induction of PKCε during muscle regeneration

We studied the *in vivo* expression levels of PKCε during skeletal muscle regeneration experiments in cardiotoxin (CTX) treated mice. Figure 4.11a shows a spontaneous up-regulation of PKCε expression in the damaged muscle starting from day 3 after the CTX injury. Morphological analysis shown in figure 4.11b confirms the expression of PKCε in most fibers of the injured region including the new regenerating fibers (centrally-nucleated fibers) (Fig. 4.11c).

To study the *in vivo* the effects of PKCε modulation on muscle regeneration, we first injected mouse tibialis muscle with CTX together with the PKCε inhibitor peptide (εV1-2) or the PKCε activator peptide (ψεRACK). Subsequently, protein levels of the myogenic factors MYOG and MYOD and PKCε phosphorylation levels (p-PKCε) were studied at 3 and 7 days after treatment. At day 3 we did not observe a difference in MYOG and MYOD expression (data not shown), while at day 7 both MYOG and MYOD decreased in muscles injected with PKCε inhibitor peptide, confirming the role of PKCε in in vivo muscle regeneration (Fig 4.11 d-e).

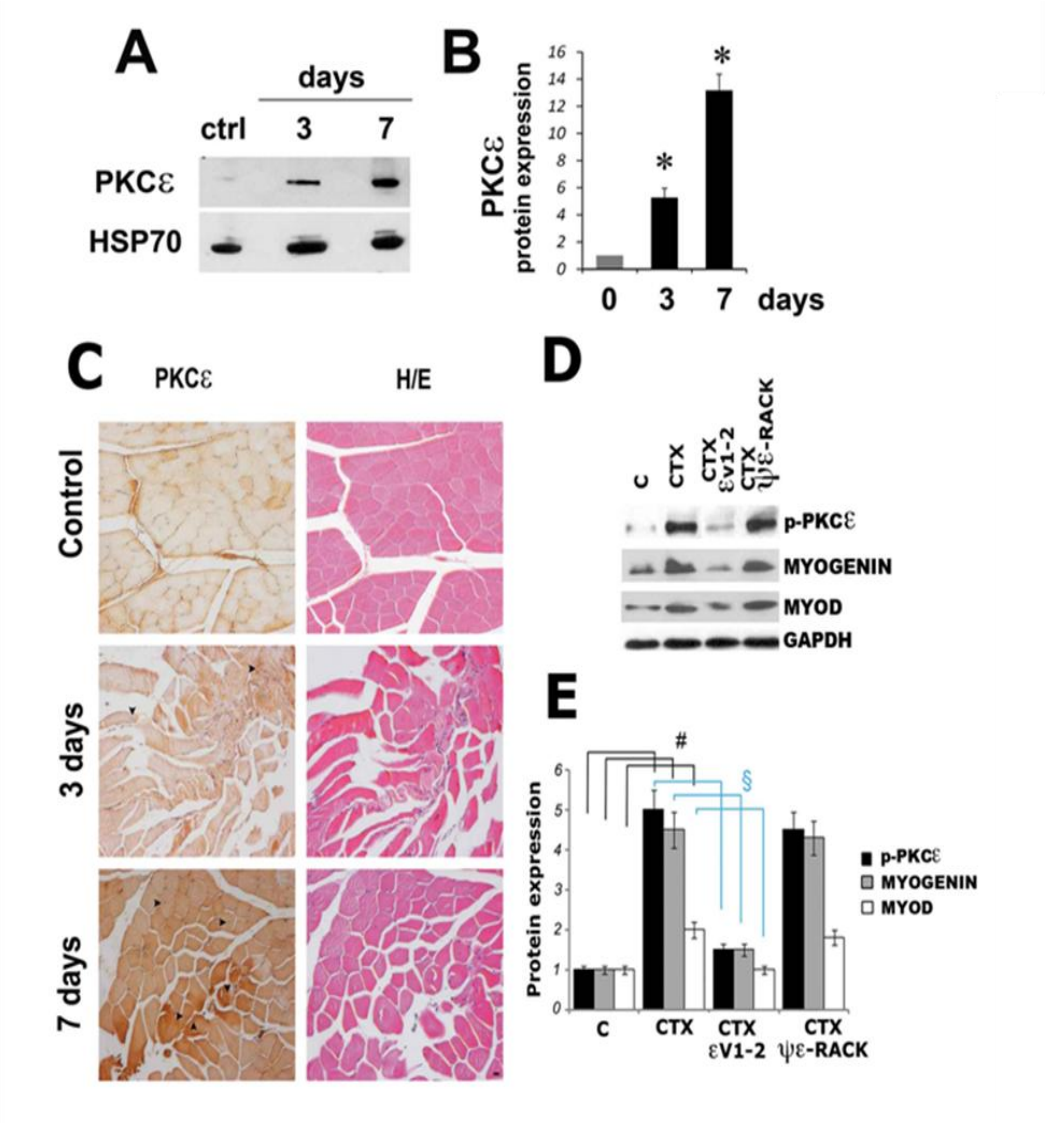

Figure 4.11

Panel A: Western blot analysis of protein extracts from regenerating tibialis muscle at 3 and 7 days after cardiotoxin induced injury in CD1 adult mice. The blot was incubated by anti-PKCε and anti-myogenin antibodies. HSP70 confirmed equal loading samples. **Panel B:** Densitometric analysis of PKCε protein levels. Values, normalized by HSP70 expression levels, are mean of 3 independent experiments ± standard deviations. **Panel C:** Immunohistochemical detection of PKCε and haematoxilin/eosin (H/E) staining of serial muscle section of CD1 untreated adult mice (control) and treated with CTX (3 and 7 days). Centro-nucleated regenerating fibers expressing PKCε are indicated (arrow heads). Scale bar corresponds to 40 μm and it is the same for all panels. **Panel D:** p-PKCε, Myogenin and MYOD western blot analysis of protein extracts from regenerating tibialis muscles at 7 days after cardiotoxin (CTX), cardiotoxin with εV1-2 (CTX εV1-2) and cardiotoxin with ψε RACK (CTX ψεRACK) injection. GAPDH was used as a loading control. **Panel E:** Densitometric analysis of p-PKCε, Myogenin and MyoD expression levels. The values, normalized with respect to GAPDH, are mean of 3 independent experiments ± standard deviations. *p<0,05 Anova-Dunnett test of PKCε expression vs untreated mucle; # p≤ 0,05 and § p≤0,03 Anova-Dunnett-test.

DISCUSSION

