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Original article

Cross-talk between glycogen synthase kinase 3β (GSK3β) and p38MAPK regulates myocyte enhancer factor 2 (MEF2) activity in skeletal and cardiac muscle

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ABSTRACT

Characterizing the signaling network that controls MEF2 transcription factors is crucial for understanding skeletal and cardiac muscle gene expression. Glycogen synthase kinase 3β (GSK3β) regulates MEF2 activity indirectly through reciprocal regulation of p38MAPK. Cross-talk between GSK3β and p38MAPK regulates MEF2 activity in skeletal and cardiac muscle. Understanding cross-talk in the signaling network converging at MEF2 control has therapeutic implications in cardiac and skeletal muscle pathology. Glycogen synthase kinase 3β (GSK3β) is a known regulator of striated muscle gene expression suppressing both myogenesis and cardiomyocyte hypertrophy. Since myocyte enhancer factor 2 (MEF2) proteins are key transcriptional regulators in both systems, we assessed whether MEF2 is a target for GSK3β. Pharmacological inhibition of GSK3β resulted in enhanced MEF2A/D expression and transcriptional activity in skeletal myoblasts and cardiac myocytes. Even though *in silico* analysis revealed GSK3β consensus (S/T)XXX(S/T) sites on MEF2A, a subsequent *in vitro* kinase assay revealed that MEF2A is only a weak substrate. However, we did observe a post-translational modification in MEF2A in skeletal myoblasts treated with a GSK3β inhibitor which coincided with increased p38MAPK phosphorylation, a potent MEF2A activator, indicating that GSK3β inhibition may de-repress p38MAPK. Heart specific excision of GSK3β in mice also resulted in up-regulation of p38MAPK activity. Interestingly, upon pharmacological p38MAPK inhibition (SB203580), GSK3β inhibition loses its effect on MEF2 transcriptional activity suggesting potent cross-talk between the two pathways. Thus we have documented that cross-talk between p38MAPK and GSK3β signaling converges on MEF2 activity having potential consequences for therapeutic modulation of cardiac and skeletal muscle gene expression.

1. Introduction

Mitogen activated protein kinase (MAPK) signaling pathways are prominently involved in many cellular processes including cell proliferation and growth [1], development, migration [2] and differentiation [3,4]. Deregulation of MAPK signaling almost invariably leads to developmental defects and diseases including cardiac hypertrophy, muscular atrophy and cancer [5]. Proper regulation of this pathway in the context of elaborate and highly complex signaling networks within the cell is strongly dependent on communication with other signaling molecules, resulting in either synergistic or antagonistic relationships that produce a spectrum of biological outcomes. Understanding the nature of cross-talk between signaling pathways is indeed a major hurdle to understanding the molecular basis of all cellular processes. In the studies described here, we take advantage of the convergence of several signaling pathways on the MEF2 family of transcriptional regulators in order to gain insight into how cross-talk between GSK3β and p38MAPK signaling influence a single effector molecule which functions as a signaling conduit for the control of cardiac and skeletal muscle gene expression.

MEF2 proteins belong to the MADS (MCM1, agamous, deficiens, serum responsive factor) superfamily of transcription factors. There are four isoforms of MEF2 in vertebrates, MEF2A–D, that contain a highly conserved 57aa MADS-box domain at their amino-termini immediately adjacent to their 29aa MEF2 domain. Collectively these two domains are involved with DNA-binding, dimerization and interaction with co-factors. MEF2 factors regulate transcription as homo- or heterodimers by binding to the consensus DNA sequence (C/T)TA(A/T)$_4$TA(G/A) found in the regulatory regions of most cardiac and muscle specific genes [9,10]. Less conserved amongst the MEF2 isoforms are the C-termini which are subject to alternative splicing [11,12] and a variety of posttranslational modifications such as acetylation [13–15], sumoylation [16,17] and phosphorylation, many of which have proved important in regulating MEF2.

Previous studies have identified several kinases that regulate MEF2 transactivation properties. Casein kinase II (CK2) phosphorylates MEF2C at serine 59 enhancing its DNA-binding capacity and

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hence transcriptional activity [18]. ERK5 interacts with the N-termini of MEF2A/C/D [19] and can phosphorylate serine 387 in the transactivation domain [20]. Of the kinases that target MEF2, p38MAPK has been most extensively studied and deemed to be a key requisite for skeletal and cardiac muscle differentiation. Defects in this pathway have also been associated with muscle related diseases, such as embryonal rhabdomyosarcoma (ERMS) [21]. MEF2A has multiple p38MAPK phosphoacceptor sites as indicated by mass spectrometric analysis [8] and all four isoforms have been repeatedly demonstrated to be activated by this kinase. During embryogenesis, p38MAPK activation of MEF2 is necessary for proper heart development [23] and is also involved in cardiac hypertrophy in adult heart tissue, both *in vivo* and *in vitro* [22,23]. Similarly, as well as being a key regulator of skeletal myogenesis *in vitro*, p38MAPK critically interacts with and activates MEF2 in the somite myotome during development [24]. Thus MEF2 is a key convergence point for several cellular signaling pathways in the control of striated muscle gene expression.

Two kinases that actively repress skeletal and cardiac muscle differentiation are PKA and GSK3β. Whilst the effect of PKA has been shown to be mediated through repression of MEF2 transactivation properties [25], the effect of GSK3β on this process is less clear. GSK3β is involved in multiple cellular processes including glycogen metabolism, embryonic development, cell proliferation and apoptosis [26,27]. Several unique features distinguish GSK3β from other protein kinases; it is constitutively active in unstimulated cells and paradoxically, it is inhibited in response to cellular signals such as growth factors [28]. More importantly phosphorylation of its substrates often leads to their subsequent ubiquitylation followed by proteasomal degradation [26]. GSK3β usually targets proteins that have already been phosphorylated by another kinase at a serine or threonine residue located four amino acids C-terminal to a consensus (S/T)XXX(S/T)-PO_4 motif [26,27]. In addition to this canonical consensus recognition sequence, GSK3β has been shown to phosphorylate KSP motifs in neurofilament proteins [29] and microtubule associated proteins [30] leading to their inactivation.

GSK3β has been studied extensively in insulin and Wnt signaling. Upon insulin binding to its receptor, activation of the phosphatidylinositol-3-kinase (PI3K) pathway occurs, leading to phosphorylation and hence inactivation of serine 9 on GSK3β via protein kinase B (PKB) [31] as well as C-terminal phosphorylation on serine 389 by p38MAPK [38]. In a myogenic context, PI3K activation has been shown to lead to cardiac and skeletal muscle hypertrophy [6,7,26,28,32,33] as well as being an activator of p38MAPK [34] and a co-requisite for p38MAPK induced chromatin remodeling [35,36]. Defects in the PI3K pathway lead to activation of GSK3β and consequently repression of myogenic differentiation [35]. In kidney cells induction of GSK3β results in the repression of JNK and p38MAPK through the inhibition of their upstream mitogen activated protein kinase kinase kinase (MAPKKK), MEKK4 [37]. Thus there is considerable circumstantial evidence suggesting an intersection between GSK3β and p38MAPK signaling pathways.

