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Depletion of mammalian target of rapamycin (mTOR) via siRNA mediated knockdown leads to stabilization of β-catenin and elicits distinct features of cardiomyocyte hypertrophy

Marco Hagenmueller *, Pratima Malekar, Christiane Fieger, Celine S. Weiss, Sebastian J. Buss, David Wolf, Hugo A. Katus, Stefan E. Hardt

Department of Cardiology, University of Heidelberg, INF 410, 69120 Heidelberg, Germany

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ABSTRACT

Cardiac myocyte growth is under differential control of mammalian target of rapamycin (mTOR) and glycogen-synthase-kinase-3β (GSK3β). Whereas active GSK3β negatively regulates growth and down-regulates cellular protein synthesis, activation of the mTOR pathway promotes protein expression and cell growth. Here we report that depletion of mTOR via siRNA mediated knockdown causes marked down-regulation of GSK3β protein in cardiac myocytes. As a result, GSK3β target protein β-catenin becomes stabilized and translocates into the nucleus. Moreover, mTOR knockdown leads to increase in cardiac myocyte surface area and produces an up-regulation of the fetal gene program. Our findings suggest a new type of convergence of mTOR and GSK3β activities, indicating that GSK3β-dependent stabilization of β-catenin in cardiac myocytes is influenced by mTOR.

1. Introduction

Cardiac hypertrophy is an adaptive response to an increased workload of the heart. The enlargement of the heart muscle is predominantly a result of an increased size of individual cardiomyocytes and is not controlled by proliferation [1]. Although a recent study from Bergmann et al. provides evidence that cardiomyocytes are able to renew, it is not clear whether this renewal is due to cardiomyocyte proliferation or stem cell differentiation [2]. Cell growth and proliferation are subjected to many intracellular control points. Mammalian target of rapamycin (mTOR) and glycogen-synthase-kinase-3β (GSK3β) are crucial cellular modulators of protein synthesis and cell growth, but apparently have contrary roles. Among other functions GSK3β acts as an important negative regulator of cellular protein synthesis [3], while activation of mTOR promotes protein expression and cell growth [4].

Although mTOR and GSK3β operate in different signaling pathways possible points of convergence have been described. Under low cellular energy conditions GSK3β, a constitutively active serine/threonine kinase, inhibits the mTOR pathway by phosphorylating tuberous sclerosis complex (TSC2). This activity depends on AMP kinase (AMPK)-mediated priming phosphorylation. Hyperphosphorylated TSC2 has an increased activity and acts as a GTPase activating protein on Rheb, thereby inhibiting mTOR activated cell growth [5]. In response to Wnt signaling, mTOR is activated through inhibition of GSK3β [6]. Moreover, in this pathway active GSK3β is a component of the destruction complex consisting of GSK3β, adenomatous polyposis coli (APC), casein kinase 1 (CK1) and the scaffolding protein Axin. This complex continuously phosphorylates β-catenin, thus activating its degradation through the ubiquitin system. In turn, activation of Wnt signaling pathway disrupts the destruction complex via dishevelled (Dvl) and thereby stabilizes β-catenin, enabling its translocation into the nucleus and interaction with transcription factors of the TCF/LEF family. This activity finally induces the expression of a number of growth promoting genes such as c-myc and PPARδ [7]. Furthermore, Haq et al. described Wnt independent stabilization of β-catenin and consecutive myocyte hypertrophy after inhibition of GSK3β [8]. Both mTOR and GSK3β play major roles in the cellular response to insulin stimulation via PI3K/Akt pathway [9]. Insulin stimulates protein synthesis by promoting phosphorylation of the eIF4E-binding protein, 4EBP1. This rapamycin-sensitive effect is mediated by mTOR [10]. GSK3β activity decreases after insulin stimulation. This relieves the inhibition of glycogen synthase and leads to glycogen synthesis. The regulation of insulin response of mTOR as well as GSK3β is mediated by protein kinase B (PKB/Akt) [11].

* Corresponding author. Address: Department of Cardiology, Angiology and Pulmology, University of Heidelberg, Im Neuenheimer Feld 410, 69120 Heidelberg Germany. Fax: +49 6221 56 8068.
E-mail address: marco.hagenmueller@med.uni-heidelberg.de (M. Hagenmueller).

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In this study we investigated the effects of reducing mTOR activity via siRNA mediated knockdown on cardiac myocyte growth and its implications for intracellular signal transduction.

2. Methods

2.1. Primary cultures of ventricular cardiac myocytes

Primary cultures of ventricular cardiac myocytes were prepared from 1–3-day-old BR-Wistar rats (Charles River Laboratories) and purified using a discontinuous Percoll gradient. Cells were cultured in cardiac myocyte culture medium containing Dulbecco's modified Eagle's medium (DMEM)/F-12 supplemented with 5% horse serum, 4 μg/mL transferrin, 0.7 ng/mL sodium selenite, 2 g/L bovine serum albumin (fraction V), 3 mM pyruvic acid, 15 mM HEPES, 100 μM ascorbic acid, 100 μ/mL ampicillin, 5 μg/mL linoleic acid and 100 μM 5-bromo-2′-deoxyuridine. We obtained cultures in which more than 95% of cells were myocytes. Culture medium was changed to serum-free at 24 h. The investigation was carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the local animal ethics review board (Approval No. 40631).

