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MEF2C exon α: Role in gene activation and differentiation

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ABSTRACT

Myocyte enhancer factor 2C (MEF2C) belongs to the MEF2 transcription factors. All products of MEF2 genes have a common amino-terminal DNA binding and dimerization domain. All four vertebrate MEF2 gene transcripts are also alternatively spliced. In the present study we identify two novel MEF2C splice variants, named VP and VP2. These variants are generated by the skipping of exon α. The identified α− variants are ubiquitously expressed, although at very low levels compared to the α+ variants. The existence of MEF2C α− variants gave us the opportunity to study for the first time the function of exon α. Transactivation experiments show that the presence of exon α induces a reduction of transcription levels. Moreover, α− variants are significantly expressed during neuronal cell differentiation, indicating a putative role of these variants in development.

1. Introduction

Myocyte enhancer factor 2 (MEF2) proteins are members of the MADS (MCM1, agamous, deficiens, serum response factor)-box family of transcriptional regulators (Shore and Sharrocks, 1995). Four distinct vertebrate genes encoding MEF2 isoforms, referred to as *mef2a*, *mef2b*, *mef2c*, and *mef2d*, are located on different chromosomes in vertebrate genomes. MEF2 transcription factors recognize and bind to the consensus DNA sequence YTA(A/T)$_4$TAR as homo- or heterodimers via a 57-amino acid domain (i.e., MADS-box) (Black and Olson, 1998). Adjacent to the MADS-box is a 29-amino acid extension, known as the MEF2 domain, which contributes to high-affinity DNA binding and dimerization with other homologous MEF2 proteins and facilitates interactions with other cofactors (Potthoff and Olson, 2007). The C-terminal of MEF2 proteins, which is subject to complex patterns of alternative splicing, contains the transcriptional activation domain to promote signal transduction and/or regulate target gene transcription (Wu et al., 2011).

It has been reported that MEF2 proteins control multiple biological processes, including skeletal muscle and neural differentiation, and heart and bone development (Potthoff and Olson, 2007). The four MEF2 genes are differently expressed both spatially and temporally during development and in mature tissues (Edmondson et al., 1994). However, MEF2 isotype functions partially overlap and distinct roles for the different genes remain to be fully elucidated. Murine gene disruption studies provide genetic evidence in support of discrete MEF2 isotype-specific functions. For example, *mef2c* null mice die at embryonic day 10 due to failure of cardiac development and also exhibit vascular defects (Bi et al., 1999; Lin et al., 1997, 1998). In the *mef2c* null animals, the other MEF2 genes (*mef2a*, *mef2b*, and *mef2d*) are expressed at normal or supraphysiological levels highlighting the lack of compensation and a unique role for MEF2C in cardiac and vascular development (Zhu and Gulick, 2004; Zhu et al., 2005).

In contrast to the *mef2a*, *mef2b*, and *mef2d* genes, the *mef2c* gene is expressed in a tissue-specific manner and is restricted to brain, skeletal and cardiac muscle (Edmondson et al., 1994; Lyons et al., 1995). The *MEF2C* gene consists of 13 exons and the primary transcript is alternatively spliced. The mutually exclusive alternative splicing takes place in exons α1 and α2 immediately adjacent to the MEF2 domain. Exon β, located in transcriptional activation domain II (TAD II), is a cassette exon. The 3′ splice site selection-type of alternative splicing, the γ region, is located in the terminal coding exon of the *MEF2C* gene. It has been demonstrated that this γ region encodes a transcriptional repression domain (Zhu and Gulick, 2004).

In this study we identified two novel MEF2C splice isoforms lacking exon α (α−), termed VP and VP2, focusing on their expression patterns and biological functions. The VP variant excludes the β exon (β−) and includes the γ domain (γ+). On the contrary, the VP2 variant includes the β exon (β+) and excludes the γ domain (γ−). The α− expression pattern in numerous adult tissues and α−/total MEF2C mRNA ratio in

Abbreviations: db-cAMP, dibutyryl-cAMP; FOX1, RNA binding protein fox-1 homolog 1; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; LUC, luciferase; MEF2C, myocyte enhancer factor 2C; RA, retinoic acid.

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fetal and adult brain and heart tissues are described. Finally, the function encoded in exon α, which has been unknown so far, is reported herein.

2. Materials and methods

2.1. Construction of plasmids

A threefold repeat canonical MEF2 site (3×MEFSITE) (Table 1) was cloned into the pGL3 promoter-LUC vector (Promega, Madison, WI, USA) upstream of the firefly luciferase cDNA (pGL3-3×MEFSITE) as described previously (Infantino et al., 2007). *MEF2C* coding regions were obtained using reverse transcription-PCR with human heart, brain, or skeletal muscle RNAs as templates. All amplification reactions were performed in a 50 μl volume containing iProof High Fidelity DNA Polymerase (Bio-Rad Laboratories, Hercules, CA, USA), 10 pmol of each primer (see Table 1), 50 ng of cDNA template and 1 μl of dNTPs (10 mM for each nucleotide) (Abate et al., 2012).

The MEF2C α1, α1γ, α1β, γ (VP) and β (VP2) splicing variant expression vectors were prepared by cloning *MEF2C* coding regions into the TOPO-TA cloning vectors (Life Technologies, Paisley, UK) and then transferred to the pcDNA3.1 expression vector (Life Technologies). The VP and VP2 amplified products were also cloned into a modified pcDNA3 expression vector (pcDNA3.1/CT-GFP-TOPO, Life Technologies) in-frame with the GFP coding sequence, following the manufacturer's protocol. Human FOX1 cDNA (Accession No. NM_145891.2) was amplified from human embryonic kidney (HEK293) cDNA using forward and reverse primers (Table 1) and subsequently cloned in the pcDNA3.1 expression vector (pcDNA3-FOX1). The fidelity of the final inserts in pcDNA3 plasmid was verified by DNA sequencing using BigDye Terminator Kit (Life Technologies), pcDNAF and pcDNAR primers (Table 1).

