Three fingers on the brake: Kruppel-like factor 15, a repressor of cardiac gene expression
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Regulation of Cardiac Gene Expression by KLF15, a Repressor of Myocardin Activity


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SUMMARY

Pathological forms of left ventricular hypertrophy (LVH) often progress to heart failure. Specific transcription factors have been identified that activate the gene program to induce pathological forms of LVH. It is likely that apart from activating transcriptional inducers of LVH, constitutive transcriptional repressors need to be removed during the development of cardiac hypertrophy. Here we report that the constitutive presence of krüppel-like factor 15 (KLF15) is lost in pathological hypertrophy and that this loss precedes progression towards heart failure. We show that TGFβ mediated activation of p38 MAPK is necessary and sufficient to decrease KLF15 expression. We further show that KLF15 robustly inhibits myocardin, a potent transcriptional activator. Loss of KLF15 during pathological LVH relieves the inhibitory effects on myocardin and stimulates the expression of SRF target genes, such as ANF. This uncovers a novel mechanism where activated p38 MAPK decreases KLF15, an important constitutive transcriptional repressor whose removal seems a vital step to allow the induction of pathological LVH.
INTRODUCTION

Pathological forms of LVH often progress to heart failure. Many transcription factors have been identified that play a role in the development of pathological LVH. Most of these transcription factors are inducers of hypertrophy, for instance GATA4, MEF2, NFAT and SRF and their function and regulation are increasingly understood. Myocardin is an extraordinarily potent transcriptional coactivator expressed exclusively in cardiomyocytes and smooth muscle cells. Myocardin stimulates transcription from CArG-dependent muscle enhancers, but does not bind DNA directly. Instead, myocardin forms a stable ternary complex on CArG-boxes by associating with SRF. Recently, Parmacek and colleagues showed that ablation of myocardin in the adult mouse heart leads to rapid onset of heart failure, which was accompanied by dissolution of sarcomeric organization. Moreover, patients with dilated or hypertrophic cardiomyopathy have been reported to harbor causal mutations in myocardin-regulated genes such as ACTN2, MYH7, ACTC and TPM1. Altogether, these studies have shown that myocardin regulates the organization of the contractile unit and adaptive responses of the cardiomyocyte to stress.

In contrast to these activators, repressors of cardiac gene expression and hypertrophy are less well explored. One of the best studied repressors of cardiac hypertrophy is the family of histone deacetylases (HDACs). For instance, HDAC9 null mice display spontaneous cardiac hypertrophy and are hypersensitive to pressure overload. In addition, Nab1 has been identified to repress hypertrophy by direct inhibition of Egr-dependent transcription. Recently it was also shown that KLF15 acts as a transcriptional repressor of pathological cardiac hypertrophy. KLF15 belongs to the family of krüppel-like factors, which has 17 members. KLF15 is widely expressed but the highest expression levels are found in liver, kidney, pancreas and cardiac and skeletal muscle. The zinc finger transcriptional regulator KLF15, was first identified as a repressor of the kidney specific chloride channel CLC-K1. KLF15 can also act as an activator of transcription as illustrated by the increased GLUT4 promoter activity and GLUT4 expression in response to KLF15 overexpression. Expression of KLF15 in the mouse heart is very low during development but increases after birth to reach high levels in the adult heart. Mice lacking KLF15 have higher baseline expression of ANF and BNP. Interestingly, when these mice are exposed to pressure overload they develop rapid and severe hypertrophy and heart failure, suggesting that KLF15 may repress cardiac hypertrophy. Here we sought to understand how KLF15 is regulated and how it represses cardiac hypertrophy. Our results reveal that the TGFB p38 MAPK axis down-regulates KLF15 and that KLF15 acts as a potent competitive inhibitor of myocardin, thereby preventing transcription of SRF target genes.
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MATERIAL AND METHODS

Animal models
Ren-2 rats were obtained from Möllegard Breeding Center, Lille Skensveld, Denmark and were studied as described.\(^\text{20}\)