In this report, based initially on informatics analysis, we hypothesized that GSK3β is involved in the regulation of cardiac and skeletal muscle gene expression either by directly phosphorylating and hence destabilizing MEF2, or indirectly abrogating MEF2 activity through inhibition of p38MAPK. Whilst we report that MEF2A is a weak substrate of GSK3β *in vitro* we document that GSK3β activity represses MEF2 transactivation properties in both skeletal and cardiac myocytes both *in vitro* and *in vivo*. Pharmacological inhibition of GSK3β resulted in (i) increased MEF2 activity and (ii) de-repression of p38MAPK. Heart specific excision of GSK3β also resulted in up-regulation of p38MAPK activity. Gain of function assays using constitutively active GSK3β (S9A) repressed MEF2 activity which can be counteracted by exogenous activation of p38MAPK. Based on these data we propose integration of GSK3β and p38MAPK into the signaling network converging on the MEF2 transcription factors regulating both skeletal and cardiac gene expression.

2. Materials and methods

2.1. Plasmids

MEF2 and MCK reporter constructs (pMEF2, pMCK, pMCKΔMEF2) in pGL3 and expression vectors for MEF2A in pMT2 were used in reporter gene assays. The Gal4-MEF2A fusions have been described previously [43]. HA tagged Pax3-fkhr was cloned into pcDNA3.1 and kindly donated by Dr. Malkin and Adam Durbin at MaRS, Toronto. HA tagged GSK3β(S9A) was cloned in pcDNA3 ORF 995–2305. p38 and MKK6(EE) expression vectors were previously described [8].

2.2. Antibodies

Anti-MEF2A rabbit polyclonal antibody was produced with the assistance of the York University Animal Care Facility; anti-MEF2D (1:1000; BD Biosciences); β-catenin, phospho-β-catenin, p38, phospho-p38, ATF2, phospho-ATF2 and GSK3β (1:1000; Cell Signaling); actin, α/β-tubulin (1:2000; SantaCruz) were used for immunoblotting experiments.

2.3. Cell culture and transfection

C2C12 and RH30 cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone), 1% L-glutamine and 1% penicillin–streptomycin. Cells were maintained in a humidified, 37 °C incubator with a 5% CO_2 atmosphere. For transfections, cells were seeded 1 day prior to transfection and transfected according to the standard calcium phosphate method previously described by Perry et al. A mixture of 50 μl 2.5 M $CaCl_2$ per 25 μg DNA with an equal volume of 2× HeBS (2.8 M NaCl, 15 mM Na_2HPO_4, 50 mM HEPES, pH = 7.15) was used and the cells were and incubated overnight followed by washing and addition of fresh media [54]. Neonatal cardiomyocytes were isolated from 2- to 5-day-old rats. Whole hearts were separated and minced in a buffer solution (calcium and bicarbonate free Hanks with Hepes) and then dissociated into single cells by trypsin enzyme (Gibco) during repeated digestion with slow stirring. 10% FBS (Sigma) DMEM F12 (w/1% penicillin/streptotocin, 50 mg/L gentamycin sulfate) (Invitrogen) was added to the suspended cells and centrifuge for 10 min in 1200 rpm. The pellet was resuspended in medium. The isolated cells were plated for 30–60 min at 37 °C, allowing differential attachment of non-myocardial cells. The cardiomyocyte cells were counted and transferred to pre-gelatin coated plates.

The HL1 cardiac cell line was cultured in Claycomb Medium (Sigma Aldrich) supplemented with 100 μM norepinephrine (Sigma Aldrich), 10% FBS and 4 mM L-glutamine (Invitrogen). Cells were maintained in a humidified 37 °C incubator with 5% CO_2. The HL-1 cell line was originally established from an AT-1 subcutaneous tumor excised from an adult female Jackson Laboratory inbred C57BLy6J mouse.

Transient transfections in neonatal cardiomyocytes and HL-1 cells were performed using lipofectamine 2000. A 1:2.5 mixture ratio of DNA to lipofectamine in 250 μl Opti-Medium (Gibco) was prepared for a 4 h incubation.

2.4. Protein extractions, immunoblotting and reporter gene assays

Cells were harvested using an NP-40 lysis buffer (0.5% NP-40, 50 mM Tris–HCl [pH 8.0], 150 mM NaCl, 10 mM sodium pyrophosphate, 1 mM EDTA [pH 8.0], 0.1 M NaF) containing 10 μg/ml leupetin and aprotinin, 5 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride and 0.5 mM sodium orthovanadate. Protein concentrations were determined using the Bradford method (Bio-Rad) with bovine serum albumin (BSA) as a standard. Total protein extracts (20 μg) were used for immunoblotting, diluted in sample buffer containing 5% β-mercaptoethanol and boiled.

Transcriptional assays were done using luciferase reporter plasmids. The cells were harvested for these assays using 20 mM Tris, (pH 7.4) and 0.1% Triton-X 100 and the values obtained were normalized to β-galactosidase activity expressed from a constitutive SV40 driven expression vector and represented as relative light units (RLU) or in some cases corrected Luciferase values for control, reporter alone transfections were arbitrarily set to 1.0, and fold activation values were calculated. Bars represent the mean ($n=3$) and error bars represent the standard error of the mean ($n=3$).

Independent two sample *t*-tests of all quantitative data were conducted using R software. *P*-values are indicated with respect to controls where appropriate.

2.5. In vitro kinase assay

Purified recombinant GST-MEF2A (2.5 μg) (1–507) was mixed with 0.5 μg purified recombinant GST-GSK3β (1–433; Cell Signaling) and with [γ-^{32}P] ATP and incubated for 30 min at 37 °C. Samples were denatured for 5 min at 95 °C in SDS sample buffer. Protein samples were then separated by 10% SDS-PAGE and exposed on X-ray film (Kodak X-Omat) for 21 h to detect ^{32}P incorporation.