2.2. siRNA transfection

For at least 5 h myocytes were cultured in serum and antibiotic free medium. For transfection of 2×10^6 cardiac myocytes in a 6 cm dish 250 pmol siRNA was diluted in 650 μL serum- and antibiotic free OptiMEM (Invitrogen). In another tube 4.5 μL of Lipofectamin 2000 (Invitrogen) was diluted in 200 μL OptiMEM and incubated for 5 min. After complex formation the solution and OptiMEM were added to the cells. The cells were incubated for 72 h at 37 °C. siRNAs used were mTOR 5′-CUUCGAGACAUGAGUCAGCUUTT-3′, GSK3β 5′-UAACGCCGCUUCUGCAUCATT-3′ and non-specific control siRNA 5′-AGGUAGUGUAAUCGCCUUGTT-3′. In all siRNA mediated knockdown experiments myocytes transfected with non-specific (scrambled) siRNA were used as controls. siRNAs were synthesized by MWG (Ebersberg, Germany).

2.3. Western immunoblot analyses

Myocytes were washed twice with ice cold phosphate-buffered saline and scraped from the culture dish. Cells were lysed and incubated for 30 min in ice cold RIPA buffer containing 150 mM NaCl, 50 mM Tris (pH 7.5), 0.5% deoxycholic acid, 1% NP-40, 0.1% sodium dodecyl sulphate (SDS), 1 mM Na_3VO_4, 10 mM NaF and protease inhibitors AEBSF 0.5 mM, aprotinin 5 μg/mL, Leupeptin 5 μg/mL. Protein concentration was measured using BCA protein assay (Interchim). Equal amounts of protein were separated with SDS–PAGE and transferred to a nitrocellulose membrane (Milipore). The membranes were incubated overnight at 4 °C with a primary antibody. Antibodies used were anti-mTOR, anti-Phospho-p70S6 Kinase (Thr389), anti-p70S6 Kinase, anti-ubiquitin, anti-HDAC1 (Cell Signaling Technology), anti-GSK3β (BD), anti-β-catenin (Santa Cruz) and anti-α-actin (Sigma–Aldrich). Anti-rabbit IgG and anti-mouse IgG horseradish peroxidase-conjugated antibodies (Cell Signaling Technology) were used as secondary antibody. Bands were quantified by densitometry using the Image J program.

2.4. Immunoprecipitation

Whole cell lysates were prepared as described above. Protein concentration was measured using BCA protein assay (Interchim). About 100 μg of cell lysates were incubated with anti-GSK3β antibody (250 μg/mL) overnight at 4 °C. At least 3 h after addition of Protein-G-Agarose (Roche) samples were washed three times with RIPA buffer. Samples were analyzed by immunoblotting using anti-GSK3β (BD Biosciences) and anti-ubiquitin (Cell Signaling Technology) specific antibody.

2.5. Quantitative real-time PCR

Total RNA was isolated from cardiac myocytes using the Trizol reagent (Invitrogen). cDNA was synthesized with Revert Aid First strand cDNA synthesis kit (Fermentas). The copy numbers of HPRT housekeeping gene, GSK3β, β-catenin, c-myc, PPARδ, ANF and β-MHC were determined using the LightCycler system (Roche Diagnostics). Primers and specific probes were designed using the Rat Universal Probe Library from Roche Diagnostics. Following primers were used. For HPRT 5′-GTCAAGGGGGACATAAAAG-3′ and 5′-TGCATTGTTTTACCAGTGTCAA-3′, probe #22; for GSK3β 5′-CAGCTTTTGGTAGCATGAAAGTT-3′ and 5′-CAGGAGTTGCCACCACTGT-3′, probe #121; for β-catenin 5′-GCAATCAGGAAAGCAAGCTC-3′ and 5′-TCAGCACTCTGCTTGTGGTC-3′, probe #128; for PPARδ 5′-GGACCAGAGCACACCCTTC-3′ and 5′-GAGGAAGGGGAGGAATTCTG-3′, probe #120; for c-myc 5′-CCTAGTGCTGCATGAGGAGA-3′and 5′-TCTTCCTCATCTTCTTGCTCTTC-3′, probe #75; for ANF 5′-CAACACAGATCTGATGGATTTCA-3′ and 5′-CCTCATCTTCTACCGGCATC-3′, probe #25 and for β-MHC 5′-GAGGAGAGGGCGGACAT-3′ and 5′-ACTCTTCATTCAGGCCCTTG-3′, probe #95. All real-time PCR sample reactions were normalized to HPRT mRNA expression. A standard curve was run with the dilution series of the amplified fragment allowing for mRNA copy number calculation.

2.6. Myocyte cell surface area

Myocytes were cultured on coverglasses and transfected with siRNA as described. Four days after transfection myocytes were washed twice with ice cold phosphate-buffered saline and fixed in PBS containing 4% paraformaldehyde for 30 min. For permeabilization cell membrane cells were incubated for 10 min in phosphate-buffered saline containing 0.1% Triton X. Fixed cells were blocked with 2% BSA in phosphate-buffered saline for 1 h. Immunostaining was performed using Texas Red phalloidin (Molecular Probes) and DAPI staining for 30 min. After staining coverglasses were fixed on microscope slides. For myocyte surface area microscope images were captured by a digital camera. Analysis was performed using the Image J program.