Neonatal rat cardiomyocyte isolation and transfection.
1-2 day old Lewis neonatal rats were sacrificed by decapitation. Hearts were carefully removed en left ventricles were cut into small pieces. Cardiac cells were then isolated by enzymatic disassociation using 0.05% collagenase I (Invitrogen, Breda the Netherlands, #17100-017) and 0.05% pancreatin (Sigma-Aldrich, Zwijndrecht, the Netherlands #p3292) dissolved in 1xHanks Balanced Salt Solution (Sigma-Aldrich, Zwijndrecht, the Netherlands #H4641). Cells were pre-plated for 1 hour in DMEM (Invitrogen, Breda, the Netherlands #11966) (supplemented with 10% horse serum (HS), 5% New born calf serum (NBCS), 0.16% glucose and antibiotics) to separate myocytes from fibroblasts. After 1 hour, cardiomyocytes were collected, counted and plated in plates coated with 1% gelatin (Fluka, via Sigma Aldrich, Zwijndrecht, the Netherlands). Overnight, cells were grown in DMEM supplemented with 10% horse serum, 5% NBCS, 0.16% glucose and antibiotics). Dharmacon ON-TARGETplus siRNA SMARTpools for non-targeting control (D-001810-10) and KLF15 (L-080131-01) were transfected (lipofectamine 2000, Invitrogen) to a final concentration of 300 nM and incubated for 5 hours. Medium was changed to medium containing 2% BSA and after 72 hours cells were fixed with 4% PFA, permeabilised with 0,1% triton X-100 in PBS and stained with phalloidin 1:40 (Invitrogen F432) in PBS for actin. Images were taken with a Leica inverted microscope and cell-size (actin-positive area) was analysed with Scion image.

Stimulation of cardiomyocytes
Prior to stimulation of cardiomyocytes with TGFβ1 (10ng/ml) (Peprotech, via Tebu-Bio, the Netherlands), cells were serum starved for 24hrs with DMEM supplemented with 0.16% glucose and antibiotics. Cells were stimulated for 24hrs. One hour prior to TGFβ1, cells were treated with two indepenent p38-MAPK inhibitor SB202190 or SB203580 (Sigma-Aldrich, Zwijndrecht, the Netherlands). Endothelin-1 (100nM) and phenylephrine (50\(\mu\)M), Insulin (100nM) (Sigma Aldrich), IGF-I (100nM) and IGF-II (100nM) (Peprotech, via Tebu-Bio) were used to stimulate cardiomyocytes as described for TGFβ1.

\(^{3}\)H leucine measurements
TGFβ1 (10ng/ml) and SB202190 (10\(\mu\)M) stimulated cells were exposed to \(^{3}\)H leucine (1\(\mu\)Ci) for 24 hours. After 24 hours of TGFβ1 stimulation, cells were scraped in 5%
trichlore acetic acid (TCA), centrifuged and pellets were dissolved in 0.5 NaOH. Activity was measured in 4 ml high ionic count fluid in a scintillation counter.

**Phalloidin staining**
Cardiomyocytes were fixed with 4% paraformaldehyde for 10 min at RT. Cells were washed with 0.3% Triton-X (in PBS) for 10 min at RT. Cells were washed with PBS and exposed to fluorescent phalloidin (Invitrogen) (1 ul stock in 200 ul PBS) for 20 min at RT.

**Adenoviral infection**- infection of cardiomyocytes with AdMKK6 was performed to phosphorylate p38 MAPK as described. For infection of cardiomyocytes, 200 ul of viral supernatant (AdMKK6 and AdGFP) was added to 5x10^5 cardiomyocytes that were plated in a 6-well gelatinized six-well plate and cultured as described above. Three days after infection, cells were harvested for protein analyses by scraping the cells in a SDS buffer containing 10% 2-Mercaptoethanol and 0.5 mM sodium orthovanadate. Western blotting was performed using phospho-p38(thr180/Tyr182) (1:1000) and p38 MAP kinase (1:1000) (Cell Signaling Technology, via Bioké, the Netherlands).

