# The transcription factor MEF2C mediates cardiomyocyte hypertrophy induced by IGF-1 signaling

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ABSTRACT

Myocyte enhancer factor 2C (MEF2C) plays an important role in cardiovascular development and is a key transcription factor for cardiac hypertrophy. Here, we describe MEF2C regulation by insulin-like growth factor-1 (IGF-1) and its role in IGF-1-induced cardiac hypertrophy. We found that IGF-1 addition to cultured rat cardiomyocytes activated MEF2C, as evidenced by its increased nuclear localization and DNA binding activity. IGF-1 stimulated MEF2 dependent-gene transcription in a time-dependent manner, as indicated by increased MEF2 promoter-driven reporter gene activity; IGF-1 also induced p38-MAPK phosphorylation, while an inhibitor of p38-MAPK decreased both effects. Additionally, inhibitors of phosphatidylinositol 3-kinase and calcineurin prevented IGF-1-induced MEF2 transcriptional activity. Via MEF2C-dependent signaling, IGF-1 also stimulated transcription of atrial natriuretic factor and skeletal α-actin but not of fos-lux reporter genes. These novel data suggest that MEF2C activation by IGF-1 mediates the pro-hypertrophic effects of IGF-1 on cardiac gene expression.

## Introduction

Cardiac hypertrophy is an adaptive response of the heart to an array of different conditions, including hemodynamic overload, neurohumoral factors, ischemic diseases or intrinsic defects in cardiac genes coding for structural proteins. At the cellular level, the cardiomyocyte hypertrophic response is characterized by an increase in cell size and protein content, myofibrillar reorganization and changes in gene transcription, including increased immediate early gene expression (e.g. *c-fos*), re-expression of atrial natriuretic factor (ANF), β-myosin heavy chain (β-MHC), skeletal α-actin (SKA) and upregulation of other contractile protein genes [1].

Different intracellular signaling pathways contribute to cardiac hypertrophy, such as the mitogen activated kinase (MAPK) cascades, calcineurin and calmodulin-dependent protein kinase pathways [2]. Given that multiple signaling pathways can elicit similar cellular responses, it appears likely that during hypertrophy different transduction pathways ultimately converge on common downstream targets. The transcription factor myocyte enhancer factor 2 (MEF2), which integrates multiple $Ca^{2+}$/calmodulin-dependent signaling pathways in cardiomyocytes and other cell types [3], is a major candidate for a common endpoint target. MEF2C has been implicated in cardiac and skeletal muscle differentiation [4] and activation of genes that promote cardiac hypertrophy [5].

Distinct evidences suggest that insulin-like growth factor-1 (IGF-1) participates in the initiation and development of cardiac hypertrophy [6]. We and others have shown that IGF-1 activates multiple signal transduction pathways in cardiomyocytes, including activation of phosphatidylinositol 3-kinase (PI3-K), ERK and JAK–STATs cascades, and recently, activation of a cascade comprising a G protein, PI3-K and phospholipase C, which enhances $IP_3$ generation and promotes $Ca^{2+}$ release [7,8]. The aim of this study was to determine if IGF-1 regulates MEF2C activity, and whether MEF2C participates in IGF-1-induced transcriptional activation associated to hypertrophy.

We found that IGF-1 activated MEF2C and induced MEF2 dependent-gene transcription. We also observed that IGF-1 activated p38-MAPK, whereas inhibitors of p38-MAPK, PI3-K and calcineurin all decreased IGF-1-induced MEF2C transcriptional activation. Transcription of ANF and SKA, but not of fos-lux reporter genes, required IGF-1-induced MEF2C signaling. Our results show for the first time that IGF-1 stimulates cardiac MEF2C activity, which mediates the genetic program underlying IGF-1-induced hypertrophy.