2.2. Cell culture and transient transfection

Human embryonic kidney HEK293 (ATCC: CRL-1573) and human epithelioid cervix carcinoma HeLa (ATCC: CCL-2) cells were grown as described in (Iacobazzi et al., 2005, 2009). Human neuroblastoma SH-SY5Y cells (ATCCID: CRL-2266) were grown in a 1:1 mixture of DMEM and minimum essential medium (MEM) supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin/streptomycin (100 U/100 μg/ml), 1 mM sodium pyruvate and 0.1 mM non-essential amino acids. All cell culture processes were carried out in a humidified atmosphere of 5% CO_2, at 37 °C. Transient transfection experiments were performed using FuGENE® HD Transfection Reagent (Promega) (Infantino et al., 2011b). HEK293 cells were transfected with pcDNA3-FOX1 or cotransfected with pGL3-3×MEFSITE and MEF2C splicing variant expression vectors. Luciferase (LUC) activity in cell extracts was measured in a 96-well plate format by using a VICTOR^3 multilabel plate reader (PerkinElmer, Waltham, MA, USA). The extent of transfection was normalized by β-galactosidase activity (Laghezza et al., 2013). HeLa cells were transfected with pcDNA3.1/CT-GFP-TOPO plasmid containing or void of the VP and VP2 sequences. For SH-SY5Y cell differentiation, growth medium was replaced with serum-free medium for 16 h. The cells were then stimulated with differentiation medium (DM) consisting of serum-free medium supplemented with 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, St Louis, MO, USA), 10 μM retinoic acid (RA; Sigma) and 1 mM dibutyryl-cAMP (db-cAMP; Sigma) (Tio et al., 2010). SH-SY5Y cell differentiation was monitored by evaluating cell morphology under phase-contrast microscopy and by measuring the expression levels of neuron specific synaptophysin and synapse-associated protein-97 (SAP97), two neuronal differentiation markers. Cells were harvested at the indicated time points and total RNA was isolated and analyzed.

2.3. Reverse transcriptase-PCR and real-time PCR

Total RNA was extracted from 1 × 10^6 HEK293 and SH-SY5Y cells as described previously (Infantino et al., 2011a). The RNAs from cell lines and human RNAs purchased from Clontech (Mountain View, CA, USA), which include skeletal muscle, liver, kidney, fetal brain and heart, adult brain and heart, and diseased heart were reverse-transcribed with GeneAmp® RNA PCR Core Kit (Life Technologies). RT-PCR was carried out using three sets of primers (Table 1). Real-time PCR was conducted as previously described (Iacobazzi et al., 2008). TaqMan probes for total human MEF2C (Hs00231149_m1) and human β-actin (4326315E) were purchased from Life Technologies. A specific TaqMan probe for α-MEF2C isoforms was designed by using the IDT SciTools (Table 1) and provided by IDT (Coralville, USA). MEF2C transcript levels were normalized against the β-actin expression levels.

2.4. Western blotting

Thirty micrograms of HEK293 total proteins were heated at 100 °C for 5 min, separated on 4–12% SDS polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then blocked for 1 h in a PBS solution containing 2% bovine serum albumin and 0.1% Tween 20, and then treated at room temperature with anti-MEF2C

Table 1
Oligonucleotides used in this study.

Primer name	Primer sequence 5′–3′
αFOR	ACAAACTCAGACATCGTGGAG
αREV	AAGTTGGGAGGTGGAACAG
βFOR	TCTGAGGATGTCGACCTGCT
βREV	TGAAATGGCTGATGGATATCC
γFOR	GAGCTTGCACTAGCACTCATT
γREV	TGTTGTGGGTATCTCGAAGG
MEF2CFORNHE	ACGGCTAGCATGGGGAGAAAAAAGATTCAGATTACGA
MEF2CREVHIND	CGAAAGCTTTCATGTTGCCCATCCTTCAGAAAG
pcDNAF	AATACGACTCACTATAGGGA
pcDNAR	AGAAGGCACAGTCGAGGC
3×MEFSITE	ACGGCTAGCTGTTGCTAAAAATAGAATGTTGCTAAAAATAGAATGTTGCTAAAAATAGAA AGATCTTAC
FOX1 FOR	CAGGCTAGCATGCTGGCGTCTCAAGGAGTTC
FOX1 REV	CGAAAGCTTTTAGTATGGAGCAAAACGGTTG
FOR real-time	AAC AGC ACC AAC AAG CTG TTC CAG
REV real-time	ACA GCC TCC ACG ATG TCT GAG TTT
PROBE real-time	AG CAC CGA C A TGG ACA AAG TGC TTC T
MEF2CFOR GFP	ACCATG GGG AGA AAA AAG ATT CAG ATT A
MEF2CREV GFP	TGTTGCCCATCCTTCAGAAAGT

and anti-β-actin antibodies (Santa Cruz, Santa Cruz, CA, USA). The immunoreaction was detected by Immobilon Western Chemiluminescent HRP (horseradish peroxidase) Substrate (Millipore, Billerica, MA, USA).