**Neonatal mouse cardiomyocyte isolation and transfection.**
Mouse neonatal cardiomyocytes were isolated from 1-3 day old FVB mice. Heart tissue was incubated overnight at 4°C in 0.5 mg/ml trypsin (USB 22715) HBSS (sigma H4641) supplemented with 1 g/l D-glucose (merck). Tissue was digested in collagenase buffer (150 u/ml collagenase type II (Worthington 4176) in DMEM 41965 (Gibco) for 45’ at 37°C. Individual cells were obtained by trituration and filtering over a 100 μm filter. Cells were centrifuged and pre-plated for 1.5 hours at 37°C and 5% CO₂. Cells were seeded in plating medium (10% FCS, 20 μM L-glutamine and 10 μM AraC in DMEM) on 1% gelatin (Sigma-Aldrich (G1890)) coated culture plates at a final density of 100,000 cells/cm². After 48 hours medium was changed to DMEM 41965 supplemented with 25 mM L-glutamine, 2% BSA (Sigma-Aldrich (A6003)), 0.25 μU/ml Insulin (Sigma-Aldrich I6634), 250 μM L-carnitine (Sigma-Aldrich C0283) and 10 μM AraC (Sigma-Aldrich C6645)). 24 hours later medium was changed to DMEM 41965 without pen/strep for transfection. All solutions were supplemented with 100 u/ml pen/strep and 10 mM HEPES. Transfection was performed according to manufacturers protocol. Dharmacon ON-TARGETplus siRNA SMARTpools for non-targeting control (D-001810-10) and KLF15 (L-059453-01) were added with lipofectamine 2000 (Invitrogen 11668) as described above.

**Quantitative Real Time PCR**
For quantitative Real Time PCR, RNA was isolated from cardiomyocytes or left ventricles using the RNeasy mini kit (Qiagen) or Trizol (Invitrogen) according to manufacturers protocols. cDNA was synthesised using the iScript cDNA Synthesis Kit (Bio-Rad)
Laboratories. Quantitative Real Time was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories), 20ng of total RNA and 10 pmol/μl forward and reverse primers (Suppl table 1a). Quantification was performed using LinRegPCR analysis software. Transfections and luciferase assays - Transfection of COS7 cells for luciferase assays were performed as described. SM22(505bp) luciferase reporter was a kind gift of Dr. JM Miano (Rochester, NY, USA). The 3xCArG- and ANF(638bp) luciferase reporter and the expression plasmids encoding Myocardin (935aa) and SRF were provided by Dr. Eric Olson (Dallas, Tx, USA). A pcDNA3.1 based expression vector encoding Flag-tagged KLF15 was donated by Dr. MK Jain (Cleveland, OH, USA). Transfection of COS7 cells and luciferase assays were performed as described. In short, twenty-four well plates with COS7 cells were transfected per well with 30ng of pcDNA-β-gal, 75ng of luciferase reporter and 50ng of pcDNA-KLF15 and myocardin(935). Transfection of vectors was facilitated using GeneJammer (Stratagene, via Bio-Connect, Huissen, the Netherlands). For titration experiments we transfected 50ng ANF- or Sm22- luciferase, 50 ng pcDNA-myocardin and increasing concentrations of KLF15 (1-100ng) or pcDNA-SRF (0,5-25ng) as indicated. Luciferase assays were performed using the luciferase Assay System (Promega). Measurements were performed in duplicate and repeated at least three times.

GST pull-down
Glutathion S-Transferase (GST)-KLF15 fusion proteins were generated by subcloning the KLF15 open reading frame into the pGEX-KG vector. GST fusion proteins were produced and isolated by standard procedures. Deletion mutants of myocardin were constructed through PCR-based mutagenesis and subcloning of these DNA fragments into FLAG-tagged pcDNA3.1 expression vector. Myocardin proteins were translated in vitro and labeled with [35S]methionine in a coupled transcription-translation T7 reticulocyte lysate system (Promega) and assayed for binding to GST-fusion proteins.

Statistical analyses
Data are shown as mean ± SEM. Unpaired t-test was used. P-values of ≤0.05 were considered statistically significant.

RESULTS

Loss of KLF15 expression is specific for pathological LV hypertrophy.
To explore whether KLF15 expression, besides in the TAC model, is also regulated in other models of hypertrophy and failure we studied KLF15 expression in the homozygous TGR(mRen2)27 rat (Ren-2). In this model KLF15 is down-regulated already in an early
stage of LVH. We employed this model to evaluate whether down-regulation of KLF15 could distinguish the hypertrophied hearts that will quickly progress to failure versus those where hypertrophy remains prolonged compensated. We obtained cardiac biopsies at a time when all Ren-2 rats displayed similar cardiac hypertrophy and were well compensated. After cardiac biopsies were taken, we followed each rat to see whether it would progress to failure or not (see also chapter 3). This revealed that KLF15 expression was decreased significantly more in the hypertrophied hearts that later progressed towards failure (Figure 1a). To evaluate whether KLF15 is also down-regulated in physiological LVH, we assessed KLF15 expression levels in rats with exercise induced LVH. Heart weights from trained rats were significantly increased compared to those from sedentary rats. Training resulted in hypertrophy of individual cardiomyocytes as shown by an increased length, but not width, of these cells. (Supplemental Figure 1a-d). Despite this pronounced cardiac remodeling no difference in KLF15 gene expression
between the sedentary group and the trained rats was noted (Figure 1b).