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## Materials and methods

*Plasmids and adenoviruses.* The ANF-lux reporter construct pANF(−638) LΔ5′ and pON249, encoding cytomegalovirus (CMV) promoter-driven β-galactosidase, were generously provided by Dr. K.R. Chien (Harvard Medical School). Lux reporter constructs for SKA and c-fos serum responsive element (SRE) were gifts from Dr. M.D. Schneider (Baylor College of Medicine). The plasmids encoding dominant negative pcDNA MEF2C 1-105 (mtMEF2C 275), constitutively active pcDNA MEF2C 1–117 fused to VP16 (mtMEF2C 219) and MEF-lux constructs were kindly donated by Dr. E.N. Olson (University of Texas Southwestern). Adenovirus CAIN (AdCAIN) was kindly provided by Dr. J.D. Molkentin (University of Cincinnati, Cincinnati). Human recombinant IGF-1 was donated by Dr. C. George-Nascimento (Austral Biological, San Ramon, CA).

*Culture of rat cardiomyocytes.* All studies conform to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health, Publication No. 85–23, revised 1996), and were approved by our Institutional Ethics Review Committee. Cardiomyocytes were isolated from neonatal rat ventricles and cultured as described [7]. Cell cultures were at least 95% pure. Serum was withdrawn 24 h before the cells were further incubated with 10 nM IGF-1. Before incubation with IGF-1, cardiomyocytes were preincubated for 30 min with inhibitors of PI3-K (LY294005, 50 μM), p38-MAPK (SB302580, 10 μM), MEK1/2 (PD98059, 50 μM) or calcineurin (cyclosporine A, CsA, 250 nM). IGF-1 receptor tyrosine kinase inhibitor AG538 (50 μM) was added 24 h before incubation with IGF-1.

*Immunocytochemistry.* Cardiomyocytes grown on coverslips were fixed with PBS containing 4% paraformaldehyde and permeabilized with 0.3% Triton X-100. Nonspecific sites were blocked with 5% BSA in PBS. Cells were subsequently incubated overnight with MEF2C antibodies (Santa Cruz Biotechnology) 1:1000 and incubated with anti-rabbit IgG-FITC antibodies. Nuclei were stained with 5 μg/mL propidium iodide. The resulting fluorescence was evaluated in a scanning confocal microscope (Carl Zeiss Axiovert).

*Transient transfection and reporter assay of cultured cardiomyocytes.* Cardiomyocytes were transfected using lipofectamine according to manufacturer's instructions. 0.5 μg of lux reporter plasmid, 0.5 μg of control pON249 and 0.5 μg of test plasmid, as indicated, were transfected for 24 h. Transfected cells were incubated with 10 nM IGF-1 for an additional 24 h period. Cardiomyocytes were washed twice with ice-cold PBS and luciferase activity was determined with Luciferase Assay System and normalized with β-galactosidase activity.

*Electrophoretic mobility shift assay (EMSA).* Nuclear fractions from cultured cardiomyocytes were prepared as described [9]. Fraction purity was assessed using lactic dehydrogenase activity as cytosolic marker and TFIIB transcription factor as nuclear marker. MEF2 binding activity was determined using the MEF2 consensus oligonucleotide 5′-GATCGCTCTAAAAATAACCCTGTCG-3′. Supershift assays were performed incubating 5 μg of nuclear extracts with anti-MEF2C antibody for 2 h. As controls, 100-fold excess of non-radioactive MEF2 consensus sequence and 1000-fold excess of mutated (5′-GATCGCTGTAAACATAACCCTGTCG-3′) oligonucleotide were used.

*p38-MAPK activity*. Endogenous p38-MAPK, diluted in a solution containing 50 mM Tris, pH 7.5, 150 M NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 2 μg/mL aprotinin, 2 μg/mL leupeptin, 1 μg/mL pepstatin and 100 μg/mL PMSF, was immunoprecipitated at 4 °C with a p38-MAPK antibody, followed by protein A/G-Sepharose precipitation. Immunoprecipitates were washed with kinase assay buffer (50 mM Hepes, 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT, pH 7.5). Immunocomplex activity was assayed for 30 min at 30 °C in the presence of 1 μCi [$\gamma$-$^{32}$P]-ATP and 10 μg of myelin basic protein (MBP) as substrate. The p38-MAPK activity was analyzed by autoradiography after SDS–PAGE in 12% gels.

*Calcineurin activity.* Calcineurin activity was assessed using a commercial colorimetric assay (Calbiochem).