2.5. Fluorescence imaging

For microscopic imaging HeLa cells were plated on glass coverslips. The expression vectors were transfected into HeLa cells as described above. Empty pcDNA3.1/CT-GFP-TOPO plasmid was transfected as control. Forty-eight hours post-transfection, coverslips were incubated with 1 μg/ml DAPI (Life Technologies) according to the manufacturer's protocol. The cells expressing GFP alone or GFP fusion proteins were directly used for fluorescent visualization. Fluorescent images were acquired through a Zeiss Axiovert 200 Inverted Microscope (Zeiss, Jena, Germany) equipped with a CoolSNAP HQ CCD camera (Roper Scientific, Trenton, NJ, USA). The images were analyzed using Metamorph software (Universal Imaging Corporation, Downington, PA, USA).

3. Results

3.1. Identification of two novel human MEF2C variants

We amplified MEF2C cDNA from human heart, skeletal muscle and brain, cloned the PCR products in TOPO-TA cloning vectors and analyzed each clone with three sets of primers encompassing the alternatively spliced regions (Fig. 1A, Table 1). Among the screened clones, two positive clones showed an amplification band containing a β or γ exon but not α exon amplification (Fig. 1B). Subsequent sequencing analyses revealed that these clones represent two novel MEF2C splicing variants, which were named VP and VP2. The nucleotide sequences of these new splice variants were deposited in GenBank with the accession numbers FM163484 and FM180475, respectively. Compared to the full-length MEF2C isoform, VP and VP2 skip the alternative third exons 3α1 and 3α2 and include the β exon (VP2) and γ region (VP), respectively (Fig. 1C). Thus, besides the eight MEF2C variants described previously (Zhu and Gulick, 2004; Zhu

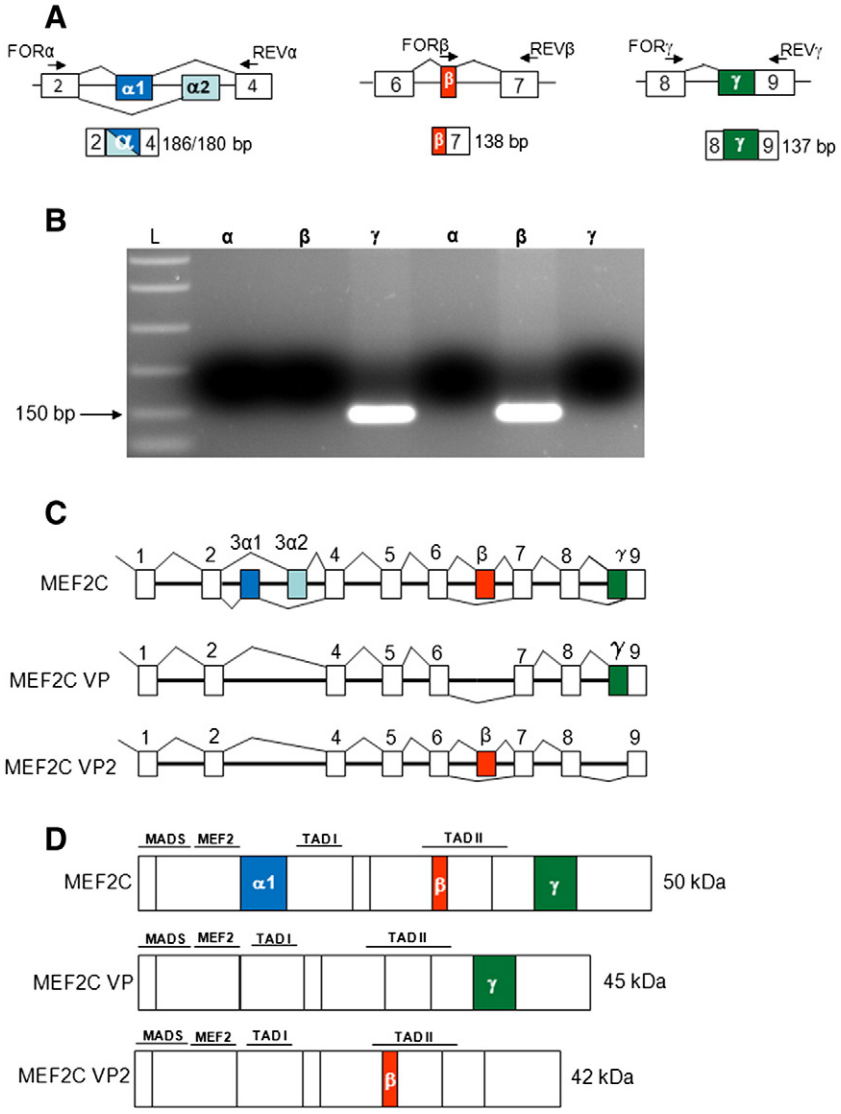

Fig. 1. Alternative splice isoforms of human MEF2C. (A) Schematic representation of MEF2C alternative exons, including the mutually exclusive exons α1 and α2 (blue and light blue boxes, respectively), exon β-skipping (red box) and 3′ splice site selection of the γ region (green box). Arrows show the primer annealing sites. Numbers indicate the length of the PCR products. (B) Amplification of the α, β and γ regions in two clones. Lane L represents the DNA ladder. (C) MEF2C coding exons. The open boxes represent the conventional exons. The three alternative exons are indicated by blue and light blue boxes (exons α1 and α2, respectively), a red box (exon β), and green box (γ region). The coding exons of the VP and VP2 splicing isoforms are also shown. (D) MEF2C protein structure. The N-terminal region of the MEF2C protein contains the MADS-box domain and MEF2 domain involved in DNA binding and dimerization. Two transcriptional activation domains (TAD I and TAD II) are located in the middle of the MEF2C protein. Numbers indicate the molecular weight of proteins in kilodaltons. VP and VP2 protein structures are also displayed.

et al., 2005), there are two additional MEF2C variants that exclude the exon α (Fig. 1C). The predicted structure of the MEF2C protein variants compared to the structure of the full-length MEF2C protein is depicted in Fig. 1D. VP and VP2 are identical to the full-length MEF2C protein within the MADS boxes and MEF2 domains as well as within the transcriptional activation domains, TAD I and TAD II.