KLF15 expression is regulated by the TGFβ-p38 MAPK pathway. That KLF15 is specifically down-regulated in pathological hypertrophy prompted us to search for the stimuli and pathways that repress KLF15. We used cultured neonatal rat cardiomyocytes to study the effect of several hypertrophic stimuli such as phenylephrine, endothelin-1, TGFβ and fetal calf serum (FCS) on the expression of KLF15. All stimuli were able to decrease KLF15 expression levels (Figure 1c). In contrast, when cardiomyocyte growth was stimulated with stimuli that are known to induce physiological hypertrophy, (insulin, IGF-1 and IGF-2) KLF15 levels did not decrease (Figure 1d). This further supports the in vivo findings that KLF15 is down-regulated only in pathological hypertrophy. Since TGFβ was the most robust inhibitor of KLF15 expression we explored this pathway more extensively. The observed TGFβ-mediated downregulation of KLF15 was completely abolished in cultured cardiomyocytes after knockdown of transforming growth factor beta receptor 1 (TGFBR1 or ALK5) (Figure 2a and Supplemental Figure 2), demonstrating that TGFβ signaling via ALK5 is vital to regulate KLF15 expression.

It is known that during hypertrophy of cardiomyocytes, TGFβ induces p38 MAPK phosphorylation via TAK1, suggesting that TGFβ may decrease KLF15 expression by activation of p38 MAPK. In order to investigate this hypothesis we treated cultured cardiomyocytes with two different specific p38 MAPK inhibitors: SB202190 and SB203580. Indeed, TGFβ-induced down-regulation of KLF15 was dose-dependently abolished by either inhibitor (Figure 2b and Supplemental Figure 3). Strikingly, KLF15 expression was inversely correlated with ANF expression (Supplemental Figure 3).

Apart from preventing KLF15 downregulation, inhibition of p38 phosphorylation blunted the TGFβ-induced hypertrophic response of cardiomyocytes as assessed by [3H]leucine incorporation and phalloidin staining of F-actins (Figures 2c and d). From this we conclude that in cardiomyocytes p38 MAPK signaling is necessary for TGFβ induced hypertrophy and KLF15 down-regulation. To elucidate whether activation of p38 MAPK is not only necessary, but also sufficient to repress KLF15 expression downstream of TGFβ, we activated p38 MAPK by adenoviral overexpression of the upstream kinase of p38 MAPK, MKK6 which induced a robust increase of phosphorylated p38 (Figure 2e). This resulted in an almost 80% decrease in KLF15 mRNA levels and induced expression of the hypertrophy marker BNP in cultured cardiomyocytes (Figure 2e). In conclusion, we show that activation of p38 MAPK is necessary and sufficient to decrease KLF15 expression in cardiac myocytes, providing one of the first examples of a cardiac transcription factor that is actually down-regulated by activated p38.

KLF15 inhibits the activity of myocardin, a transcriptional co-activator of SRF. Earlier
studies have shown that loss of KLF15 aggravates cardiac hypertrophy and dysfunction. We confirmed these observations, after transfecting cultured neonatal rat and mouse cardiomyocytes with two independent smartpool siRNAs against KLF15 or a non-targeting control siRNA. This resulted in a 16-30% increase in cardiomyocyte size and shows that loss of KLF15 alone is sufficient to induce cardiomyocyte hypertrophy and elevate ANF expression (Supplemental figure 4). The opposite also holds true; overexpression of KLF15 using a lentiviral approach seems to inhibit cardiomyocyte hypertrophy, as demonstrated by decreased mRNA levels of ANF in rat neonatal cardiomyocytes (Supplemental figure 4). It remains unknown how KLF15 inhibits LVH.