*Expression of results and statistical methods.* Data are presented either as means ± SEM of the number of independent experiments indicated ($n$) or as examples of representative experiments performed on at least three separate occasions. Data were analyzed by ANOVA and comparisons between groups were performed using Tukey's $t$ test. A value of $p < 0.05$ was set as the limit of statistical significance.

## Results

### *Effect of IGF-1 on nuclear localization and transcriptional activity of MEF2C in cardiomyocytes*

To investigate whether IGF-1-induced nuclear translocation of MEF2C, cardiomyocytes were incubated with IGF-1 and MEF2C location was visualized by immunofluorescence microscopy. Under basal conditions, MEF2C exhibited punctuate staining both in cytoplasm and nucleus. MEF2C nuclear location increased after 5 min of incubation with IGF-1 and this nuclear location became more prominent after 30 min incubation with IGF-1 (Fig. 1A). IGF-1 increased MEF2 DNA binding activity at 45 min with a maximum at 60 min (Fig. 1B). The identity of MEF2C was verified by supershift assays using a specific anti-MEF2C antibody (Fig. 1B). Competition experiments using an excess of unlabeled or mutated MEF2 binding oligonucleotide confirmed the specificity of the assay (Fig. 1B). Transcriptional activation of MEF2C was assessed using a *MEF-lux* reporter gene. After 24 h incubation, 10 nM IGF-1-induced significant luciferase activity that was slightly lower than that induced by 50 nM IGF-1 (Fig. 1C). AG538, a specific IGF-1 receptor tyrosine kinase inhibitor, prevented MEF-lux reporter gene activation induced by 10 nM IGF-1 (Fig. 1C). Cotransfection of MEF2 reporter and MEF2C dominant negative genes significantly suppressed IGF-1-induced promoter activity (Fig. 1C), while cotransfection of MEF2 reporter with MEF2C dominant positive genes significantly stimulated luciferase activity, independent of IGF-1 (Fig. 1C). Taken together, these results show that IGF-1-induced MEF2C nuclear translocation and activated its transcriptional activity in cardiomyocytes.

### *Effect of IGF-1 on p38-MAPK and calcineurin activity*

Previous reports have shown that MEF2C transcriptional activity increases as a result of enhanced MEF2C phosphorylation at serine 387 by p38-MAPK [10]. Consequently, IGF-1 may stimulate MEF2C via p38-MAPK activation. To test this point, cardiomyocytes were incubated with IGF-1 and p38-MAPK activation was assessed by phosphorylation and enzymatic activity. IGF-1 significantly increased phospho-p38-MAPK levels (1.4-fold over control) after 5 min incubation, returning to basal values at 15 min (Fig. 2A). Likewise, p38-MAPK activity, measured by immunoprecipitation followed by determination of MBP phosphorylation, increased 2-fold following 5 min incubation with IGF-1 and decreased subsequently (Fig. 2B). These results show fast and transient IGF-1-dependent p38-MAPK activation in cultured cardiomyocytes.

As demonstrated previously in our laboratory, IGF-1 induces a transient $[Ca^{2+}]_i$ increase in cardiomyocytes [8]. To explore if IGF-1 activates pro-hypertrophic signaling pathways by engaging $Ca^{2+}$-activated calcineurin, we measured calcineurin activity in