The ε isoform of the novel group of PKC family is a serine-threonine kinase that has been implicated in many biological processes such as proliferation, differentiation, carcinogenesis and cell death (Newton and Messing, 2010). PKCε is expressed in a wide variety of tissues and organs, including brain, skin, liver, adipose tissue, kidney, heart and skeletal muscle. We and others have shown its role in the differentiation of hematopoietic (Gobbi et al., 2007; Mirandola et al., 2006; Gobbi et al., 2009) and intestinal (Gobbi et al., 2012) cells, but to date very little information is available on its role in both cardiac and skeletal muscle differentiation.

Role of PKCε in Bone Marrow Mesenchymal Stem Cells (BMMSCs) cardiac differentiation

The prevailing paradigm that the heart is a terminally differentiated organ and cardiomyocytes are all non-dividing cells is outdated. Also if the proliferative ability of adult cardiomyocytes is very low (Senyo et al. 2013), de novo cardiomyogenesis after injury was proved (Malliaras et al. 2013). However, little is known about the molecular mechanisms driving cardiomyocyte or other stem cell sources to complete cardiomyogenic differentiation.

In the heart, PKCε was heavily characterized for its cardioprotective effects and its ability to mediate the preconditioning in ischemia-reperfusion injury. Its chemical activation before heart ischemia results in a strong cardioprotective effect (Inagaki et al. 2005), suggesting that it is needed in this process. Several mechanisms were proposed. The activation of PKCε induces the expression of mitochondrial Aldehyde Dehydrogenase 2 (ALD2), resulting in a cardioprotective effect on the damaged heart (Dorn et al. 1999; Chen et al. 2008). PKCε also increases sarcK_{ATP} channel activity after preconditioning (Aizawa et al. 2004) and directly phosphorilates connexin43, a well known component of cardiomyocyte Gap junctions. (Doble et al., 2000; Bowling et al., 2001).

The current work has led to the identification of a new PKCε pathway implicated in the modulation of cardiac transcription factors nkx2.5 and gata4. We chose 5-azacytidine

treated - Bone Marrow Mesenchymal Stem Cells as *in vitro* model of cardiac differentiation (BMMSCs) (Makino et al.1999). The results show that PKCε has a peculiar kinetic of expression, with maximum expression occuring two days after 5-azacytidine induction of differentiation. This transient up-regulation of PKCε is followed by a strong down-modulation until day 7. Interestingly, both the cardiac transcription factors nkx2.5 and gata4 show similar expression profiles that are opposite to that of PKCε. This evidence supported the thesis that PKCε could be a negative modulator of nkx2.5 and gata4 transcription genes.