![Figure 2](image-url)

*Figure 2.* In vitro activation of p38 MAPK is both necessary and sufficient to decrease KLF15 levels. (a) TGFβ regulates KLF15 expression in vitro. Neonatal rat cardiomyocytes were infected with a lentivirus containing shRNA against ALK5 or with a control lentivirus. TGFβ decreased expression of KLF15 after control virus, but had no effect in the cells treated with the shRNA. (n=3/group,*:p<0.01 compared to control group; †:p<0.01 compared to TGFβ treated cells without shRNA against ALK5; ‡ p<0.05 compared to TGFβ treated control cells). (b) The specific inhibitor of p38MAPK, SB203580 (SB) abolishes the TGFβ-induced downregulation of KLF15 in cardiomyocytes in a dose-dependent manner. (c) Inhibition of p38 MAPK prevents TGFβ-induced hypertrophy, measured by [3H]-leucine incorporation (n=3/group,*:p<0.05 compared to control group; †:p<0.05 compared to TGFβ treated cells without SB treatment) and (d) by phallolidin staining of F-actins. Bars in panels represent 50 μm. (e) Western blot analysis shows an increase in phosphorylated p38 MAPK after infection of cardiomyocytes with constitutively active adMKK6, the upstream kinase of p38. (f) Adenoviral overexpression of MKK6 resulted in decreased KLF15 mRNA levels and increased expression of the hypertrophy marker BNP (n=3/group, *:p<0.05 compared to control group).
It has been shown that KLF15 interacts with different major transcriptional regulators of cardiac hypertrophy, like the MADS box transcription factor MEF2A and the zinc finger transcription factor GATA4, but it remains unclear whether KLF15 displays specificity to certain cardiac transcription factors or whether it represses the general transcriptional machinery through other mechanisms. This prompted us to investigate whether KLF15 could repress SRF, “the other” MADS box transcription factor with an established role in cardiac gene expression. We first performed GST pulldown assays using in vitro translated MEF2A and SRF and a GST-KLF15 fusion protein but did not observe a direct interaction of KLF15 to either SRF or MEF2A (Supplemental Figure 5a). This suggested to us that the previously reported repression of MEF2 activity by KLF15 is indirect. We subsequently tested if KLF15 could physically interact with myocardin, an extraordinarily potent coactivator of the MADS box transcription factors, which has recently been reported to play a role in cardiomyocyte hypertrophy and cardiac failure. Indeed, GST-pulldown assays showed that KLF15 directly binds to myocardin (Figure 3d). We next tested whether KLF15 could affect the transcriptional activity of myocardin. Indeed, luciferase assays showed that KLF15 virtually abolished myocardin activity on SRF dependent promoters such as ANF(638), SM22(505) and an artificial promoter that contains three SRF binding domains (3xCarG) in COS7 cells. Repression of these promoters by KLF15 only took place in the presence (Figure 3a-c) and not in the absence of myocardin (supplemental figure 5b), indicating that KLF15 does not affect the basic transcriptional machinery in our assays, but specifically functions through its interaction with myocardin. To map the region of myocardin that is required for the interaction with KLF15, we compared binding of several deletion mutants of myocardin to KLF15 using GST pulldown assays. As shown in Figure 3d and e, KLF15 binds to a 50 amino acid region within amino acids 232-282 of myocardin. Interestingly, this region contains the basic domain of myocardin, which is also required for binding to SRF. Binding of SRF and KLF15 to the same region within myocardin implicates that KLF15 can trigger the displacement of myocardin from SRF by competition for a common docking site. Indeed, competition experiments in COS7 cells using the ANF(638) reporter shows that repression of myocardin activity by KLF15 is relieved in a dose-dependent manner by addition of SRF (Figure 3f). In conclusion, competition of SRF and KLF15 for myocardin provides a mechanism whereby lower KLF15 levels in response to TGFβ signaling can enhance the expression of SRF dependent genes during the hypertrophic response of the heart (Figure 5).