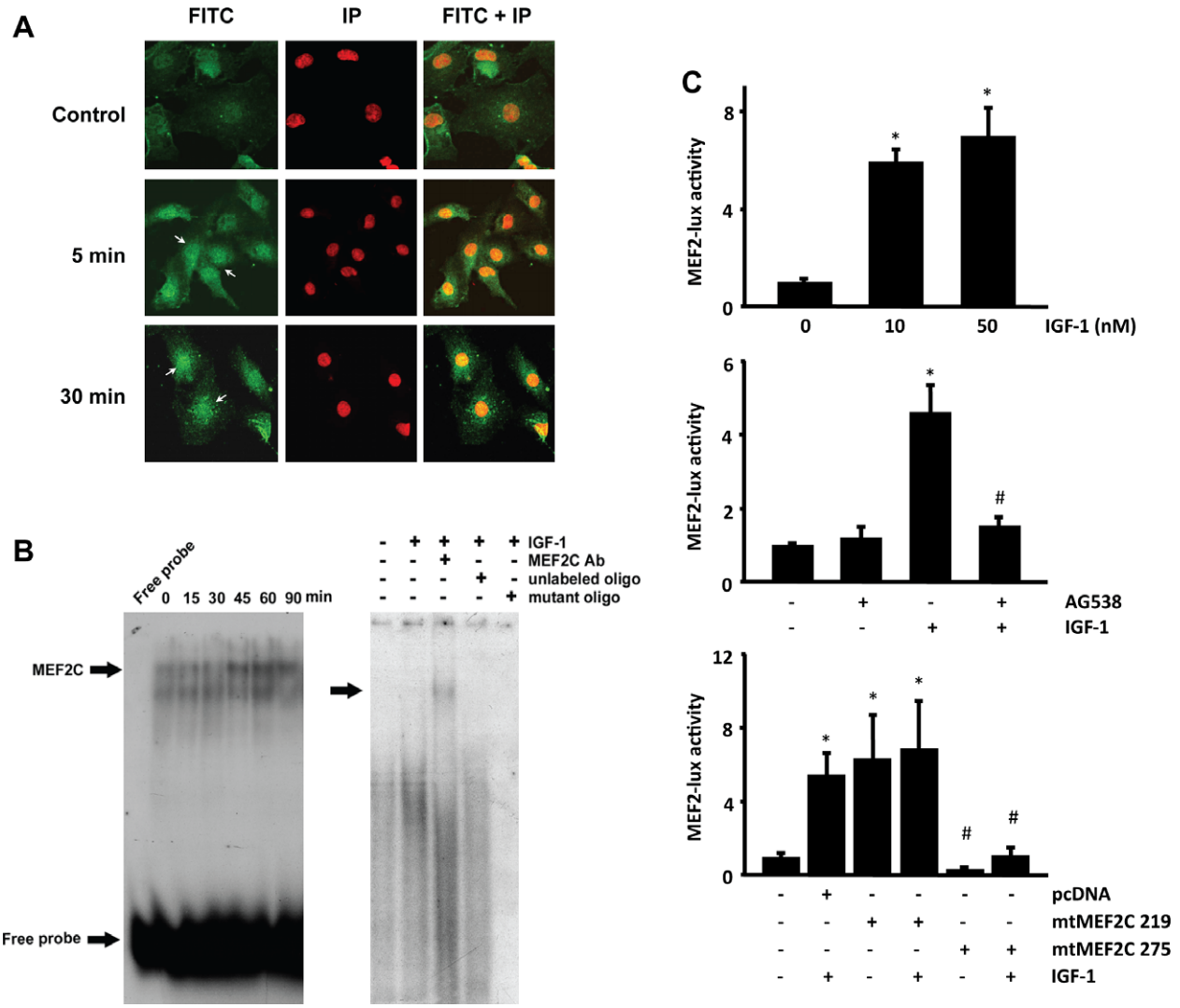


**Fig. 1.** IGF-1 activates MEF2C in cultured cardiomyocytes. (A) Cells were incubated with 10 nM IGF-1 for 5 or 30 min and MEF2C was detected by indirect immunofluorescence using anti-MEF2C antibody (green). Nuclei were stained with propidium iodide. (B) Electrophoretic mobility shift assays (EMSA, upper panel) were carried out using nuclear protein extracts prepared from cardiomyocytes incubated with 10 nM IGF-1 for up to 90 min. The arrow shows the MEF2/DNA complex. Supershift assays (lower panel) were carried out using nuclear protein extracts prepared from cardiomyocytes incubated with 10 nM IGF-1 for 1 h, and subsequently incubated with MEF2C specific antibody. The arrow indicates the supershift complex. As control, 100-fold molar excess of the unlabeled or mutant MEF2 oligonucleotides was used. (C) Cells were cotransfected with reporter MEF2-lux and β-galactosidase expression plasmids (pON) and incubated with IGF-1 (10 or 50 nM) for 24 h (upper panel). Cells were preincubated with AG538 (a specific IGF-1 receptor inhibitor) for 24 h before incubation with IGF-1 (10 nM) (middle panel). Cells cotransfected with MEF2-lux and β-galactosidase plasmids, together with constructs expressing either control vector (pcDNA 3.1), dominant positive MEF2C (mtMEF2C VP16) or dominant negative MEF2C (mtMEF2C 1–105), were incubated with or without 10 nM IGF-1 for 24 h (lower panel). Lux activity was normalized by β-galactosidase activity. Values are expressed as means ± SEM of triplicates from 3 independent experiments. $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. IGF-1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cultured cardiomyocytes incubated with 10 nM IGF-1. After 5 or 20 min of IGF-1 addition, calcineurin activity increased 1.8- and 2.2-fold over control, respectively (Fig. 2C). In summary, these data show that IGF-1 activated p38-MAPK and calcineurin signaling pathways in cardiomyocytes.