2.6. Animal treatment with GSK3 inhibitor in vivo

LacZ-MEF2 transgenic mice were used in this study. Two groups of male mice ($n=4$/each group) at 3 months old were used. The mice received i.p. injections of 0.6 M LiCl or 10 mM sterile PBS daily for 30 days. Mice were sacrificed by cervical dislocation. The apexes of heart and 10 μm transverse sections of the skeletal muscles were fixed with 2% paraformaldehyde in PBS for 30 min. After being washed three times with PBS, the samples were incubated with X-Gal solution (5 mM ferrocyanide, 5 mM ferricyanide, 2 mM $MgCl_2$, and 1 mg/ml X-Gal) at 37 °C.

2.7. Embryo treatment with GSK3 inhibitor in vitro

The MEF2-LacZ transgenic mouse embryos were dissected at 9.5 dpc from timed pregnant mice and cultured in a 24-well plate with 1 ml DMEM/10% FBS containing 50 μM GSK3β inhibitor TD-8 or solvent only (served as the control) at 37 °C for 24 h. After incubation, the embryos were fixed with 2% paraformaldehyde for 30 min. The embryos were rinsed twice with PBS, and then immersed in X-gal staining solution (5 mM ferrocyanide; 5 mM ferricyanide; 2 mM $MgCl_2$; 1 mg/ml X-gal) at 37 °C.

3. Results

3.1. Pharmacological inhibition of GSK3β enhances MEF2 transcriptional activity in skeletal myoblasts

Given that GSK3β suppresses myogenic differentiation [39,40] and that phosphorylation by GSK3β is generally inhibitory to protein function, it was hypothesized that the pharmacological inhibition of GSK3β might regulate MEF2A transcriptional activity. To test this hypothesis, C2C12 myoblasts were transfected with MEF2A and were treated with increasing concentrations (1–50 μM) of AR-A014418, a GSK3β inhibitor [41]. MEF2 activity was assessed using a 3× MEF2-Luciferase construct, a reporter gene containing three copies of the MEF2 *cis* element. These data illustrate enhanced transcriptional activity of both endogenous ($p<0.001$) and ectopically expressed MEF2 (Supplementary data) in a dose dependent manner upon GSK3β inhibition. The optimum concentration of AR-A014418 treatment was determined to be 10 μM ($p<0.001$) and the effect of this treatment on MEF2A transcriptional activity is depicted in Fig. 1A. To further corroborate this, the effect of GSK3β on the muscle creatine kinase (MCK) enhancer, a physiological MEF2 target, was also analyzed (Fig. 1B). The MCK enhancer is useful in studying muscle specific gene expression as it is highly dependent on MEF2, CARG-box and E-box *cis* elements during myogenesis. Therefore, myoblasts were co-transfected with MEF2A and either the wild type MCK-Luciferase construct or an alternate version containing mutated MEF2 binding sites (MCK-Luc ΔMEF) and treated with 10 μM AR for 19 h. Data revealed that GSK3β inhibition enhanced MCK-Luc activity ($p<0.001$) and that this effect was abrogated when the MEF2 sites are mutated, hence demonstrating that the effect of GSK3β is primarily through the MEF2 cis element (Fig. 1B). As a consequence it was hypothesized that the effects of GSK3β inhibition were mediated by modulation of MEF2A *trans*activation properties. GSK3β is predicted to phosphorylate the first serine or threonine in the consensus sequence (S/T)XXX(S/T) of its substrates preferably with the +4 serine or threonine already primed by phosphorylation by a different kinase [26,27], although this is not absolutely required. In human MEF2A, *in silico* analysis revealed several potential GSK3β phosphoacceptor sites. To begin to determine whether MEF2A is indeed a substrate for GSK3β, an *in vitro* kinase assay was performed using GST-MEF2A 1–507, purified GST-GSK3β and γ-^{32}P ATP. Bands were resolved using SDS-PAGE and revealed radio-labeled bands for autophosphorylated GSK3β and MyBP (a positive control). A very weak radio-labeled band for MEF2A was detected (Supplementary Fig. 2). However, compared to the positive control (MyBP) this phosphorylation was very weak and we therefore concluded that MEF2A was at best a weak GSK3β substrate *in vitro*. We subsequently hypothesized that any effects caused by manipulation of GSK3β on MEF2A activity were indirect.

3.2. GSK3β inhibition causes a posttranslational modification in MEF2A and this correlates with increased p38MAPK phosphorylation in skeletal myoblasts

Since a common mode of GSK3β regulatory activity is through modification of protein stability (e.g. β-catenin) [42], we also tested the effect of GSK3β on MEF2A protein expression levels. If GSK3β can indeed directly phosphorylate MEF2A, then GSK3β inhibition should either result in an increase in MEF2A expression or enhanced stability. To test this hypothesis, skeletal myoblasts were cultured and treated with increasing concentrations (0.1–50 mM) AR-A014418 for 4 h. Western immunoblotting revealed increasing MEF2A expression levels with increasing concentration of GSK3β inhibition, 0–50 μM AR-A014418 and that also corresponded with an obvious change in MEF2A gel migration (Fig. 2A). A lower mobility, high molecular weight form of MEF2A that is posttranslationally modified has already been identified [43] and at 50 μM AR-A014418, the faster migrating band shifted and merged with this slower migrating band forming a single, high molecular weight MEF2A band. This strongly suggests that MEF2A underwent a posttranslational modification [8,43]. Modification of MEF2A was obviously not due to GSK3β phosphorylation (since it was inhibited) and was reminiscent of effects that we have observed and documented before with p38MAPK [8]. Therefore we went on to test the possibility that GSK3β inhibition might cause p38MAPK phosphorylation using 50 μM AR-A014418 as well as a second GSK3β inhibitor, TDZD-8 [44]. The results are depicted in Fig. 2B and illustrate that the MEF2A band shift observed at 50 μM AR-A014418 corresponds with increased levels of phospho-p38MAPK. Similarly increasing concentrations (1–10 μM) of TDZD-8 resulted in increased phospho-p38MAPK protein levels as well a MEF2A band shift (Fig. 2B). Finally, we conducted a time course experiment with 50 μM AR-A014418 treatment for 0–24 h and looked at the effect of GSK3β inhibition on (i) p38MAPK activity (ii) ATF (a known p38MAPK substrate) and, (iii) β-catenin (a known GSK3β substrate, Fig. 2C). The results show activation of p38MAPK within 30 min of GSK3β inhibition and subsequent phosphorylation of its substrate, ATF which also coincided with the observed MEF2A band shift. These effects become increasingly prominent with time. De-phosphorylation of the GSK3β target,