3.2. Cellular localization of VP and VP2 proteins

To analyze the subcellular localization of VP and VP2, fusion proteins of both isoforms to GFP were generated and used for HeLa cell transient transfection experiments. Fluorescence was measured after 48 h using a fluorescence microscopy CCD system. MEF2C VP and VP2 clearly colocalized with the nuclear marker (DAPI), indicating that they are primarily located in the nucleus (Fig. 2). To confirm these data, the constructs were also transfected into SK-N-SH cells and the same distribution of GFP was detected (data not shown). Subcellular localization of VP and VP2 was equivalent to that of full-length MEF2C suggesting that exon α is not involved in cellular localization process.

3.3. Role of exon α in promoter activation

Given that MEF2C is a transcription factor, the transactivation ability of the newly identified VP and VP2 splice variants, lacking exon α, was tested. To this end, HEK293 cells were transiently transfected with several MEF2C transcripts, including the VP (γ) and VP2 (β) expression vectors, in the presence of the pGL3-3×MEFSITE vector containing a threefold repeat of the MEF2C binding site. MEF2C α1γ was about 2.5 fold less active than MEF2C α1 (Fig. 3A). Conversely, MEF2C α1β was more transcriptionally active than MEF2C α1. These results are in line with previous studies (Zhu and Gulick, 2004; Zhu et al., 2005) confirming that exon β is an activation domain whereas the γ region is an inhibitory domain. Furthermore, MEF2C VP (γ) and MEF2C VP2 (β) activity were compared to that of MEF2C α1γ and MEF2C α1β, respectively. Interestingly, MEF2C VP (γ) exhibited an increased activity by about 200% when compared to MEF2C α1γ. Moreover, MEF2C VP2 (β) displayed maximal transcription activity among all the MEF2C splice variants. Similar results were obtained when MEF2C VP (γ) and MEF2C VP2 (β) activity were compared to that of MEF2C α2γ and MEF2C α2β (data not shown). MEF2C splicing isoforms showed similar expression levels in HEK293 cells (Fig. 3B). A more accurate evaluation is not possible, since a specific antibody against each isoform is not available. Thus, both new isoforms lacking exon α (α− isoforms) were substantially more active than the corresponding α+ isoforms in activating the MEF2 responsive reporter gene.

In another set of experiments, HEK293 cells were cotransfected with different ratios of MEF2C α1γ and VP (γ) expression vectors and with pGL3-3×MEFSITE (Fig. 3C). Expression of the MEF2C VP (γ) protein alone produced more than nearly 200% induction of gene reporter activity compared to MEF2C α1γ activity. Gene reporter activity also increased up to a maximum of 40% when HEK293 cells were cotransfected with different ratios of MEF2C α1β and MEF2C VP2 (β) and with pGL3-3×MEFSITE (Fig. 3D). The MEF2C α1γ and α1β isoforms alone were set to 100% (Figs. 3C and D, respectively). In both MEF2C α1γ:γ and MEF2C α1β:β coexpression, intermediate responses were seen with expression of the two isoforms together, indicating that neither of the isoforms is dominant and that any unexpected novel transcriptional activity stems from MEF2C splicing isoform coexpression. Since MEF2 transcription factors recognize and bind to the consensus DNA as homo- or heterodimers, it is not excluded that the presence of exon α may affect the complex formation. Reduction of the presence of isoform α leads to an increase of luciferase activity. In addition, it could also be hypothesized that exon α affects interactions with other cofactors. However, further investigations are needed to elucidate how exon α reduces transcriptional activity.

All together, these results clearly indicate that the presence of exon α induces a reduction of transcription levels.

3.4. Tissue distribution of exon α

Once the two MEF2C α− variants were identified, we proceeded to investigate their expression patterns in various human tissues. To quantify the relative amounts of VP and VP2 α− transcripts, real-time PCR assays were conducted. Because VP and VP2 lack exon α, specific primer/probe sets to detect the presence of this exon were designated. We compared the relative levels of α− variants in human skeletal muscle, liver, heart, kidney and brain tissue to total MEF2C. As shown in Fig. 4A, the α− variants were differently expressed in all samples examined, but always much less than total MEF2C. Notably, the α− variants were more highly expressed in brain and heart tissue (9- and 3-fold more, respectively) than in liver (Fig. 4B).