3. Results

3.1. siRNA mediated knockdown of mTOR decreases GSK3β protein content and stabilizes β-catenin

Depletion of mTOR ($-83 \pm 4\%$, $P < 0.01$ vs. control) via specific siRNA was associated with decreased phosphorylation of the mTOR downstream target p70S6 kinase ($-35 \pm 11\%$, $P < 0.01$ vs. control), indicating an inhibition of the mTOR signaling pathway (Fig. 1A and B). Since activation of mTOR leads to increased protein synthesis and active GSK3β is a negative regulator of cell growth, we hypothesized that knockdown of mTOR is associated with a compensatory effect on GSK3β protein level. Indeed, in cardiac myocyte cultures a reduction in GSK3β protein content compared to controls was observed ($-33 \pm 8\%$, $P < 0.01$ vs. control) (Fig. 2A and B). By contrast, after GSK3β knockdown no difference in mTOR protein content was noted (data not shown). In order to evaluate whether reduced GSK3β is a consequence of lower GSK3β transcription we measured mRNA levels. However, no significant differences compared to controls were detected. Therefore, assuming accelerated

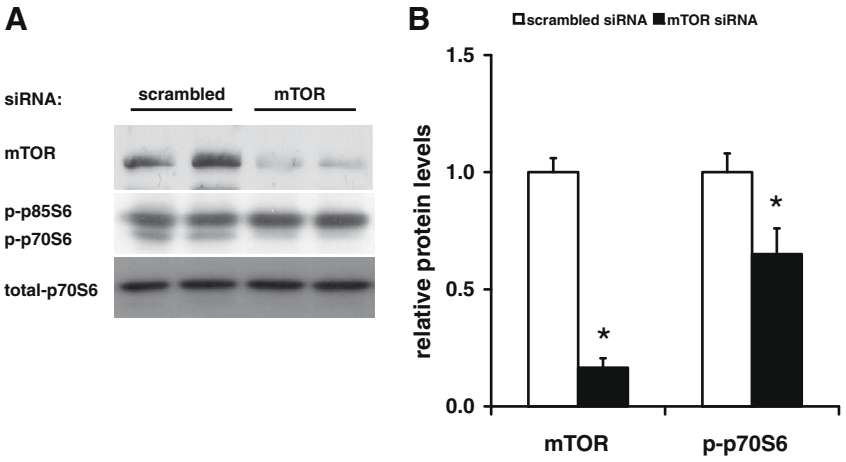

Fig. 1. Efficiency of siRNA mediated knockdown. (A) Shown is a representative Western Blot with mTOR, p70S6 and phospho-p70S6 specific antibodies. Cardiomyocyte lysates were prepared 72 h after mTOR specific siRNA transfection. (B) Left bars indicate effective protein knockdown of mTOR. Data are presented as the mean and SEM from three experiments in duplicate ($^{*}P < 0.01$ vs. control). Right bars show reduction of phosphorylated mTOR downstream component p70S6 kinase, as a consequence of mTOR knockdown. Graph shows mean and SEM from four individual experiments in duplicate ($^{*}P < 0.01$ vs. control).