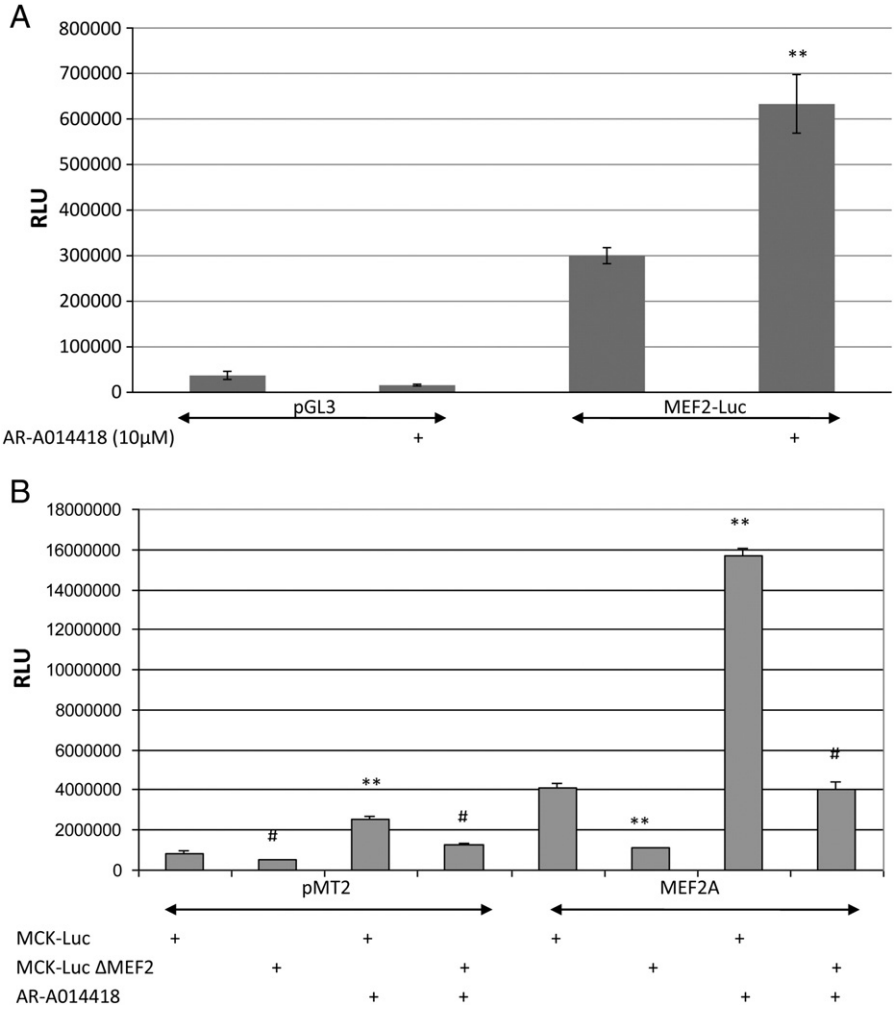

Fig. 1. Pharmacological GSK3β inhibition enhances (A) MEF2-Luc and (B) MEF2A transactivation of the MCK enhancer. C2C12 myoblasts were maintained in GM and transiently transfected with either pMT2 or pMT2-MEF2A and MCK-Luc with or without the MEF2 binding sites mutated. Luciferase activity was assessed using the respective reporter genes mentioned above and normalized to β-galactosidase activity. Cells were either treated with 10 μM AR-A014418 (unless otherwise depicted) or solvent (DMSO) for 19 h prior to harvesting. Data are the mean ± S.E. ($n = 3$). ** indicates a significant difference with respect to the control ($p < 0.001$) and # indicates no significant change.

β-catenin was also observed at 2–24 h indicating that the drug treatment worked.

3.3. Pharmacological inhibition of p38MAPK antagonizes the enhancement of MEF2 transcriptional activity caused by GSK3β inhibition in myoblasts

Based on our observations we next tested the possibility that GSK3β inhibition indirectly activates MEF2 through a corresponding de-repression of p38MAPK. To test this idea, we transfected C2C12 myoblasts with MEF2A, using 3×MEF2-Luc to assess MEF2 transcriptional activity and β-galactosidase activity as a control for transfection efficiency. We then treated the transfected cells with or without 10 μM AR-A014418 with either 5 μM p38MAPK specific inhibitor, (SB203580) or its negative control, (SB202474) for 6 h prior to harvesting. We hypothesized that if GSK3β indeed enhances MEF2 transcriptional activity through p38MAPK, then AR-A014418 would lose its effect when p38MAPK is inhibited. The results show that a 6 h treatment with 10 μM AR-A014418 enhances exogenous MEF2A transcriptional activity ($p < 0.001$) and that effect is reduced in the presence of 5 μM SB203580 (Fig. 3A). This effect is clear considering that in the presence of 5 μM SB202474 (which was used as a negative control) 10 μM AR-A014418 still enhanced MEF2 transcriptional activity ($p > 0.01$). To confirm that indeed this effect was caused by MEF2 transactivation and not an increase in protein levels, western blot analysis was included and revealed no enhanced MEF2A/D protein expression levels.

3.4. Constitutively active GSK3β(S9A) mutation reduces MEF2 transcriptional activity and this effect is rescued by p38MAPK

Since MEF2 activation by GSK3β inhibition was determined to be dependent on p38MAPK activity, we wanted to determine whether p38MAPK could rescue MEF2 inhibition by GSK3β. Myoblasts were therefore co-transfected with either pMT3 or p38/MKK6(EE) and pcDNA3.1 or GSK3β(S9A). Endogenous MEF2 activity was assessed using MEF2-Luc as described above. The data revealed that MEF2 transcriptional activity was potentiated by p38/MKK6(EE) ($p < 0.01$) and repressed by GSK3β(S9A) ($p < 0.01$, Fig. 3B). Interestingly, the combination of the two activated kinases resulted in neither activation nor repression of MEF2 compared to the control. This could mean one of two things: either activation of p38MAPK partially rescues MEF2 repression by GSK3β or that GSK3β represses p38MAPK induced activation of MEF2. Regardless, cross-talk between these two signaling pathways converges at MEF2 activity. However to