It is well known that MEF2C is a key transcription factor in heart and brain development (Leifer et al., 1994; Lin et al., 1997; Lyons et al., 1995). Therefore, MEF2C α− variant expression pattern was evaluated in fetal and adult brain and heart by using real-time PCR analysis. The exon α-skipping event is represented as α−/total MEF2C mRNA ratio (Fig. 4C). In adult brain and heart tissue we observed an increase of about 50% of exon α exclusion when compared to fetal tissue

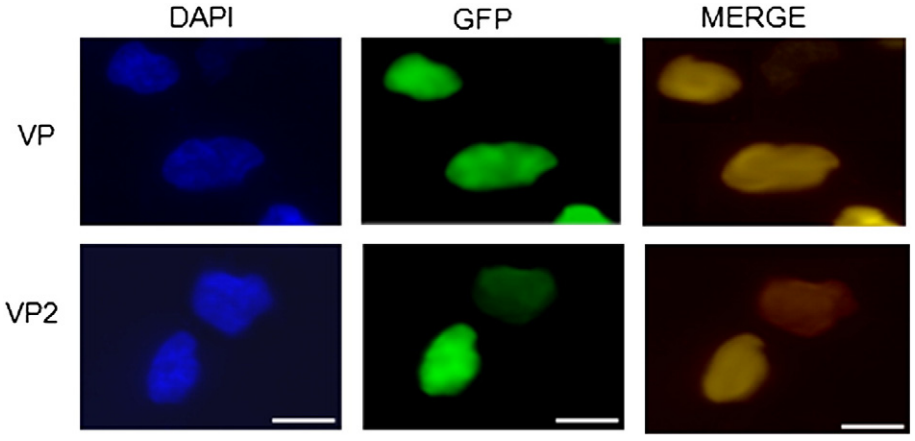

Fig. 2. Subcellular localization of MEF2C α− splicing isoforms. HeLa cells were transiently transfected with pcDNA3.1/CT-GFP-TOPO vectors carrying the cDNA sequence coding VP and VP2 in-frame with the GFP DNA sequence, treated with 5 mM DAPI and analyzed by fluorescence microscopy. Magnification of photomicrographs is ×60. Lane DAPI, DAPI nuclear staining; lane GFP, fluorescence of GFP fused with the VP or VP2 coding sequence; lane MERGE, overlapping images of nuclear stained cells with GFP fused to the VP and VP2 proteins. Scale bar, 10 μm.