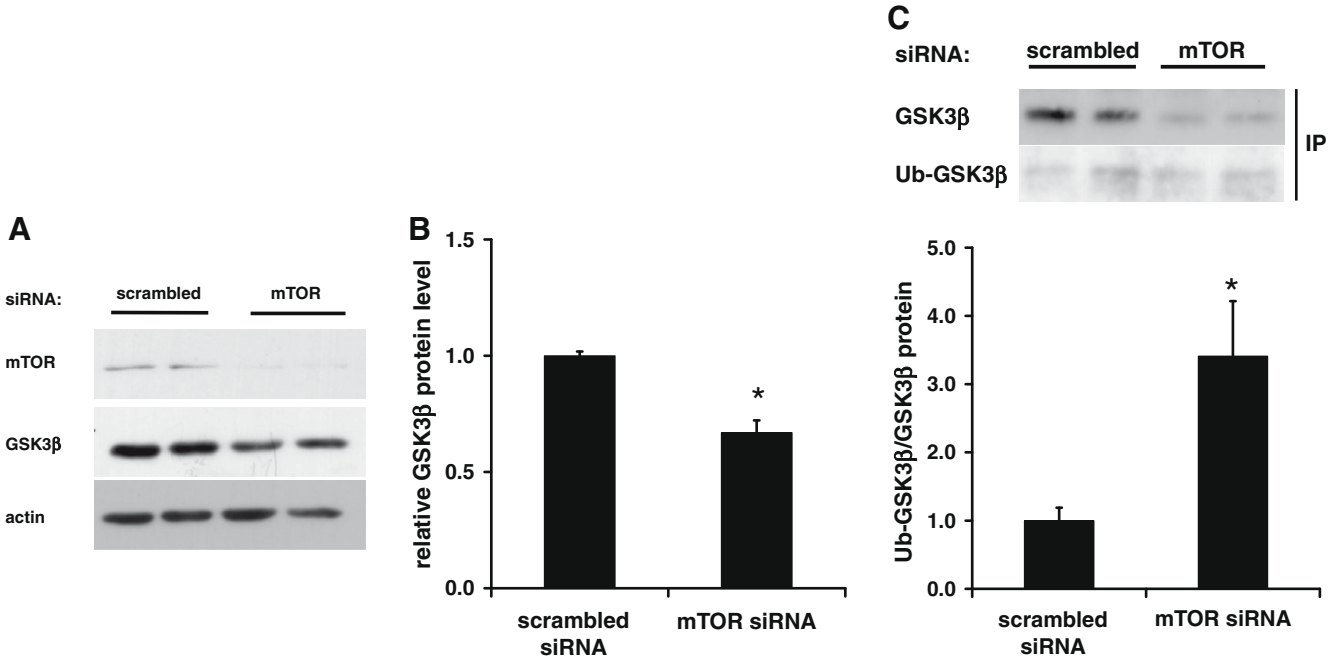

Fig. 2. mTOR siRNA knockdown and effects on GSK3β. (A) About 72 h after siRNA transfection of cultured cardiac myocytes protein lysates were subjected to immunoblot analysis with specific anti-mTOR and anti-GSK3β antibodies. Anti a-actin was used to control equal loading of SDS–PAGE. GSK3β protein content was decreased after mTOR knockdown. (B) Graph shows mean and SEM of six individual experiments in duplicate of control and mTOR knockdown cardiomyocytes ($^{*}P < 0.01$ vs. control). (C) Degradation of GSK3β via Ubiquitin/Proteasom pathway. About 72 h after siRNA transfection 80 μg protein was utilized for immunoprecipitation with GSK3β specific antibody. Western Blot and subsequent densitometric analysis was performed to determine the ratio of ubiquitinated GSK3β and total GSK3β. To avoid cross reaction with immunoglobulins, antibodies from different species were used to detect GSK3β and Ubiquitin. Increase of Ub-GSK3β/GSK3β ratio in mTOR knockdown cells was observed. Graph shows mean and SEM of three individual experiments in duplicate ($^{*}P < 0.05$ vs. control).

ubiquitin (Ub) mediated degradation of GSK3β, we performed further mTOR knockdown experiments. Immunoprecipitation with GSK3β specific antibody, followed by Western Blot analysis showed an increase in Ub-GSK3β/GSK3β ratio (+240% ± 80%, $P < 0.05$ vs. control) (Fig. 2C).

Next we investigated whether degradation of GSK3β after mTOR knockdown affects target proteins of GSK3β. β-Catenin is phosphorylated by GSK3β, leading to its degradation [8]. Activation of Wnt signaling as well as inhibition of GSK3β leads to accumulation of β-catenin in the cytoplasm and its translocation into the nucleus. This represents an important step to regulate target gene transcription [12]. In concordance with these findings, we observed that reduction of GSK3β after mTOR knockdown led to elevation of levels of β-catenin in both cytoplasm (+159% ± 57%, $P < 0.01$ vs. control) and nucleus (+53% ± 24%, $P < 0.05$ vs. control) (Fig. 3A). In Wnt signaling β-catenin interacts with TCF/LEF and thereby activates expression of c-myc and PPARδ [13]. We determined whether β-catenin translocation into the nucleus involves