*Effect of inhibition of p38-MAPK, PI3-K, ERK/MEK and calcineurin on IGF-1-stimulated MEF2C activation*

To assess the influence of ERK/MEK, p38-MAPK, PI3-K and calcineurin signaling on MEF2 transcriptional activity, cultured cardiomyocytes transfected with MEF2-lux reporter were preincubated with different inhibitors and were then incubated with IGF-1. The p38-MAPK inhibitor SB302580 and the PI3-K inhibitor LY294002 significantly reduced IGF-1-dependent MEF2 reporter gene activation (Fig. 3A). In contrast, the MEK inhibitor PD98059 did not modify IGF-1-induced MEF2-lux activation, strongly suggesting that the MEK/ERK pathway is not involved in the activation of MEF2C-dependent transcription induced by IGF-1. The calcineurin inhibitor Ad-CAIN suppressed while cyclosporin A, another less specific calcineurin inhibitor, significantly reduced IGF-1-dependent activation of the MEF2-lux reporter gene (Fig. 3B and C). These data directly implicate p38-MAPK, PI3-K and calcineurin signaling pathways in the regulation of MEF2C transcriptional activity by IGF-1.

*Role of MEF2C in cardiomyocyte hypertrophy induced by IGF-1*

Transcriptional activation of immediately early genes, i.e. *c-fos*, and of genes involved in the fetal expression program—such as ANF, SKA and β-MHC—is a classical hallmark of cardiac hypertrophy [1]. IGF-1 increased 4-fold *c-fos-lux*, 10-fold *SKA-lux* and