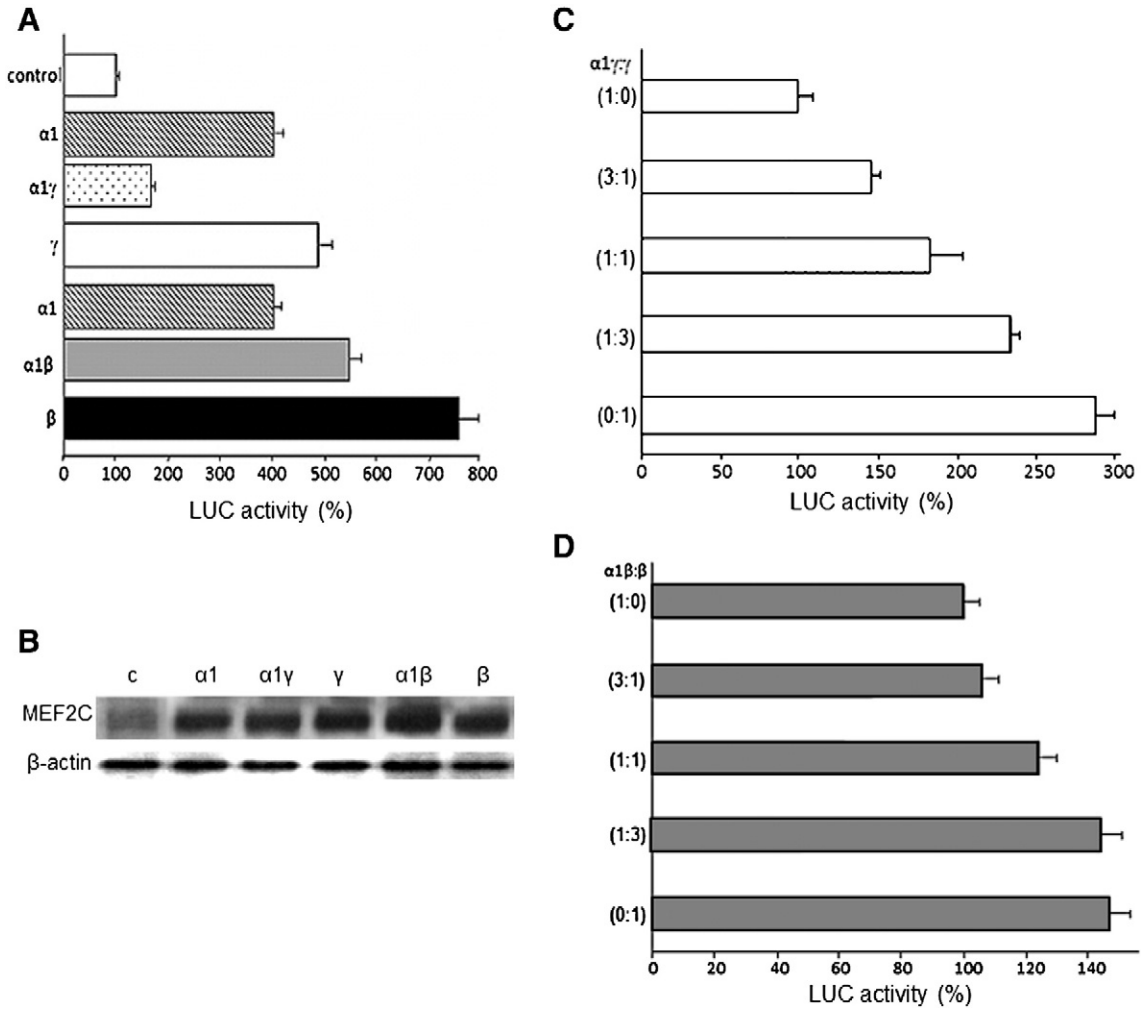

Fig. 3. MEF2C α− transcriptional activity. (A) HEK293 cells were transiently cotransfected with the pcDNA3.1 empty vector (control), pcDNA3.1 carrying the cDNA sequence coding α1, α1γ, γ (VP), α1β, and β (VP2), and the pGL3 promoter-LUC vector containing a threefold repeat canonical MEF2 site (pGL3-MEF), respectively. (B) HEK293 cells transfected as in (A), were used to quantify MEF2C protein levels. MEF2C and β-actin were immunodecorated with specific antibodies. (C) HEK293 cells were cotransfected with pGL3-MEF and either the pcDNA3.1 empty vector or the indicated ratios of pcDNA3-α1γ (white bars) to pcDNA3-γ (white dotted bars). (D) HEK293 cells were cotransfected with pGL3-MEF and either the pcDNA3.1 empty vector or the indicated ratios of pcDNA3-α1β (gray bars) to pcDNA3-β (black bars). Data are shown as means ± SD of five duplicate independent experiments. In (A) and (C) differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA); in (D) difference between (1:0) and (3:1) and difference between (1:3) and (0:1) were not significant.

(Fig. 4C). Because previous studies demonstrated that MEF2C is also involved in cardiac hypertrophy, cardiomyopathy and heart failure (Cortes et al., 2012; Xu et al., 2006), exon α skipping was also evaluated in diseased heart. Fig. 4C shows a considerable decrease of the α−/total MEF2C mRNA ratio in diseased heart compared to a healthy heart. These data suggest a possible physiological role for MEF2C α− variants in brain and heart.

3.5. Exon α during differentiation

In light of the findings outlined above, we assessed the exon α skipping during SH-SY5Y neuronal cell differentiation. The cells were differentiated for 1 and 3 days with IBMX, RA and db-cAMP. As shown in Fig. 5A there were no significant changes in MEF2C exon α-skipping after 1 day of differentiation, but after 3 days a strong increase of exon α-skipping event (about 50%) was detected. Thus, MEF2C α-skipping can be upregulated during neuronal cell differentiation.

To gain insight into the molecular mechanisms underlying the observed exon α skipping event, we searched for splicing protein binding sites in introns adjacent to exon 3. A computer analysis by a web server for motif analysis and prediction of splicing factor binding sites (http://sfmap.technion.ac.il/index.html) revealed the presence of seven specific sites for FOX1 in the intron upstream of exon 3. FOX1 is a well-known tissue-specific alternative splicing regulator, which specifically binds to the GCAUG sequence (Hakim et al., 2010). Because FOX1 is highly expressed in the heart (Park et al., 2011) and brain (Hakim et al., 2010) and previous studies implicated its role in the heart (Kalsotra et al., 2008) and brain (Hakim et al., 2010) development, we focused on its impact in alternative splicing of MEF2C.

To this end, we evaluated the FOX1 expression levels in SH-SY5Y differentiated as described above. FOX1 mRNA did not show significant changes at the first day of differentiation induction, whereas it significantly increased about 2 fold at the third day (Fig. 5B), in agreement with the pattern of MEF2C α-skipping event (Fig. 5A).

Moreover, the effect of FOX1 overexpression on MEF2C α-skipping event, was analyzed. HEK293 cells were transiently transfected with pcDNA3.1-FOX1 expression vector and exon α-skipping event was quantified. Efficiency of FOX1 activity was followed by testing for MEF2C exon β inclusion (data not shown). By means of RT-PCR, maximum FOX1 overexpression was observed 24 h after transfection (point 0 h in Figs. 5C and D). At this time the effect of FOX1 overexpression on exon α-skipping was measured. Real-time PCR experiments were performed using primers and probe sets encompassing