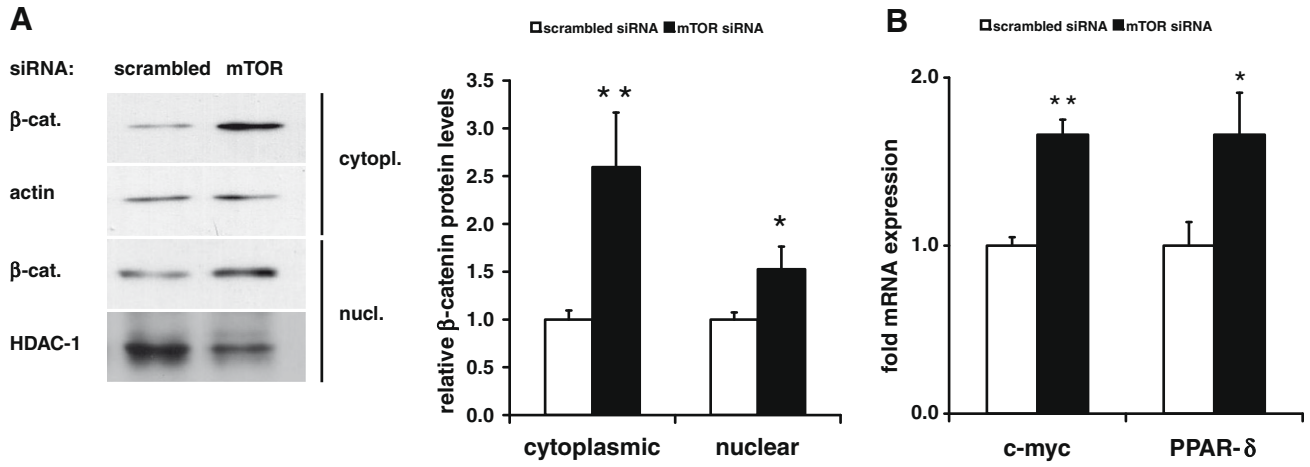

Fig. 3. β-Catenin accumulation in the cytoplasm, translocation into the nucleus and expression of target genes. (A) About 72 h after mTOR siRNA or scrambled siRNA transfection translocation of β-catenin into the nucleus was determined by immunoblotting of separated nuclear and cytoplasmic lysates using specific anti-β-catenin antibody. HDAC-1 was used as loading control for nuclear-, α-actin for cytoplasmic lysates. Depletion of mTOR leads to increased β-catenin level in the cytoplasm and in the nucleus. Bar Graph shows mean and SEM of four individual experiments in duplicate ($^{**}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. control). (B) mRNA copy numbers of c-myc and PPARδ were analysed by real-time PCR. Graph shows mean and SEM of four individual experiments in duplicate ($^{**}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. control). All measurements were normalized to HPRT expression. Compared to controls, c-myc (left bars) and PPARδ (right bars) mRNA expression in mTOR depleted myocytes were increased 72 h after mTOR siRNA transfection.

expression of these target genes. Increased mRNA levels in both c-myc (+66 ± 9%, $P < 0.01$ vs. control) and PPARδ (+63 ± 28%, $P < 0.05$ vs. control) (Fig. 3B) were detected.

3.2. mTOR knockdown elicits distinct features of cardiomyocyte hypertrophy

It has been demonstrated that inhibition of GSK3β using GSK inhibitor 1 or LiCl leads to increased cell size [14]. Furthermore, activation of Wnt signaling stabilizes β-catenin and promotes cell growth [15]. Due to the antihypertrophic effects of mTOR inhibition [16] we subsequently investigated the net effect of mTOR knockdown on myocyte size. We found an increased cell surface area (+44 ± 3%, $P < 0.01$ vs. control) (Fig. 4A) accompanied by increased expression of fetal marker genes like ANF (+124 ± 35% vs. control, $P < 0.01$) and β-MHC (+33 ± 8%, $P < 0.05$ vs. control) (Fig. 4B), suggesting a potential role in the pathogenesis of cardiac hypertrophy. Although myocyte surface area is increased and hypertrophy markers such as ANF and β-MHC are up-regulated, total-protein content in mTOR knockdown cells was not increased, compared to myocytes transfected with scrambled siRNA (+5% ± 0.9%, $P > 0.5$) (Fig. 4C). In order to determine if this hypertrophic response is a result of attenuated GSK3β signaling, we carried out GSK3β siRNA knockdown experiments (Fig. 4D). We found an increased cell surface area (+32 ± 2%, $P < 0.01$ vs. control) (Fig. 4A) as well as up-regulation of ANF (+72 ± 8%, $P < 0.01$ vs. control) and β-MHC (+42 ± 18%, $P < 0.05$ vs. control) under these conditions (Fig. 4B).

Isoproterenol (ISO) is a potent stimulus to induce sarcomeric reorganisation and hypertrophic growth in cardiac myocytes [17]. We investigated whether stimulation of mTOR knockdown myocytes with ISO (50 μM) increases hypertrophic response. Compared to ISO stimulated control cardiomyocytes no significant difference in myocyte surface area was observed (Fig. 5A). In comparison to basal cultured mTOR knockdown cells, we found an increased cell surface area in ISO stimulated mTOR knockdown cells (+30 ± 5%, $P < 0.05$ vs. unstimulated mTOR knockdown cells). Interestingly, stimulation of mTOR knockdown cells with ISO (50 μM) did not lead to a stronger ANF expression. Compared to non stimulated mTOR knockdown cells, ANF expression did not further increase after ISO stimulation (Fig. 5B). No differences between control and mTOR knockdown cells cultured under basal conditions were observed as for sarcomeric organisation. In control and mTOR knockdown cardiomyocytes, stimulation with ISO (50 μM) leads to sarcomeric reorganization (Fig. 5C and D).

3.3. Rapamycin inhibition and siRNA mediated knockdown of mTOR differentially affects downstream signaling

The anti-proliferative properties of rapamycin are frequently used to investigate regulation of cell growth. Upon entering the cytoplasm rapamycin forms a complex with FK506 binding protein (FKBP12) and then rapamycin/FKBP12 binds to mTOR thereby inhibiting its kinase activity [18]. In another set of experiments we examined whether mTOR inhibition by rapamycin causes degradation of GSK3β followed by stabilization of β-catenin as seen in mTOR knockdown experiments. Phosphorylation of p70S6 kinase was analyzed by immunoblotting to verify effectiveness of mTOR inhibition by rapamycin (Fig. 6A). After pharmacological inhibition of mTOR we measured protein levels of GSK3β and β-catenin by immunoblotting. Unlike mTOR knockdown experiments in rapamycin treated myocytes no difference in both GSK3β and β-catenin protein levels were detected compared to controls (Fig. 6B).

4. Discussion

In the present study we demonstrate that siRNA mediated knockdown of mTOR in cardiac myocytes decreases GSK3β signaling via the ubiquitin system. This results in accumulation of β-catenin in the cytoplasm and increased nuclear translocation [19]. This is not surprising since active GSK3β is known to phosphorylate β-catenin, leading to an ubiquitin mediated degradation [20]. In our study we did not observe an increase in the steady state level of β-catenin transcripts, supporting the hypothesis that the increased protein level is under post-translational control and does not result from increased transcription. Concomitantly with the increase in β-catenin we did observe increased gene expression of Wnt signaling targets like c-myc and PPARδ suggesting that Wnt components are activated in reaction to inhibition of mTOR signaling. This may act as a salvage pathway to protect cellular integrity. In addition, increased myocyte area and intensified expression of hypertrophy markers indicate a potential role of mTOR in development and regulation of pathological cardiac hypertrophy.