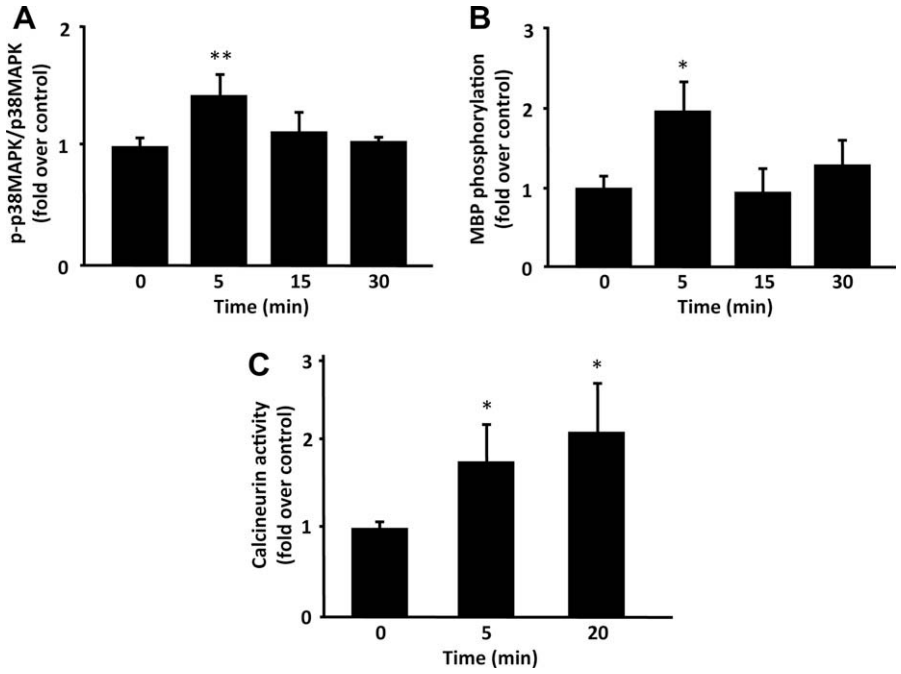


**Fig. 2.** IGF-1 receptor signaling mediates MEF2C phosphorylation in cultured cardiomyocytes. (A) Phosphorylation and (B) activity of p38-MAPK in cells incubated with 10 nM IGF-1 for 5–30 min. Myelin basic protein (MBP) is p38-MAPK substrate. (C) Calcineurin activity in cells incubated with 10 nM IGF-1 for 5–30 min. $^{*}p < 0.05$ vs. 0 min; $^{**}p < 0.01$ vs. 0 min. Values represent media ± SEM of at least 5 independent experiments.

20-fold *ANF-lux* reporter gene activities (Fig. 4A–C). Co-expression of the MEF2C dominant negative mtMEF2C 275 suppressed the enhancement of SKA and ANF reporter activities induced by IGF-1 (Fig. 4B and C). The *c-fos* promoter activity, however, did not decrease significantly in the presence of the MEF2C dominant negative suggesting that IGF-1-mediated induction of *c-fos* was independent of MEF2C activation (Fig. 4A). These results indicate that IGF-1 promotes MEF2C transcriptional activation in cardiomyocytes, which in turn activates the hypertrophic gene program that involves SKA and ANF gene enhanced transcription.

## Discussion

Several studies have shown that MEF2C is necessary for cardiac and muscular development and plays a part in the activation of several fetal cardiac genes [11,12]; MEF2C regulates as well several genes that participate in the metabolic adaptations and cytoskeleton reorganization that occur during the development of hypertrophy [5,11]. Moreover, different signaling pathways contribute to the pro-hypertrophic effects of IGF-1 in cardiomyocytes [6–8]. Accordingly, we investigated in this work if MEF2C activation forms part of the adaptive response to cardiac injury that is based on IGF-1-promoted hypertrophy.

Our results showed for the first time that IGF-1 activated MEF2C-dependent transcription in cultured neonatal rat cardiomyocytes, promoting the nuclear location of MEF2C, and its DNA binding activity. Functional assays using luciferase reporter genes revealed that nanomolar concentrations of IGF-1 enhanced MEF2C promoter activity. The specific IGF-1 receptor inhibitor AG538 prevented this activation, whereas overexpression of dominant negative MEF2C significantly decreased IGF-1-induced MEF2 reporter gene expression. These results strongly suggest that IGF-1 receptor signaling encompasses MEF2C activation in cardiomyocytes.

Previous studies have shown that IGF-1 activates several signaling pathways in cardiomyocytes, including ERK1/2, PI3-K, PKC, PKB, JAK/STAT, and PLC [7,8]. Here, we describe that IGF-1-induced p38-MAPK activation mediates MEF2C enhanced phosphorylation. MEF2C phosphorylation by p38-MAPK has been reported previously [3,10]. There are conflicting reports regarding the role of the p38-MAPK stress-activated kinase in cardiac hypertrophy. Thus, it was reported that overexpression of a heart targeted dominant negative α-p38-MAPK promotes hypertrophic cardiomyopathy through upregulation of calcineurin-NFAT signaling [13]. These results, however, contradict previous observations showing that pharmacologic inhibition of p38 kinase activity with the antagonists SB203580 or SB202190 attenuated agonist-stimulated hypertrophy in cultured cardiomyocytes [14,15]. Moreover, overexpression of a dominant negative β-p38-MAPK blunted the growth response of cultured cardiomyocytes [16], and significantly decreased agonist-induced B-type natriuretic peptide (BNP) promoter activity *in vitro* [17]. Our data do not allow us to discriminate whether α- or β-p38-MAPK, or both, is responsible for IGF-1-dependent MEF2C activation. This point remains to be clarified.

Interestingly, an inhibitor of PI3-K abolished IGF-1-stimulated MEF2C transcriptional activity. The PI3-K/PKB pathway has been recently involved in MEF2C activation in cerebral granule neurons [18]. Activation of PI3-K/PKB is associated with cell survival through several pathways, including inhibition of p53 and the Forkhead transcription factor, activation of the survival-promoting transcription factors CREB and NFκB, and phosphorylation and inactivation of BAD and caspase 9 [19]. Accordingly, the regulation of MEF2C transcriptional activity by the p38-MAPK and PI3-K pathways reported here suggests that in cardiomyocytes hypertrophic and survival signaling pathways converge at the level of MEF2C.