TGFβ regulates the expression of myocardin targets in cardiomyocytes. The observations that TGFβ represses KLF15 expression and that KLF15 inhibits myocardin activity predicts that TGFβ increases the expression of myocardin target genes. To
evaluate this we stimulated cultured cardiomyocytes under serum free conditions for 24 hours with 5 ng/mL TGFβ and measured the expression of ANF, SM22 and α-SKA (i.e. actc1), three bona fide myocardin/SRF target genes (4, 6, 27). As shown in Figure 3g, these SRF targets were significantly upregulated in response to TGFβ stimulation, while myocardin levels were unchanged (not shown). Regulation of myocardin target genes by KLF15 inhibited myocardin activity. (a-c) Myocardin activates SRF dependent reporters: ANF, SM22 and the 3xCArG-luciferase. This activity is blocked by KLF15. COS cells were transfected with the luciferase reporters and expression vectors encoding myocardin-935 and KLF15. Luciferase activity is expressed as fold change over the empty expression vector, pcDNA3.1 (n=3, mean ± SD). (d) GST pull-down assays show that KLF15 associates with myocardin. 35S-labeled myocardin mutant proteins were translated in vitro and incubated with GST-KLF15 fusion protein. Proteins were captured on glutathione agarose beads and analyzed by SDS-PAGE. The input lanes contain 10% of the amount of 35S-labeled myocardin protein in the pull-down lanes. GST-KLF15 fusion protein is shown on a Coomassie gel on the right. The shaded box represents the region that is necessary for KLF15 binding. NTD: N-terminal domain, Q: glutamine rich domain, TAD: transactivation domain. (f) KLF15 competes with SRF for myocardin interaction. COS cells were transfected with ANF luciferase and expression vectors encoding myocardin-935, and increasing concentrations of KLF15 and SRF as indicated. Luciferase activity expressed as fold change over the empty
TGFβ was fully prevented by titrating in p38 inhibitors (Figure 4). These results provide further evidence that TGFβ regulates myocardin activity in cardiomyocytes via activation of p38 MAPK as depicted in the model depicted in figure 5.

In contrast to transcriptional activators, transcriptional inhibitors of LVH are less well explored. Recently KLF15 was described as a novel transcriptional inhibitor of LVH 15. The current study aimed to elucidate how KLF15 is regulated and how it inhibits pathological LVH. There is increasing recognition of the intrinsic differences between load induced, pathological LVH and more physiological forms of cardiac hypertrophy as occurs after exercise 28. However, many factors involved in pathological LVH are also involved in adaptive LVH 29. More recently, some transcriptional mechanisms have been identified that specifically inhibit pathological LVH, like class II HDACs and the transcriptional repressor NAB1 12, 14. While NAB1 is activated during LVH, class II HDACs have been suggested to constitutively repress the expression of hypertrophy genes like MEF2 12.

**DIscussion**

In contrast to transcriptional activators, transcriptional inhibitors of LVH are less well explored. Recently KLF15 was described as a novel transcriptional inhibitor of LVH 15. The current study aimed to elucidate how KLF15 is regulated and how it inhibits pathological LVH. There is increasing recognition of the intrinsic differences between load induced, pathological LVH and more physiological forms of cardiac hypertrophy as occurs after exercise 28. However, many factors involved in pathological LVH are also involved in adaptive LVH 29. More recently, some transcriptional mechanisms have been identified that specifically inhibit pathological LVH, like class II HDACs and the transcriptional repressor NAB1 12, 14. While NAB1 is activated during LVH, class II HDACs have been suggested to constitutively repress the expression of hypertrophy genes like MEF2 12.
Here we show that KLF15 is a second constitutive transcriptional inhibitor of pathological LVH. The specific role of KLF15 in pathological forms is exemplified by the finding that a) KLF15 was not suppressed in exercise induced LVH and b) KLF15 was significantly more suppressed in the hypertrophied Ren-2 hearts that would soon progress to failure. These findings suggest a novel mechanism in pathological LVH, where activated p38 MAPK actually down-regulates a repressive transcription factor. Earlier work showed the relation between loss of KLF15 and LVH in general. KLF15 was found to be expressed less in hypertrophied hearts as compared to the healthy adult myocardium. In addition, KLF15 null mice have higher baseline levels of BNP and ANF and respond to pressure overload with exaggerated expression of these genes, accompanied by higher mortality due to heart failure. This underlines the protective role of constitutive expression of KLF15. Since activation of p38 MAPK has been shown to activate numerous mechanisms, but has not yet been shown to repress important transcriptional regulators we were surprised to find that activation of p38 MAPK was sufficient to decrease KLF15. Although there is some debate about the role of p38 in the development of LVH, published findings are concordant with those we report here. Studies in cultured cardiomyocytes conclude that p38 activation is sufficient to induce myocyte hypertrophy marked by increased cell size, and induction of hypertrophy marker genes like ANF, BNP and αSKA. In vivo studies using transgenic overexpression mice of the p38 MAPK upstream kinases, MKK3 and MKK6, do not show myocyte hypertrophy but instead they rapidly progress towards heart failure and have increased levels of embryonic genes like ANF, β-MHC and αSKA which is congruent with the loss of repression of myocardin we suggest. Finally, it has been shown that activation of p38 MAPK via TAK1 axis induces hypertrophy, which is in line with our findings. Furthermore, very recently it has been shown that KLF15 is not the only KLF that is regulated in cardiomyocytes. In cultured cardiomyocytes ET-1 upregulated several KLFs like KLF2, KLF4, KLF5 and KLF6 and downregulated KLF3, KLF11 and KLF15. The possible involvement of p38 MAPK in the regulation KLF expression was studied but blockade of p38 by a p38 inhibitor did not have an effect on the ET-1 induced expression of KLF2, KLF4, KLF5 and KLF6.