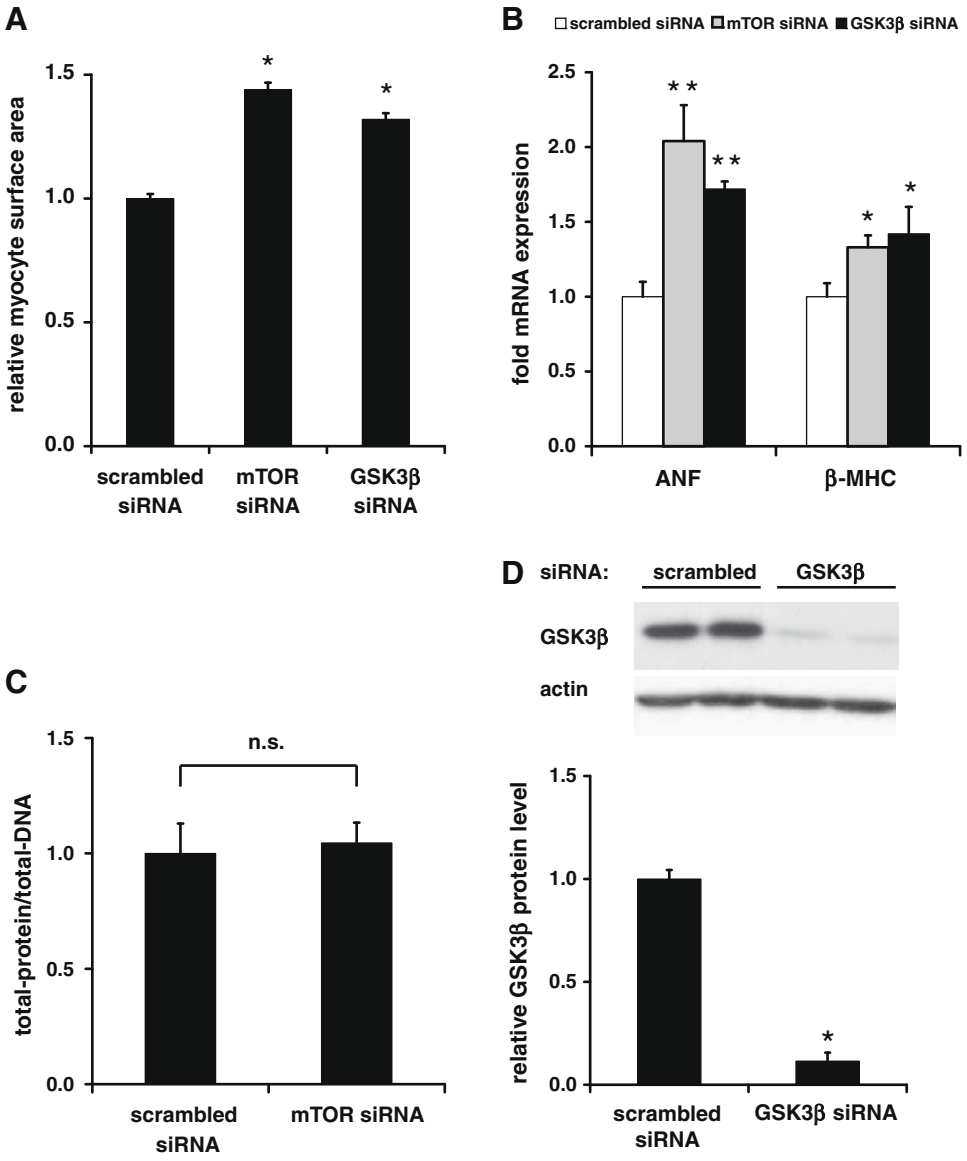

Fig. 4. mTOR knockdown elicits distinct features of cardiomyocyte hypertrophy. (A) Myocyte surface area from cells transfected with scrambled siRNA ($n = 630$), mTOR siRNA ($n = 630$) and GSK3β siRNA ($n = 400$) was determined 96 h after transfection and analyzed using Image J program. Bar graph shows mean and SEM from five individual experiments of mTOR knockdown and four individual experiments of GSK3β knockdown. Both mTOR- and GSK3β knockdown cells indicate increased myocyte surface area ($^{*}P < 0.01$ vs. control). (B) 72 h after mTOR siRNA transfection total RNA was subjected to RT-PCR. Expression levels of ANF and β-MHC mRNA were normalized by that of HPRT and expressed as 1 in unstimulated scrambled siRNA transfected myocytes. Bar graph shows mean and SEM from four individual experiments in duplicate ($^{**}P < 0.01$ vs. control; $^{*}P < 0.05$ vs. control). In mTOR knockout cells both ANF and β-MHC mRNA expression were increased. Even cells treated with GSK3β siRNA show up-regulation of ANF and β-MHC mRNA expression. (C) About 72 h after mTOR siRNA transfection, total protein was measured by BCA-assay. Protein content was normalized to DNA content measured by Hoechst 33342 staining in a fluorometer. Graph shows mean and SEM of three individual experiments in duplicate. Compared to controls, protein contend was almost unchanged in mTOR knockdown cells. (D) Efficiency of GSK3β siRNA mediated knockdown. Depicted is a representative Western Blot from myocyte lysates prepared 72 h after GSK3β specific siRNA transfection. Bar graph indicates effective protein knockdown of GSK3β. Data are presented as the mean and SEM from three experiments in duplicate ($^{*}P < 0.01$ vs. control).