Calcium-dependent calcineurin activation modifies gene expression and induces hypertrophy in cardiomyocytes [20]. Calcineurin activation also promotes skeletal muscle hypertrophy in response to IGF-1 [21] and induces MEF2 transcription in skeletal myocytes [22]. Calcineurin and MEF2 form a physical complex in cultured C2C12 cells, where MEF2 becomes hypophosphorylated when calcineurin is active and calcineurin augments the potency of the transcriptional activation domain of MEF2 [23]. Our results indicate that exposure of cardiomyocytes to IGF-1 activates calci-

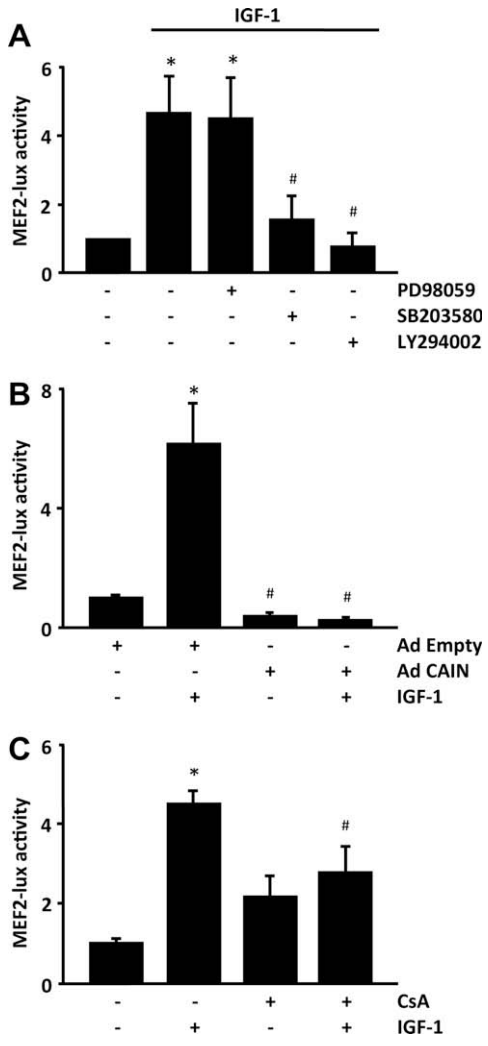


**Fig. 3.** IGF-1-dependent transcriptional activity of MEF2C is regulated by the p38-MAPK, PI3-K and calcineurin pathways in cultured cardiomyocytes. (A) Cells were transfected with MEF2-lux and β-galactosidase (pON) plasmids. Cells were preincubated with inhibitors and then incubated with or without 10 nM IGF-1. Lux activity was normalized by β-galactosidase activity. (B) Cells were transduced with adenoviruses encoding CAIN (AdCAIN) or empty (AdEmpty), at MOI = 300 for 24 h and then transfected with MEF2-lux and pON plasmids. Cells were incubated with or without 10 nM IGF-1 for 24 h. (C) Cells were preincubated with cyclosporin A (CsA) and then transfected with MEF2-lux and pON plasmids. Cells were incubated with or without 10 nM IGF-1 for 24 h. Results are given as means ± SEM of at least 5 independent experiments $^{*}p < 0.05$ vs. control; $^{\#}p < 0.05$ vs. IGF-1.