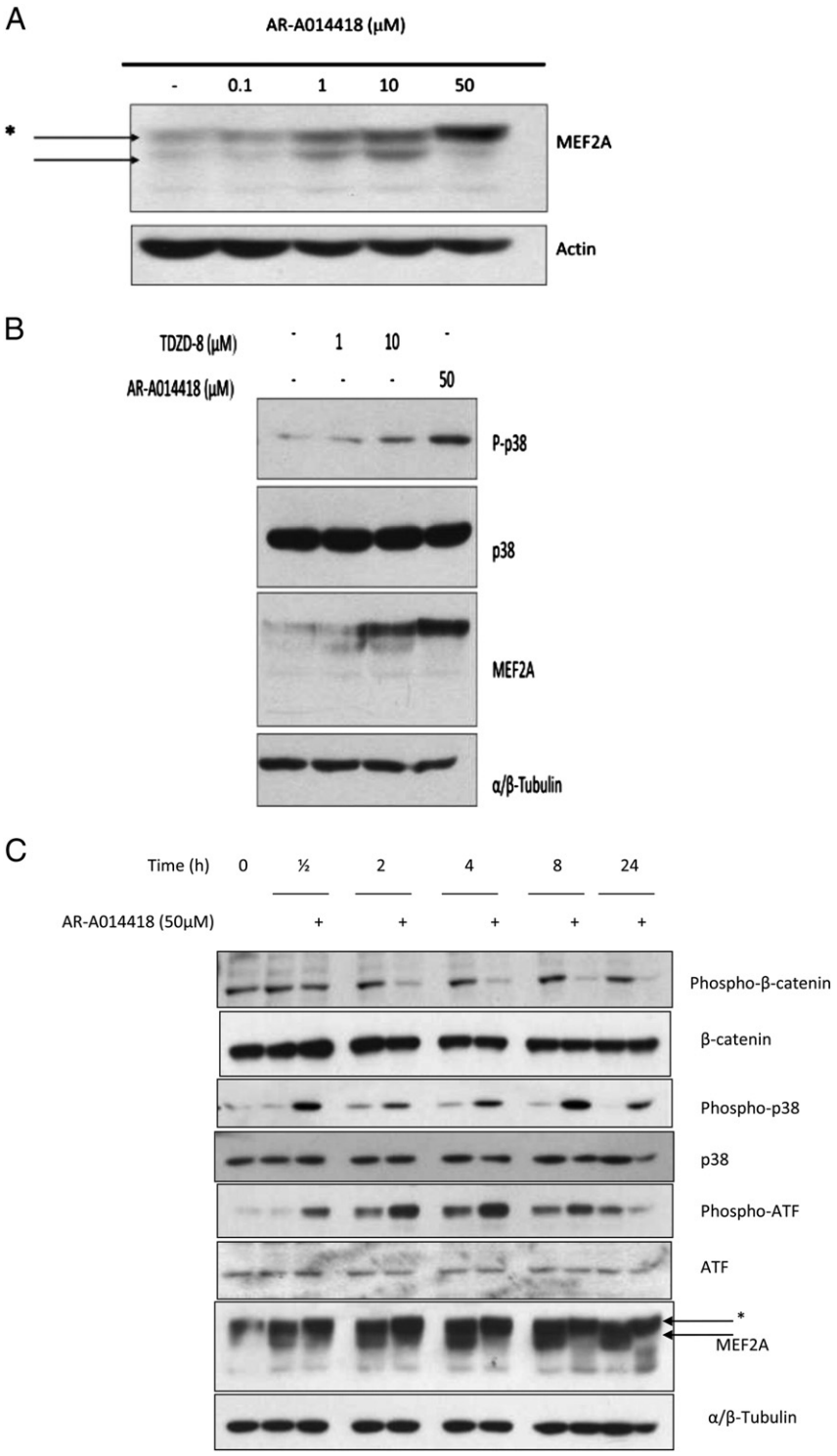

Fig. 2. GSK3β inhibition results in a (A) posttranslational modification of MEF2A in skeletal myoblasts which coincides with (B) the activation of p38MAPK and (C) activation of p38MAPK substrates. C2C12 myoblasts were maintained in growth conditions for 48 h and subsequently treated with GSK3β inhibitors, AR-A014418 (0.1–50 μM), TDZD-8 (1–10 μM) or solvent (DMSO) as indicated above. Following drug treatment, cells were lysed and equal amounts of protein (20 μg) were used for Western blot analysis. The levels of the indicated proteins were assessed by a standard immunoblotting technique using specific primary antibodies for each. α-Actin and α/β-tubulin were used as loading controls for (A) and (B) respectively. The arrows in (A) indicate MEF2A; the arrow with an asterisk points to a low mobility, high molecular weight form of MEF2A that has undergone a posttranslational modification. (B) This post-translational modification occurs upon treatment with either AR-A014418 or TDZD-8 and that this coincides with enhanced *p38* phosphorylation. (C) Total and phosphorylated protein levels of GSK3β and p38MAPK substrates were compared 0–24 h upon 50 μM treatment of AR-A014418. Activation of p38MAPK coincides with subsequent activation of its substrate, ATF as well as the observed MEF2A band shift. Similarly, GSK3β repression causes de-phosphorylation of its substrate, β-catenin.

address this question, p38MAPK protein expression and phosphorylation levels were analyzed under these conditions (Fig. 3C). MKK6(EE) increased phosphorylation of p38MAPK as expected (lane 2); however, in the presence of active GSK3β(S9A), MKK6(EE) lost its ability to phosphorylate p38MAPK (lane 3). This effect was only rescued upon 4 h treatment with 50 μM AR-A014418 (lane 4). GFP was used as a control