To better characterize this effect, we forced the modulation of PKCε by overexpressing vectors or with specific siRNAs. The results shown in this thesis demonstrate that the silencing of PKCε during the early phases of differentiation induced a significant increase of nkx2.5 and gata4. Opposite effects are shown when cells are transfected with an overexpressing vector. Surprisingly, cells expressing the K522M mutant form of PKCε - a mutation in the active site that prevents the ability of the kinase to phosphorilate its substrates- has a significant increase of nkx2.5 and gata4 expression, showing a dominant negative effect.

To understand the signaling pathway activated by PKCε during cardiac differentiation, we decided to study MAPK signaling and particularly the activation of ERK1/2 proteins. Previous studies have shown that PKCε is able to modulate cardiomyocyte proliferation and apoptosis via the ERK1/2 pathway (Basu and Sivaprasad 2007) and that the activation of ERK1/2 has a negative effect on nkx2.5 expression in cardiomyocyte cells (Marni et al. 2009). Experiments performed on cultures of 5-axacytidine - treated BMMSCs show that the activation of ERK1/2 is downstream of PKCε and that the abrogation of ERK1/2 phosphorilation, mediated by the chemical inhibitor U0126, significantly increases the expression of nkx2.5 and gata4, reverting the effect of PKCε up-regulation.

Finally, the results reported in this thesis show that the expression of PKCε during cardiac differentiation have to be transient and finely regulated. In the early stage of cardiac differentiation PKCε has a negative role to regulate the expression of two essential cardiac transcription factors, nkx2.5 and gata4, via activation of the ERK1/2 signaling pathway.

Role of PKCε in skeletal muscle differentiation

During muscle development, a complex network of signaling pathways induces the myoblasts to fuse together and form muscle fibers. After birth, postnatal muscle growth and regeneration is guaranteed by the resident stem cell called satellite cells. Little is known about PKCs involvement in muscle cell differentiation. PKCθ is required for myoblast fusion, regulating FAK activation and, in turn, the expression of the pro-fusion genes caveolin-3 and β1D integrin (Madaro et al. 2011). Recently, Gaboardi et al. have shown that PKCε participates in insulin signaling, supporting muscle cell differentiation (Gaboardi et al. 2011). In the present investigation, we demonstrate that PKCε up-regulation during myogenic cell differentiation is required for late phase gene transcription and terminal differentiation. The C_2C_{12} *in vitro* cell model helped us to understand the molecular pathway that links PKCε to the expression of myogenin, a key transcription factor of skeletal muscle differentiation. This function of PKCε involves the down-modulation of the chromatin binding protein HMGA1. The interplay between PKCε and HMGA1 was previously described to explain in part the ability of the active form of PKCε to repress the transcription of the insulin receptor, playing an important role in the induction of insulin resistance (Dey et al.2007). Our finding demonstrates that PKCε-HMGA1 axis activation also has an important implication also in skeletal muscle differentiation. The model proposed suggests that during differentiation PKCε expression and activation is up-regulated. The active form of the kinase, phosphorilated in the Ser 729 site, is able to translocate to the nucleus. Here, PKCε down- modulates the expression of HMGA1 and allows for the transcription of essential myogenic transcription factor such as Myogenin and Mrf4. Further studies will be needed to understand how PKCε modulates HMGA1 expression. Experiments conducted in other models suggest a direct interaction between these proteins and the ability of PKCε to directly phoshorilate HMGA1.

Finally, we studied the involvement of PKCε in a murine model of cardiotoxin - induced injury in muscle. We found that PKCε expression is up-modulated 7 days after injury and it is localized preferentially in regenerating fibers. Pharmacological inhibition via

intramuscolar injection of a specific PKCε inhibitor peptide (εV1-2), led to a decrease of the active phospho-PKCε, Myogenin, and Myod expression suggesting a PKCε contribution to *in vivo* muscle regeneration. The PKCε activator peptide has no effects on PKCε phosphorylation and Myod and Myogenin expression induced by CTX, maybe because PKCε activation is phisiologically very high in the injured muscle.

Overall, by showing that PKCε is an upstream key regulator of skeletal muscle cell differentiation, we believe that it might represent an attractive model to be translated into human for further studies on satellite cell-driven muscle repair and substitution, with obvious clinically relevant implications in muscle pathology as atrophy, dystrophy and sarcopenia.

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PUBLICTIONS AND ABSTRACTS

Publications:

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