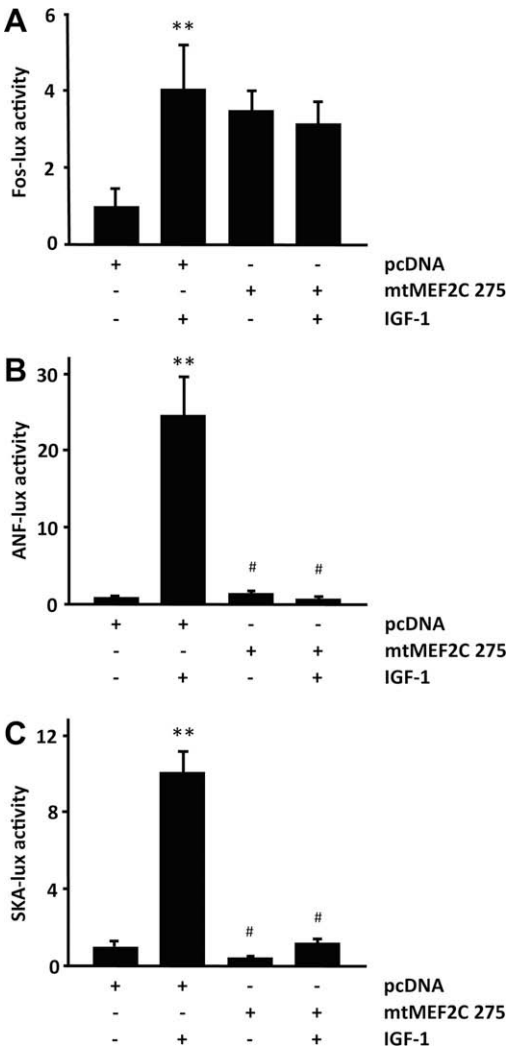


**Fig. 4.** IGF-1-dependent activation of ANF and SKA gene transcription is mediated by MEF2C in cardiomyocytes. Cells were transfected with β-galactosidase plasmid (pON) and c-fos (A), ANF (B) or SKA (C) lux reporter genes, and cotransfected with or without a dominant negative MEF2C (mtMEF2C 1–105). pcDNA 3.1 was used as mock plasmid. Cells were incubated with or without 10 nM IGF-1 for 24 h. Lux activity values were normalized by β-galactosidase activity. Results represent mean ± SEM of at least 5 independent experiments. $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ vs. IGF-1.

neurin, which could in turn dephosphorylate MEF2C, suggesting that IGF-1 can also up-regulate MEF2C activity in cardiomyocytes by hypophosphorylation of MEF2C.

The functional significance of IGF-1-dependent MEF2C activation was assessed studying gene transcription associated to hypertrophy. Pathological hypertrophy induced by pressure overload or heart failure increases intracellular $Ca^{2+}$ levels, promotes G-protein activation, calcineurin-NFAT signaling, SAPKs (JNK and p38-MAPK) and PKC signaling [24]. In contrast, the PI3-K/PKB and ERK1/2 signaling pathways mediate physiological hypertrophy [24]. Our laboratory has shown that IGF-1, a well known inductor of physiological hypertrophy, increases intracellular [$Ca^{2+}$] and activates PLC and ERK1/2 signaling in cultured cardiomyocytes [7,8]. In addition, in cardiac and renal cells, IGF-1-induced hypertrophy depends on calcineurin, suggesting that there is crosstalk between pathological and physiological hypertrophic pathways at the level of calcineurin [25]. Moreover, chondrocyte hypertrophy requires p38-MAPK signaling and this signaling route appears to be mediated in part by MEF2C [26].

We found that MEF2C activation in response to IGF-1 increased the transcriptional activities of ANF and SKA. In contrast, expression of the immediate early gene *c-fos*, which is activated via the ERK pathway [27], was not inhibited by a MEF2C dominant negative, presumably excluding its participation in the IGF-1/MEF2C signaling cascade. ANF is a neurohumoral factor with natriuretic, diuretic and vasodilatory activities that is expressed during atrium and ventricle development and is down-regulated in the ventricle after birth. Hypertrophic signals activate *ANF* gene expression in the ventricle [28], which is mediated by MEF2C [29]. The levels of SKA, an actin isoform with a MEF2 consensus sequence in its promoter [30], also increase in cardiac hypertrophy.

Taken together, the present results show that IGF-1 promotes MEF2C activation via p38-MAPK, calcineurin and PI3-K. Furthermore, since the cardiac hypertrophy-associated genes ANF and SKA were up-regulated by MEF2C, our results also suggest that the MEF2C transcription factor mediates IGF-1-dependent pro-hypertrophic signaling in cardiomyocytes.

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