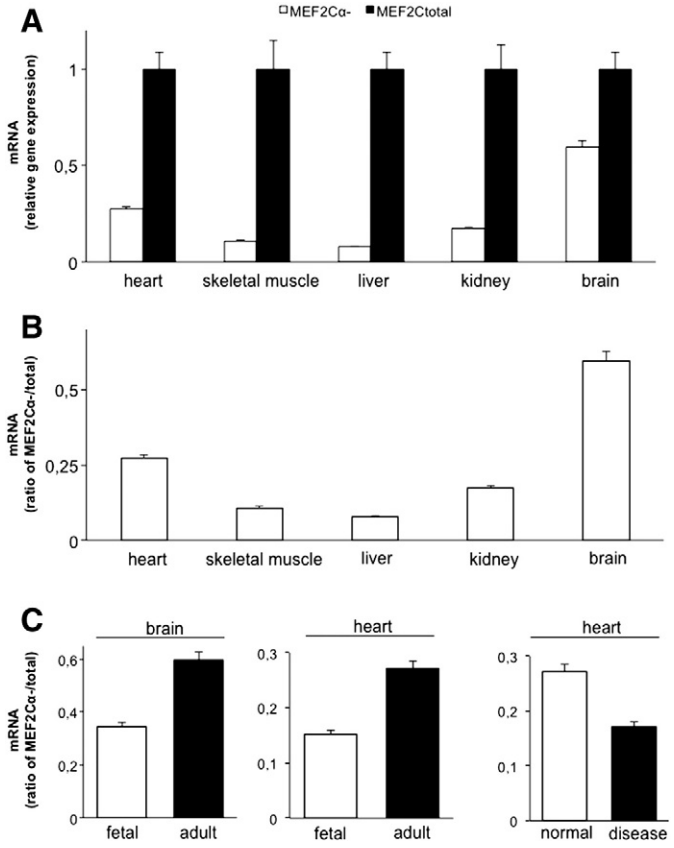

Fig. 4. Expression pattern of exon α. (A) Total RNA from various human tissues was reverse-transcribed and used to quantify total MEF2C and MEF2C α− spliced variants by real-time PCR. Quantification analysis was performed with specific TaqMan probes for total MEF2C and MEF2C α− variants (see Table 1 and Section 2.3 Materials and methods). (B) Real-time PCR analysis of α−/total MEF2C mRNA ratio in the same tissue of (A) is shown. (C) α−/total MEF2C mRNA ratio in fetal and adult brain, fetal and adult or normal and disease heart is shown. In (A), (B) and (C) data are shown as means ± SD of five duplicate independent experiments. Differences between samples and relative controls were significant ($P < 0.05$, one-way ANOVA).

the skipped region described in Table 1. The time-course of exon α-skipping in FOX1 overexpressing HEK293 cells, followed up to 12 h, shows that FOX1 overexpression increases exon α-skipping by about 70%, 150% and 100% at 3, 6 and 12 h, respectively (Fig. 5C). Interestingly, FOX1 mRNA levels at the same time-points displayed a trend similar to MEF2C exon α-skipping (Figs. 5C and D). These results suggest that FOX1 might be one of the regulators that control the exon α splicing event of human MEF2C mRNA.

4. Discussion

Alternative splicing is an important mechanism for generating transcriptional diversity and regulating gene expression (Infantino et al., 2011c; Kelemen et al., 2013). Perturbation of mRNA splicing may also influence the natural history of acquired diseases, as mRNA splicing is influenced by environmental signals and can become deregulated in disease states (Stamm, 2002). Abnormal patterns of mRNA splicing are associated with diseases such as cancer, and splicing profiles have been shown to be effective in cancer classification.

The *mef2c* gene gives rise to multiple isoforms through alternative splicing patterns that are conserved among vertebrates (Zhu and Gulick, 2004). The alternatively spliced isoforms can be tissue-specific producing multiple variants that have significant functional differences. Most notably, MEF2C mRNA was predominantly β- in all tissues, including skeletal and cardiac muscle and brain (Zhu et al., 2005). Also, alternative splicing of MEF2C exon β is highly regulated in a neural cell-specific manner and during myogenesis (Zhu et al., 2005). MEF2C β+ isoforms are more robust than β− isoforms in activating MEF2-responsive reporters despite similar expression levels, indicating that the β domain can function as enhancer. Exon β skipping occurs in all the MEF2 isoforms, whereas skipping of the γ domain takes place solely in MEF2C by splicing to a cryptic acceptor in coding exon 9 (Zhu and Gulick, 2004). This provides for MEF2C isoforms that either carry or lack a phosphoserine-dependent transrepressor γ domain. Thus, there are eight potential MEF2C variants, half of which exclude (β−) and half of which include (β+) the β domain. Alternative third exons, 3α1 and 3α2, are present in each MEF2C splice isoform described so far. Splicing to include either the α1 or α2 exon is regulated among tissues (Martin et al., 1994), but functions of the encoded alternative domains remain to be determined.

In the present work we provide evidence for the presence of MEF2C α− isoforms ubiquitously expressed in human adult tissues, although at much lower levels than the α+ isoforms. Analysis of the recently identified novel isoforms lacking exon α allows investigating the biological function of this exon for the first time. Our transactivation experiments demonstrate that the presence of exon α reduces MEF2C transcriptional activity compared to related isoforms lacking α.

Because MEF2C exon α could act as an inhibitory domain, we checked for the presence of residues joined by repressor proteins in exon α. In this respect, it has been recently reported that PIN1, a peptidyl-prolyl isomerase protein which catalyzes the isomerization of phosphorylated Ser/Thr-Pro peptide bonds, interacts with MEF2C through serine 98 and 110 contained in exon α regulating cell differentiation (Magli et al., 2010). The interaction between PIN1 and MEF2C is phosphospecific, and when bound PIN1 is able to block MEF2C activity and function. The observation that serine 98 and 110 are necessary for PIN1/MEF2C interaction and in turn for MEF2C repression (Magli et al., 2010) provides a plausible explanation to the inhibitory function of MEF2C exon α. Importantly, PIN1 overexpression negatively modulates MEF2C protein stability and activity as well as the ability of MEF2C to activate skeletal muscle differentiation (Magli et al., 2010).

In light of these findings, we measured the changes of MEF2C α-skipping in neuronal cell differentiation and in fetal and adult tissue, especially heart and brain, where the highest expression levels of α− isoforms were found. We observed that the α−/total MEF2C mRNA ratio increased in both adult brain and heart tissue compared to fetal tissue. Interestingly, in diseased heart the α−/total MEF2C mRNA ratio decreased compared to wild-type samples. These results are in agreement with activation of the "fetal gene program" (Depre et al., 1998; Razeghi et al., 2001) as well as the changes in alternative splicing (Park et al., 2011) observed in cardiac hypertrophy and in heart disease.