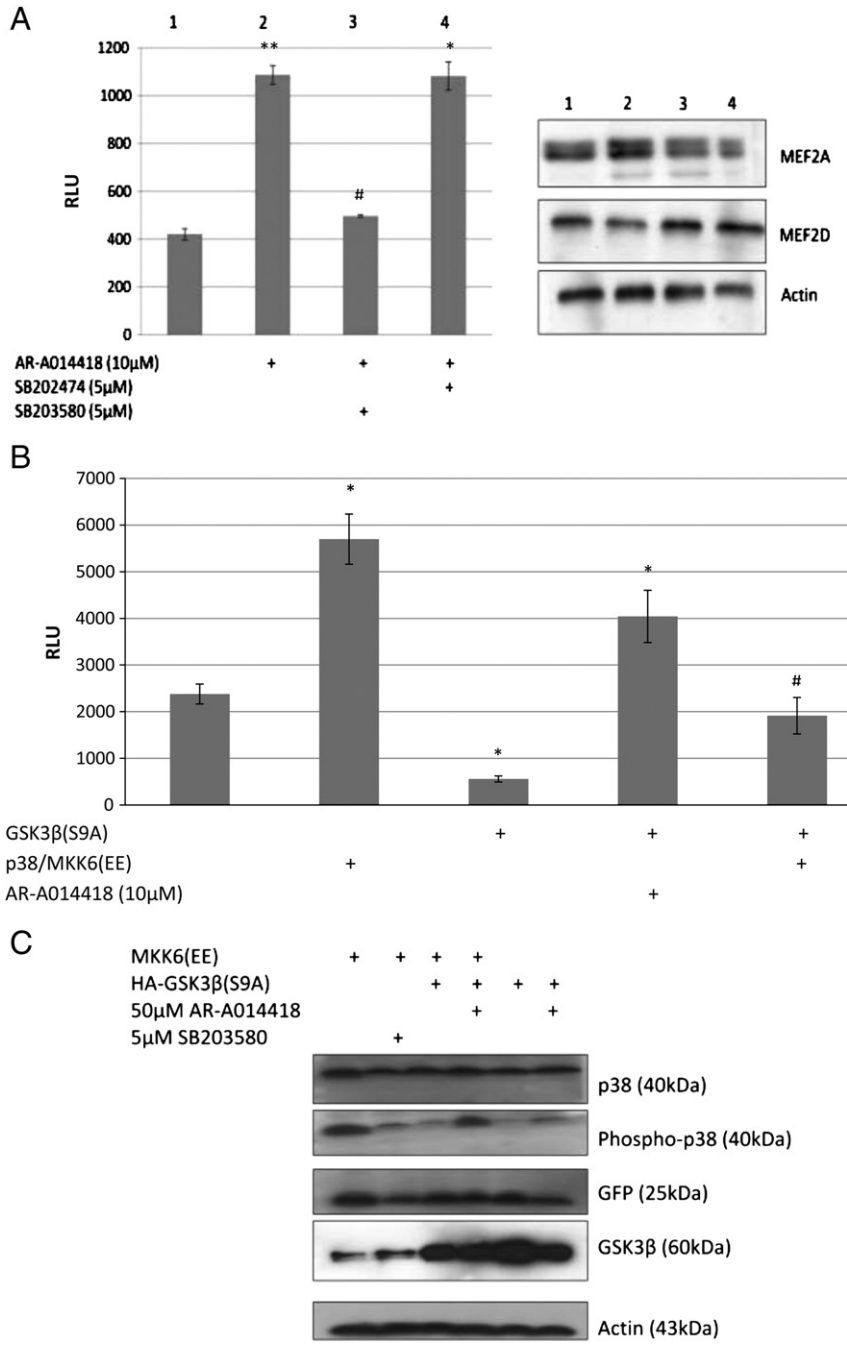

Fig. 3. MEF2-Luc assays: (A) Inhibition of p38MAPK reduces MEF2A transcriptional activity mediated by GSK3β inhibitor. C2C12 myoblasts were treated with different combinations of 10 μM AR-A014418 or solvent DMSO with or without 5 μM of p38 inhibitor, SB203580 or its negative control, SB202474 for 6 h prior to harvesting. The data is coupled with western blot analysis of MEF2A and D protein expression levels under the same conditions with actin used as a loading control. (B) MEF2 activity is repressed by constitutively active GSK3β(S9A) and enhanced by activated p38MAPK. Myoblasts were co-transfected with either pMT3 or p38/MKK6(EE) and pcDNA3.1 or GSK3β(S9A) and subsequently treated with either 10 μM AR-A014418 or DMSO as indicated, 19 h prior to harvesting. Data are the mean ± S.E. ($n = 3$). ** indicates a significant difference with respect to the control ($p < 0.001$), *indicates a significant difference with respect to the control ($p < 0.05$) and, # indicates no significant change. (C) Activated GSK3β(S9A) de-phosphorylates MKK6(EE) activated p38. Myoblasts were transfected with different combinations of active MKK6(EE), GSK3β(S9A) or both and treated with or without 50 μM AR-A014418 and 5 μM SB203580 as indicated.

for transfection efficiency and actin was used as a loading control. If GSK3β inhibited the p38MAPK pathway upstream of MAP2K as suggested in the literature [37] then ectopically expressed activated MKK6(EE) should have rescued p38 phosphorylation. However based on the data this was not the case; therefore, we conclude that regulation of p38 phosphorylation by GSK3β is at the MAPK level.

3.5. GSK3β inhibition enhances endogenous MEF2 activity in primary neonatal cardiomyocytes and HL-1 cells

Since MEF2 proteins are crucial regulators of cardiac morphogenesis [45], vascular development [46], cardiac remodeling [47,48] and cardiac hypertrophy [49] we hypothesized that GSK3β might regulate the effect

of MEF2 in the heart as well. To test this idea we used two *in vitro* models. In the first, neonatal cardiomyocytes were extracted, isolated and cultured before being transfected with pGL4-3xMEF2 Luciferase to assess MEF2 transcriptional activity upon 19 h treatment with increasing concentrations (0–10 μM) of AR-A014418 (data not shown). In the second model, endogenous MEF2 activity was measured in HL-1 immortalized cardiac cells treated with either solvent (DMSO) or 10 μM AR-A014418 (data not shown). In both cell culture models, MEF2 transcriptional activity is enhanced by GSK3β inhibition in a dose dependent manner similar to the data observed in our skeletal myoblast model.

3.6. Pharmacological GSK3β inhibition or Cre-Lox mediated GSK3β excision in the heart causes increased p38MAPK activity in cardiac myocytes both in vitro and in vivo

To assess whether GSK3β suppression of MEF2 in cardiomyogenesis is through the regulation of MEF2A/D protein expression or indirectly through p38MAPK, we analyzed endogenous protein levels by a standard western immunoblotting technique upon 4 h treatment with increasing concentrations (0–20 μM) of AR-A014418 in primary neonatal cardiomyocytes. The data clearly depicts enhanced phosphorylation of p38MAPK in a dose dependent manner up to the 10 μM drug treatment but no further elevation at 20 μM (data not shown). *In vivo* analysis of GSK3β effects on p38MAPK activity in cardiac tissue was achieved by analyzing cardiac specific Cre-Lox excision of exon 2 of the GSK3β gene. The Cre recombinase was flanked by a tamoxifen-inducible mutated estrogen receptor under the control of an α-MyHC promoter to render it cardiac specific and Cre expression was activated at 10–12 weeks by treating the mice with 20 mg/kg tamoxifen citrate for four consecutive days. Heart tissue from these mice was then isolated and lysed for protein analysis by western immunoblotting and protein levels were quantified using quantitative western blotting (LiCor odyssey system). Data analysis revealed a substantial decrease in GSK3β in three independent floxed tamoxifen treated mice; residual GSK3β likely emanates from non-cardiac cells such as fibroblasts (in which Cre would not be activated). This cardiomyocyte excision of GSK3β resulted in a 2.5–3 fold increase in phosphorylated p38 (Fig. 4A).