We explored a possible mechanism by which KLF15 can repress pathological LVH. The role of myocardin in the development of hypertrophy and failure has recently been described. Myocardin levels are reported to be increased upon induction of hypertrophy in cultured cardiomyocytes and in patients with LVH. Overexpression of myocardin in cardiomyocytes induces hypertrophy and increases ANF, BNP and β-MHC. In vivo ablation of myocardin in adult cardiomyocytes in mice results in rapid heart failure and the expression of myocardin/SRF-regulated sarcomeric genes is extinguished. Our study demonstrates that KLF15 is a very potent inhibitor of myocardin activity.
Luciferase assays show that KLF15 suppresses myocardin induced activation of three reporter vectors harboring one or more CArG-boxes (Figure 3a). In addition, binding studies revealed that KLF15 is able to bind myocardin specifically in a 50aa region where the SRF binding domain (basic domain) is also situated. Competition studies show that KLF15 mediated repression of myocardin activity is abolished by SRF in a dose dependent manner. This indicates that KLF15 and SRF compete for binding the same region within myocardin. When we studied the effect of TGFβ on the expression of established myocardin targets SM22, ANF and αSKA, we found these targets to be upregulated. Moreover, this upregulation was completely abolished by p38 inhibitors. This is congruent with the notion that TGFβ represses KLF15 expression via p38 MAPK, resulting in enhanced physical association of myocardin to SRF. Unchanged myocardin mRNA levels in response to TGFβ further underscores the interpretation that myocardin activity is not regulated at the level of transcription, but rather by interactions at the protein level. Interestingly, a recent study has shown that myocardin mRNA and protein levels are increased in human and mouse failing hearts. This indicates that in failing hearts, not only myocardin activity is enhanced by a decrease in KLF15, but also by enhancing protein levels of myocardin itself.

Taken together our data suggest a novel pathway in pathological cardiac hypertrophy. We show here that down-regulation of KLF15 is a vital step in the development of hypertrophy and possibly its progression towards heart failure. We propose a model for the role of KLF15 in the regulation of myocardin activity. The proposed mechanism is shown in Figure 5. TGFβ mediates growth factor-induced activation of transcription factors, such as SRF, which can activate the expression of cardiac hypertrophic genes. KLF15 represses myocardin-mediated gene expression by competing with SRF for a common docking site within myocardin, thereby limiting myocardin to activate cardiac hypertrophic genes in normal adult myocytes. Growth factors repress KLF15 expression, allowing myocardin to bind SRF and activate cardiac hypertrophic genes.
propose that in the healthy post-natal heart, where KLF15 levels are high, KLF15 inhibits the activity of myocardin, a potent transcriptional activator of numerous genes involved in cardiomyocyte hypertrophy like ANF, SM22 and alpha-skeletal actin. During cardiac hypertrophy, when levels of TGFβ increase, p38 MAPK signaling is activated which subsequently reduces KLF15 and releases the endogenous inhibition that KLF15 exerts on myocardin (Figure 5).