Because of the contrary functions in regulating protein synthesis it is fundamental for cells to maintain balance in GSK3β and mTOR signaling activity. Our observations of decreased GSK3β protein level and stabilization of total cellular protein content after mTOR knockdown suggest that such control is active. Without any counter-regulatory mechanisms, mTOR knockdown would reduce global protein synthesis, and finally lead to cell death. Indeed, our finding that knockdown of GSK3β does not affect mTOR protein content suggests independent regulation for mTOR. Haq et al. demonstrated that cardiac myocytes transfected with adenoviral β-catenin, which can neither be phosphorylated by GSK3β nor degraded via ubiquitin, show hypertrophic growth [8]. This is supported by the results of our study, where we found increased cardiac myocyte size in combination with stabilized β-catenin protein level. Based on our findings we propose that myocyte growth and simultaneous elevated ANF and β-MHC expression is a result of attenuated GSK3β signaling and concomitant stabilization of β-catenin. This is further corroborated by GSK3β siRNA knockdown experiments, where the same hypertrophic responses were observed. Other essential features of cardiac hypertrophy were enhanced, namely the increase of cell surface area and the induction of fetal gene program. Here it is of interest to note that ANF expression is not further increased in mTOR knockdown cells stimulated with ISO compared to mTOR knockdown cells cultured under basal conditions, although an increase in myocyte size was observed. Since mTOR knockdown leads to myocyte growth even