Furthermore, our work also attempts to explain the mechanisms by which exon α-skipping occurs. A recent study has provided evidence that FOX1 (Hakim et al., 2010) regulates exon β inclusion, which takes place in adult tissues and in differentiated cells. FOX1 is a tissue-specific splicing regulator in muscles and neuronal cells and it acts via binding to the (U)GCAUG element in mRNA precursors. It has been reported that FOX1 gene is upregulated in postnatal mouse heart development (Kuroyanagi, 2009) and downregulated in cardiac hypertrophy (Park et al., 2011). FOX1 can regulate splicing positively or negatively, most likely depending on where it binds relative to the regulated exon. In cases where the (U)GCAUG element lies in an intron upstream of the alternative exon, FOX1 protein functions as a splicing repressor to induce exon skipping (Fukumura et al., 2007). Interestingly, human MEF2C gene in silico analysis revealed specific sites for FOX1 in the intron upstream of exon 3. Noteworthy, MEF2C α-skipping increase during neuronal cell differentiation parallels FOX1 upregulation.

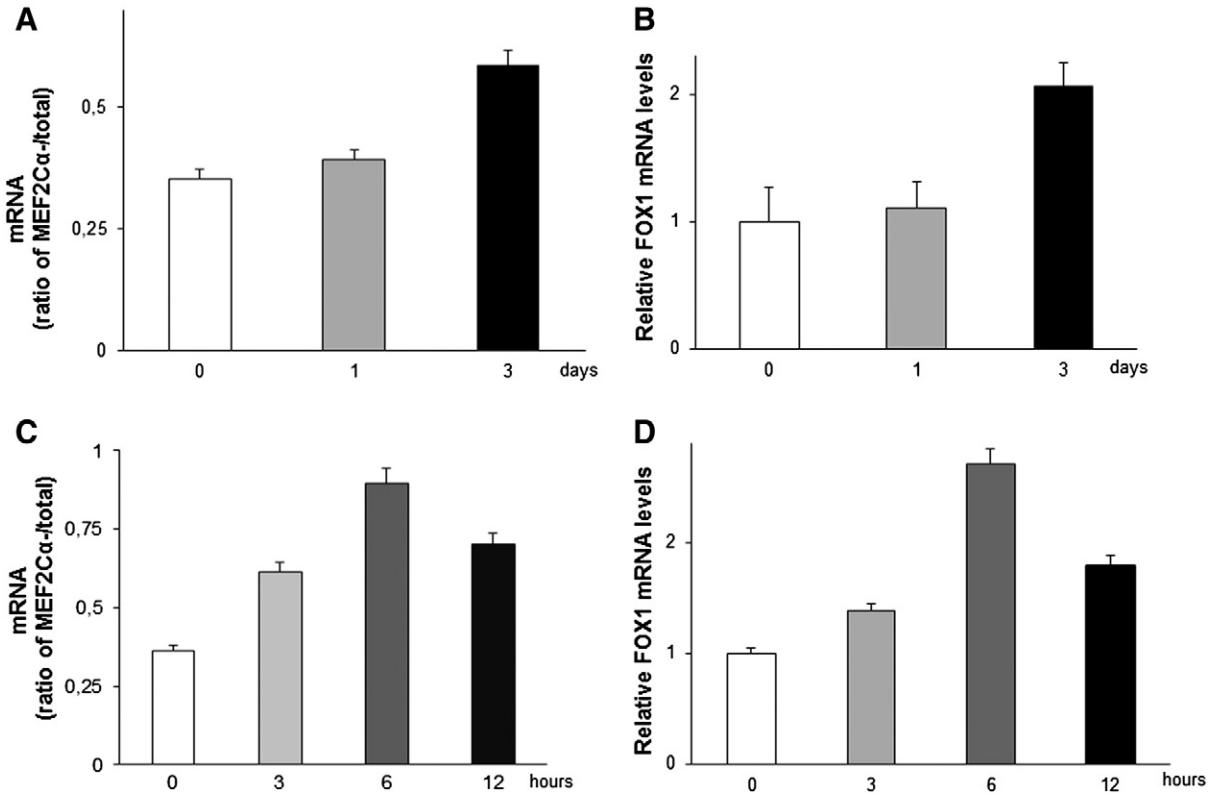

Fig. 5. Effect of FOX1 overexpression on exon α-skipping. (A) and (B) SH-SY5Y cells were seeded onto 6-well plates, grown to 50% confluence and suspended in serum-free medium for 16 h. The cells were then stimulated with differentiation medium consisting of serum-free medium supplemented with 0.5 mM IBMX, 10 μM RA and 1 mM db-cAMP (day 0). Total RNA was purified from day 0 to day 3 of neuronal SH-SY5Y differentiation. Total RNA extracted from SH-SY5Y cells was used to quantify α −/total MEF2C ratio and FOX1 mRNAs by real-time PCR. (C) and (D) HEK293 cells were transfected with pcDNA3-FOX1 for 24 h (0), 27 h (3), 30 h (6) and 36 h (12). Total RNA was extracted and used to quantify α/total MEF2C ratio and FOX1 mRNAs by real-time PCR. All values in (C) were normalized by transfecting cells with pcDNA3.1 empty vector. In (A), (B), (C) and (D) data are shown as means ± SD of five duplicate independent experiments. Differences between samples and relative control were significant ($P < 0.05$, one-way ANOVA) except for day 1 in (A) and (B).

Thus, changes in FOX1 gene expression during development and differentiation could be, at least in part, the source of the exon α-skipping event modulation.

5. Conclusions

This work describes two novel MEF2C splice variants, termed VP and VP2, lacking exon α that are ubiquitously expressed, though much less than the MEF2C α+ isoforms. Our results suggest that the presence of exon α reduces MEF2C transcriptional activity when compared to related isoforms lacking α and FOX1 may be involved in controlling the exon α-skipping event. Further studies are warranted to better elucidate the molecular mechanisms regulating exon α-skipping as well as the biological role of exon α.

Conflict of interest

The author declared that there is no conflict of interest.

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