In conclusion, our studies show that molecular mechanisms counteracting cardiomyocyte growth are dysregulated in pathological hypertrophy of the heart. The fact that KLF15 counteracts hypertrophy and is significantly down-regulated in pathological LVH suggests that therapeutic interventions aimed at preventing the decrease of KLF15 levels could be beneficial in the prevention of heart failure.

FOOTNOTES

This study was supported by grants from the Netherlands Heart Foundation to Y.M.P (2003T302) and to E.E.C (2007B077). Y.M.P is an established investigator of the Netherlands Heart Foundation.

All animal experiments using Ren2 rats and neonatal rats were approved by the Animal Care and Use Committee of the University Maastricht and the University of Amsterdam, and were performed according to the official rules formulated in the Dutch law on care and use of experimental animals.
REFERENCES


Supplemental Figure 1: (a) Heart weights of sedentary and trained rats. The heart weight of trained rats is significantly increased compared to the hearts of untrained animals. Sedentary hearts was 1.53±0.14 vs 1.22±0.11 gram (P=0.0159) (b and c). Cardiomyocyte physiological remodelling. Cells were measured for length and midpoint width on an inverted microscope. Cardiomyocyte length was increased in trained rats compared to the sedentary animals. 119.1±3.1µm vs 131.4±2.9µm, (p<0.01) Cardiomyocyte width remained unchanged. 25.9±1.4 µm in sedentary animals and 26.1±1.2 µm in trained animals. (d). Fractional shortening (FS) is increased in exercise induced hypertrophy. The FS of isolated cardiomyocytes was 13.1±3 % in sedentary animals and 19.7±6.5 in endurance trained cells (P=0.036).

Supplemental Figure 2. Lentiviral mediated overexpression of shRNA against rat ALK5 in cultured neonatal cardiomyocytes results in knockdown of ALK5. Cardiomyocytes were infected with a lentivirus containing shRNA against ALK5 or with a control lentivirus. ALK5 mRNA levels were significantly increased in the shALK5 transduced group compared to the control group. (‡: p<0.05 compared to TGFβ treated control cells, n=3 per group)
Supplemental Figure 3. Repression of KLF15 mRNA in cultured cardiomyocytes is mediated by p38-TGFβ signalling. TGFβ-induced downregulation of KLF15 is completely prevented by two independent p38 inhibitors (SB203580 and SB202190). The expression of KLF15 inversely correlates with ANF expression in these cells.

Supplemental Figure 4. Knockdown of KLF15 results in increased cell size. A) Knockdown of KLF15 in cultured neonatal mouse cardiomyocytes by using an siRNA smartpool results in a significant downregulation of KLF15, an 28% increase in cell surface area. (n=3/group **p=0.03) and an increase in ANF mRNA. The same result was observed in four other independent experiments. B) Cultured neonatal mouse cardiomyocytes transfected with a control siRNA smartpool or an siRNA smartpool targeting KLF15 were stained with phalloidin to demonstrate the increase in cell size. C) KLF15 knockdown in rat neonatal cardiomyocytes shows an increase in cell size of about 16%. D) Lentiviral overexpression of KLF15 in cultured neonatal rat cardiomyocytes represses ANF expression, as compared to cells that were transduced with a control virus. *: p<0.05, n=3 per group.
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Supplemental Figure 5. KLF15 does not directly interact with SRF and MEF2a and does not have a direct effect on reporter constructs. (a) KLF15 does not repress the ANF, SM22 and the 3xCARG reporter in the absence of myocardin. COS cells were transfected with the indicated luciferase reporters and KLF15. Luciferase activity is expressed as fold change over the empty expression vector, pcDNA3.1 (n=3, mean ± SD). (b) GST pull-down assays show that KLF15 physically associates with myocardin but not with SRF and MEF2A. 35S-labeled SRF, MEF2A and myocardin proteins were translated in vitro and incubated with GST-KLF15 fusion protein. Proteins were captured on glutathione agarose beads and analyzed by SDS-PAGE. The input lanes contain 10% of the amount of 35S-labeled SRF, MEF2A and myocardin proteins in the pull-down lanes. GST-KLF15 fusion protein is shown on a Coomassie gel on the right.

Supplemental tables. (a) Oligos used for creating plll3.7-shALK5 (b) Oligos used for quantitative Real-Time PCR