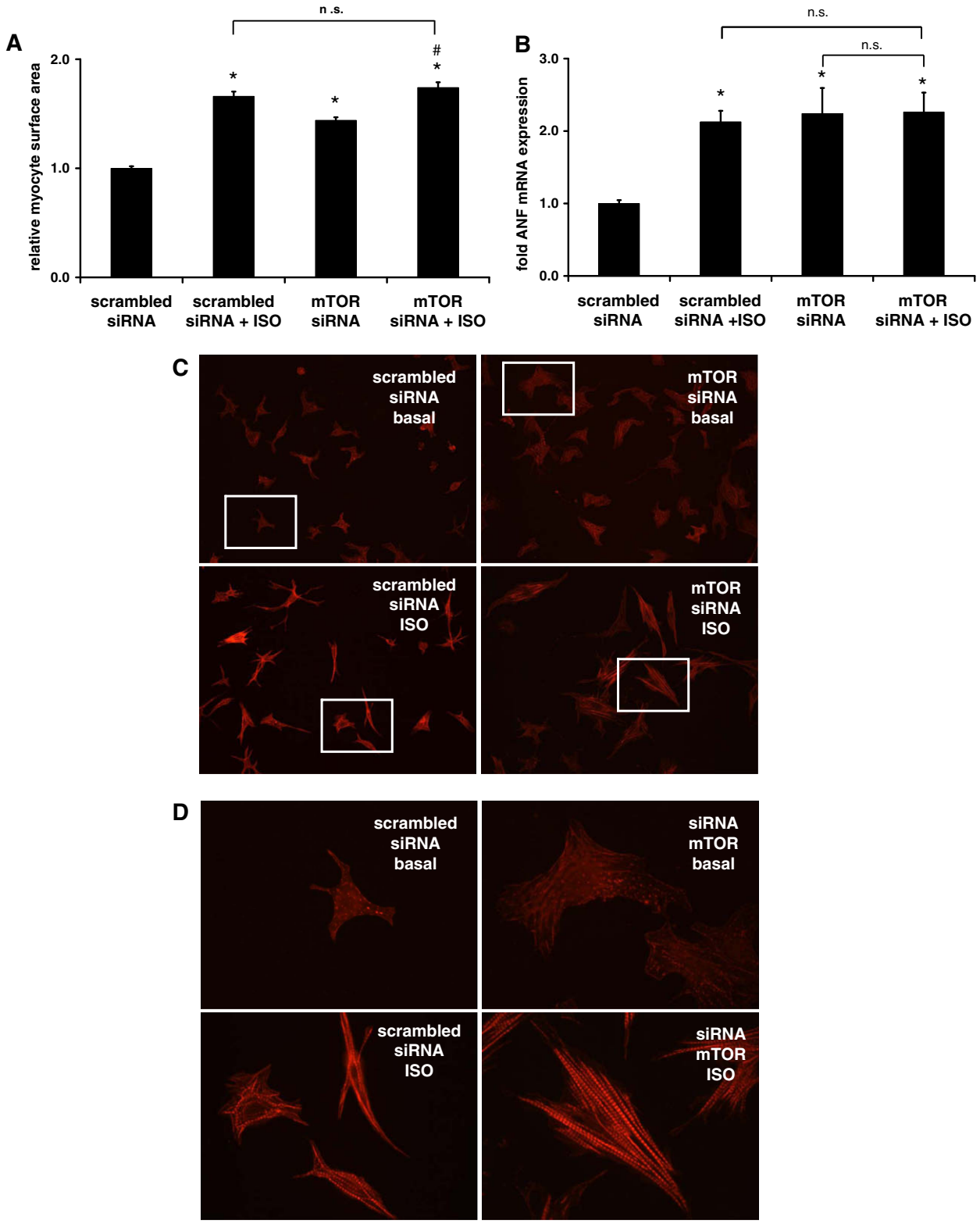

Fig. 5. mTOR depletion and ISO stimulation. About 48 h after mTOR siRNA transfection cells were stimulated with 50 μM ISO for further 48 h. Relative myocyte area was analyzed using Image J program. (A) Myocyte area of cells transfected with scrambled control siRNA ($n = 630$) and mTOR siRNA ($n = 630$) under basal conditions as well as with ISO stimulation ($n = 300$) was determined. For unstimulated cells five individual experiments and for ISO stimulated cells three individual experiments were performed. In mTOR depleted cells increased size was observed even under basal conditions. In ISO stimulated mTOR knockout cells an increased size compared to control and unstimulated mTOR knockout cells was observed ($^{*}P < 0.01$ vs. control; $\#P < 0.01$ vs. unstimulated mTOR knockout cells). (B) About 48 h after siRNA transfection myocytes were treated with or without 50 μM ISO for an additional 48 h. Total RNA was subjected to RT-PCR. Expression level of ANF was normalized by that of HPRT. Graph shows the mean and SEM of four individual experiments with unstimulated cells and three individual experiments with ISO stimulated myocytes ($^{*}P < 0.01$ vs. control). Up-regulation of ANF at mRNA level even under basal conditions was observed. ISO stimulation of mTOR depleted cells did not increase ANF copy number compared to unstimulated mTOR knockdown cells. (C) Representative immunostaining of myocytes with Texas Red phalloidin. Myocytes were transfected with scrambled control siRNA or mTOR siRNA. Four days after transfection and ISO stimulation immunostaining was performed. (D) Magnifications depicting the highlighted areas from the panels in C to evaluate sarcomeric organization.

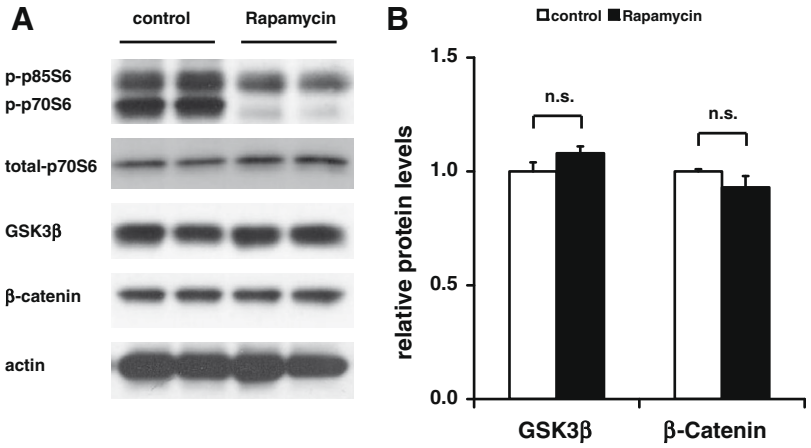

Fig. 6. Rapamycin inhibition of mTOR. Cardiac myocytes were cultured in serum free medium 48 h before treatment with rapamycin (50 ng/mL). (A) Cell lysates were prepared 24 h after rapamycin incubation and immunoblot analysis was performed with GSK3β and β-catenin specific antibody. As a demonstration of successful mTOR inhibition phosphorylation of known mTOR downstream component p70S6 was observed by immunoblotting with anti-phospho-p70S6 specific antibody. (B) Graph shows densitometric analysis of GSK3β and β-catenin protein from myocytes with rapamycin mTOR inhibition. Mean and SEM of 4 experiments in duplicate are shown.

under basal conditions, mTOR may act as a critical regulator of pathological cardiac hypertrophy.

Furthermore, our present study demonstrates that pharmacological inhibition of mTOR with rapamycin and siRNA mediated knockdown of mTOR do not have exactly similar effects. Rapamycin is a potent inhibitor of cardiac myocytes proliferation in vitro [16] and is also able to inhibit cardiac myocyte hypertrophy [22]. While knockdown of mTOR is accompanied by degradation of GSK3β as a compensatory step, which may stabilize β-catenin and finally regulate cell growth, in rapamycin treated cells no change in GSK3β and β-catenin protein content was observed. Since mTOR exists in two complexes, namely TORC1 which is rapamycin sensitive and TORC2 which is not [23], our findings can be explained by a predominant reduction of TORC2 following mTOR knockdown. A recent study from Sarbassow et al. shows that phosphorylation of the mTOR upstream component Akt/PKB depends on TORC2 in *Drosophila* [24].

In summary, siRNA mediated inactivation of mTOR pathway in cardiac myocytes is not associated with growth restriction as it is known from inhibition with rapamycin, but leads to a hypertrophic response. mTOR knockdown also results in degradation of GSK3β protein, stabilization of β-catenin and increased expression of its target genes.

5. Disclosures

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