3.7. GSK3β inhibition enhances MEF2 transcriptional activity through p38MAPK in cardiomyocytes as well as downstream target, ANF promoter activity and expression

Primary neonatal cardiomyocytes and HL-1 cells were treated with 10 μM AR-A014418 and either 5 μM p38MAPK specific inhibitor, SB203580 or SB202474 (an inactive analog) as described above, for 6 h before measuring MEF2 transcriptional activity. P38MAPK inhibition reduces the enhancement of MEF2 activity by 7-fold ($p<0.001$) in primary cardiomyocytes (Fig. 4B) resulting from GSK3β inhibition. Furthermore, we assessed that GSK3β inhibition enhanced ANF-Luc promoter activity ($p<0.01$) but not an alternate version containing mutated MEF2 sites (ANF-Luc ΔMEF2). Protein samples from GSK3β floxed mice revealed enhanced ANF protein levels but no change in MEF2A/D protein expression levels, supporting the idea that GSK3β regulates MEF2 transactivation properties and not protein expression levels (Fig. 4C).

3.8. In vivo inhibition of GSK3β enhances MEF2 activity in the hearts of MEF2-LacZ transgenic mice

To further investigate this possible cross-talk between GSK3β and p38MAPK we used a third known GSK3β inhibitor, lithium chloride (LiCl) and administered either 0.6 M LiCl or solvent (PBS) intraperitoneal daily for 30 days to MEF2 LacZ sensor mice [53,24]. Mice were sacrificed 1 h after the last treatment before (i) the apexes of heart (Fig. 5A) and, (ii) 10 μm transverse sections of the skeletal muscles (Fig. 5B) were fixed with 2% paraformaldehyde for 30 min. The tissue samples were then stained with X-Gal overnight and visualized for MEF2 activity. The data depicts a small qualitative enhancement of MEF2 activity in the hearts and skeletal muscles, illustrated by the dark blue stains in both sets of tissue samples. Lithium is already used to treat psychiatric disorders such as depression [54] and Alzheimer's [55] but our data implies that lithium treatment may also influence gene expression in cardiac and skeletal muscle. Since MEF2 is also a key player in neuronal function, lithium treatment could also influence these disorders through MEF2 activity.

4. Discussion

In this report, we document GSK3β as a negative regulator of MEF2 transcriptional activity in skeletal and cardiac muscle. This effect is mediated indirectly through repression of the p38MAPK pathway, a known positive regulator of MEF2 activity. A variety of loss of function approaches have revealed that abrogation of GSK3β signaling leads to enhanced MEF2 transcriptional activity, both *in vitro* and *in vivo* in skeletal myoblasts and cardiac myocytes. Furthermore, inhibition of GSK3β enhances p38MAPK phosphorylation *in vitro* in skeletal myoblasts, HL-1 and neonatal primary cardiomyocytes as well as *in vivo*, in heart restricted GSK3β excised mice. Thus, several lines of evidence reveal cross-talk between GSK3β and p38MAPK in the control of MEF2 activity in cardiac and skeletal muscle. Our conclusions are summarized in Fig. 6, adding a further level of complexity regarding the modulation of p38MAPK activation for the control of striated muscle gene expression through the MEF2 *cis* element. Numerous previous studies have documented a profound role of pharmacological inhibition of p38MAPK for gene expression in these cell types and it is unequivocal that a major target for p38MAPK is MEF2 [8,22–24,35,36]. Interestingly GSK3β has long been known to be an important regulator of cardiac [45,46] and skeletal muscle gene expression although its targets are much less clear. Studies reported here indicate a reciprocal relationship between GSK3β and p38MAPK at the MEF2 *cis* element that in many cases explains the phenotypic impact of modulation of GSK3β signaling in these tissues. In skeletal myogenesis p38MAPK activation is required for differentiation and inhibition abrogates myogenesis. Consistent with this, constitutive GSK3β activation inhibits myogenesis and pharmacological inhibition potentiates myogenesis. Likewise, effects of GSK3β in the heart are consistent with the modulation of p38MAPK signaling to MEF2. Constitutive activation of GSK3β in the heart protects against cardiac hypertrophy [50,51] and p38MAPK regulation of MEF2 has been strongly implicated in the hypertrophic program [22]. Thus, the idea of reciprocal antagonism between GSK3β and p38MAPK at the MEF2 cis element is supported by a variety of experimental strategies in both cardiac and skeletal muscle. Cardiac infarction caused by myocardial ischemia and reduced oxygen supply has been shown to be reduced in dnGSK3β hearts and that preconditioning loses its protective effect in when GSK3β is constitutively active [52]. It is highly likely based on our data that during oxygen deprivation GSK3β is antagonizing p38MAPK signaling in response and that upon inhibition of GSK3β, p38MAPK can carry out its stress response.

Collectively our findings display an important level of reciprocal antagonism between GSK3β and p38MAPK signaling in cardiac and skeletal muscle. In view of the central role of both signaling pathways in a variety of developmental, physiological and pathological processes in both tissue types, these findings will have important therapeutic implication for the treatment of striated muscle pathology.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2012.10.013.

Disclosures

None declared.

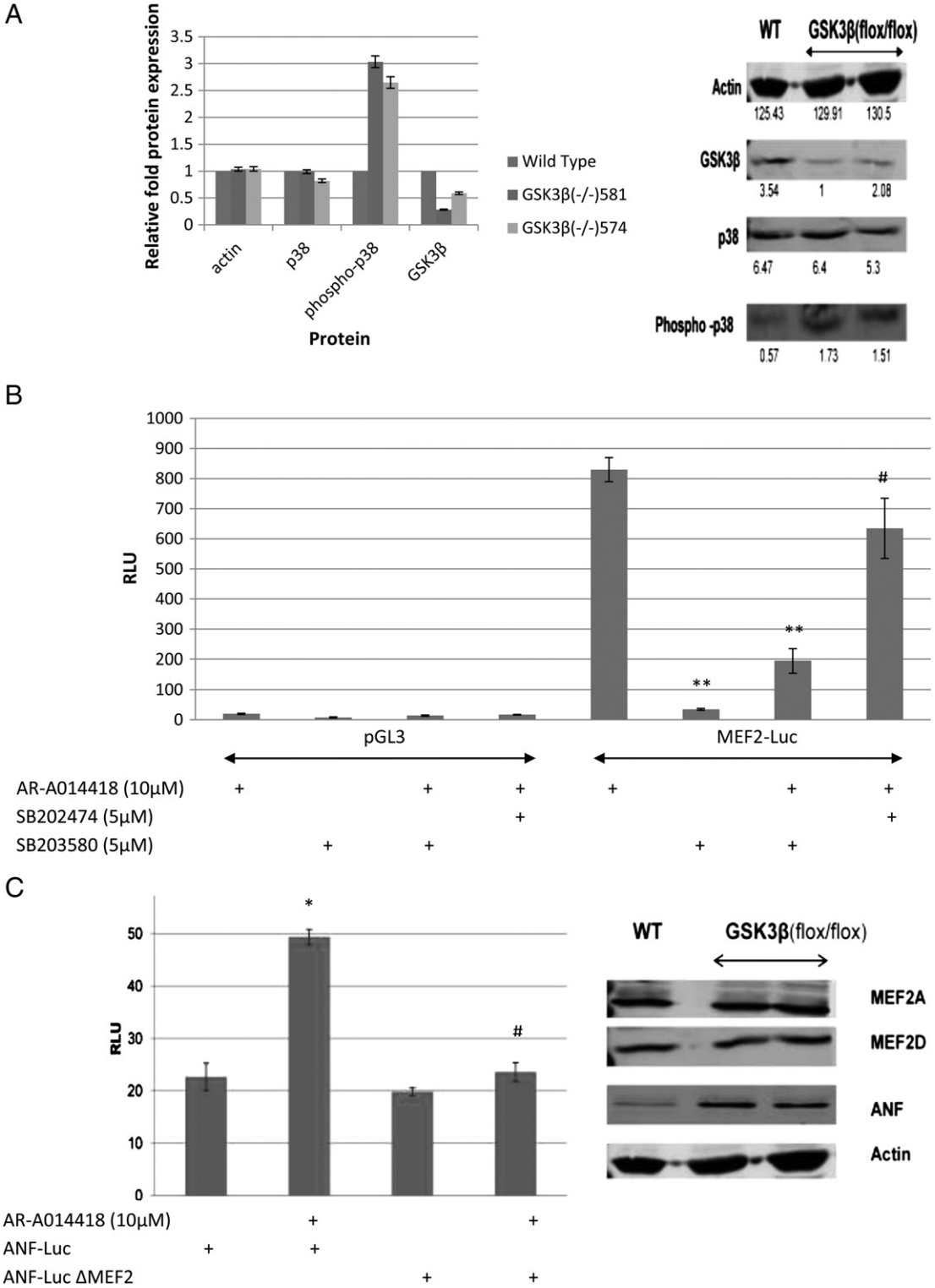

Fig. 4. (A) *In vivo*, phospho-p38 protein levels are elevated 3-fold in GSK3β knockout mice. Cardiomyocytes from GSK3β (−/−) mice were lysed and equal amounts of protein (20 μg) were used for Western Blot analysis. The levels of the indicated proteins were assessed by a standard immunoblotting technique using specific primary antibodies for each as indicated and quantified using the Odyssey system ($n=3$). (B) Inhibition of p38MAPK reduces MEF2A transcriptional activity mediated by GSK3β inhibitor. Primary neonatal cardiomyocytes were treated with different combinations of 10 μM AR-A014418 or solvent DMSO with or without 5 μM of p38 inhibitor, SB203580 or its negative control, SB202474 for 6 h prior to harvesting. Data are the mean ± S.E. ($n=3$). ** indicates a significant difference with respect to the control ($p<0.001$), *indicates a significant difference with respect to the control ($p<0.05$) and, # indicates no significant change. (C) GSK3β inhibition enhances (i) transactivation of ANF promoter through MEF2 and (ii) ANF protein expression levels. The effect of 10 μM AR-A014418 or solvent DMSO was assessed on ANF-Luc and an analog with the MEF2 site mutated (ANF-Luc ΔMEF2) together with ANF and MEF2A/D expression levels in cardiomyocytes from GSK3β (−/−) mice.

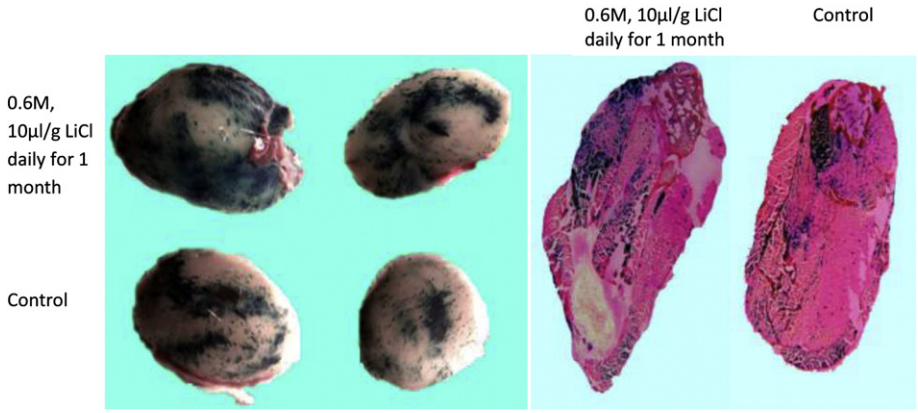

Fig. 5. Animal treatment with GSK3β inhibitor *in vivo*. Two groups ($n=4$/group) of male LacZ-MEF2 transgenic mice were used in this study. The mice received i.p. injections of either 0.6 M LiCl (10 µl/g) or sterile 10 mM PBS daily for 30 days. Mice were sacrificed 1 h after treatment and (A) the apexes of heart and (B) 10 µm transverse sections of the skeletal muscles were fixed with 2% paraformaldehyde in PBS for 30 min. The samples were then incubated with X-Gal solution overnight and visualized for MEF2 activity. The dark blue stain indicates MEF2 activity which is enhanced with GSK3β inhibition in both the heart and skeletal muscle tissue samples.

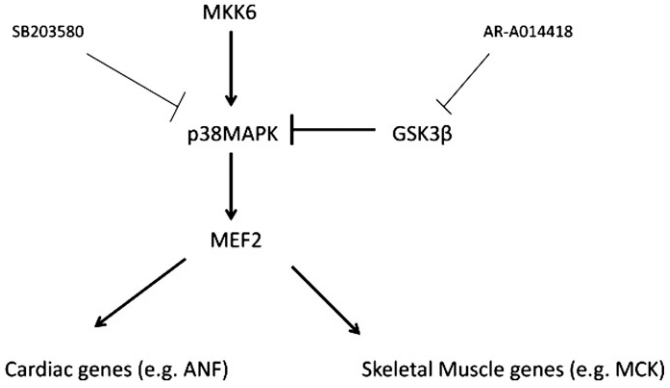

Fig. 6. Summary. Our data demonstrates that MKK6 phosphorylates p38MAPK which subsequently activates MEF2 and hence downstream cardiac genes such as ANF in cardiomyocytes and, MCK in skeletal myoblasts, hence promoting differentiation. This pathway is antagonized by GSK3β which suppresses p38MAPK activity. Pharmacological treatment with (a) SB203580 and (b) AR-A014418 results in inhibition of p38MAPK and GSK3β respectively. The latter resulted in increased p38 phosphorylation and enhanced MEF2 activity.

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