Regulation of Cardiac Gene Expression by KLF15, a Repressor of Myocardin Activity*\(^{\text{5}}\)

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 Kru¨ppel-like factor 15 (KLF15) is lost in pathological hypertrophy and that this loss precedes progression toward heart failure. We show that transforming growth factor-β (TGFβ) represses cardiac hypertrophy. Our results reveal that the essential transcriptional regulator KLF15 was first identified as a repressor of pathological cardiac hypertrophy (15). KLF15 belongs to the family of Kru¨ppel-like factors, which has 17 members (16, 17). KLF15 is widely expressed, acts as a transcriptional repressor of pathological cardiac hypertrophy (12, 13). In addition, NAB1 has been identified to repress hypertrophy by direct inhibition of Egr-dependent transcription (14). Recently, it was also shown that KLF15 acts a transcriptional repressor of pathological cardiac hypertrophy (15). KLF15 belongs to the family of Kru¨ppel-like factors, which has 17 members (16, 17). KLF15 is widely expressed, but the highest expression levels are found in liver, kidney, pancreas, and cardiac and skeletal muscle (18).

Myocardin is an extraordinarily potent transcriptional coactivator expressed exclusively in cardiomyocytes and smooth muscle cells (4). 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 (4, 5). Recently, Parmacek and co-workers (6) 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 mutations in myocardin-regulated genes such as \(\text{ACTN2}, \text{MYH7}, \text{ACTC}, \) and \(\text{TPM1} (6–10)\). 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 (11). One of the best studied repressors of cardiac hypertrophy is the family of histone deacetylases. For instance, HDAC9 null mice display spontaneous cardiac hypertrophy and are hypersensitive to pressure overload (12, 13). In addition, NAB1 has been identified to repress hypertrophy by direct inhibition of Egr-dependent transcription (14). Recently, it was also shown that KLF15 acts as a transcriptional repressor of pathological cardiac hypertrophy (15). KLF15 belongs to the family of Kru¨ppel-like factors, which has 17 members (16, 17). KLF15 is widely expressed, but the highest expression levels are found in liver, kidney, pancreas, and cardiac and skeletal muscle (18).

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–5 and Table 1.

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3. The abbreviations used are: LVH, left ventricular hypertrophy; TGFβ, transforming growth factor-β; SRF, serum response factor; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide.
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EXPERIMENTAL PROCEDURES

Animal Models—Ren-2 rats were obtained from Møllegård Breeding Center, Lille Skensved, Denmark, and were studied as described previously (20).

Neonatal Rat Cardiomyocyte Isolation and Transfection—1–2-Day-old Lewis neonatal rats were sacrificed by decapitation. Hearts were carefully removed, and left ventricles were cut into small pieces. Cardiac cells were then isolated by enzymatic dissociation using 0.05% collagenase I (Invitrogen, catalog no. 17100-017) and 0.05% pancreatin (Sigma, catalog no. p3292) dissolved in 1× Hanks’ balanced salt solution (Sigma, catalog no. H4641). Cells were pre-plated for 1 h in DMEM (Invitrogen, catalog no. 11966) (supplemented with 10% horse serum, 5% newborn calf serum, 0.16% glucose and antibiotics) to separate myocytes from fibroblasts. After 1 h, cardiomyocytes were collected, counted, and plated in plates coated with 1% gelatin (Fluka, via Sigma). Overnight, cells were grown in DMEM supplemented with 10% horse serum, 5% newborn calf serum, 0.16% glucose, and antibiotics. Dhamacon ON-TARGETplus siRNA SMARTpools for non-targeting control (D-001810-10) and KLF15 (L-080131-01) were transfected (Lipofectamine 2000, Invitrogen, catalog no. H4641) at a final concentration of 300 nM and incubated with 1 g/liter D-glucose (Merck). Tissue was digested in collagenase buffer (150 units/ml collagenase type II (Worthington 4176) in DMEM 41965 (Invitrogen) for 45 min 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 h 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, catalog no. G1890)-coated culture plates at a final density of 100,000 cells/cm². After 48 h, medium was changed to DMEM 41965 supplemented with 25 mM l-glutamine, 2% BSA (Sigma, catalog no. A6003), 0.25 milliunits/ml insulin (Sigma, catalog no. I6634), 250 μM l-carnitine (Sigma, catalog no. C0283), and 10 μM AraC (Sigma, catalog no. C6645)). 24 h later, medium was changed to DMEM 41965 without penicillin/streptomycin for transfection. All solutions were supplemented with 100 units/ml penicillin/streptomycin and 10 mM HEPES. Transfection was performed according to the manufacturer’s protocol. Dhamacon ON-TARGETplus siRNA SMARTpools for nontargeting control (D-001810-10) and KLF15 (L-059453-01) were added with Lipofectamine 2000 (Invitrogen, catalog no. 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 the manufacturer’s protocols. cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad), 20 ng of total RNA, and 10 pmol/μl forward and reverse primers (supplemental Table 1a). Quantification was performed using LinRegPCR analysis software (22).

Transfections and Luciferase Assays—Transfection of COS7 cells for luciferase assays were performed as described previously (23). SM22 (505 bp) luciferase reporter was a kind gift of Dr. J. M. Miano (Rochester, NY) (24). The 3xCARG and ANF (638 bp) luciferase reporter and the expression plasmids encoding myocardin (935 amino acids) and SRF were provided by Dr. Eric Olson (Dallas, Tx) (25). A pcDNA3.1-based expression vector encoding FLAG-tagged KLF15 was donated by Dr. M. K. Jain (Cleveland, OH). Transfection of COS7 cells and luciferase assays was performed as described previously (23). In short, 24-well plates with COS7 cells were transfected per well with 30 ng of pcDNA-β-gal, 75 ng of luciferase reporter, and 50 ng 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 50 ng of ANF- or Sm22-luciferase, 50 ng of pcDNA-myocardin, and increasing concentrations of KLF15 (1–100 ng) or pcDNA-SRF (0.5–25 ng) as indicated. Luciferase assays were performed using the luciferase assay system (Promega). Measurements were performed in duplicate and repeated at least three times.

GST Pulldown—Glutathione S-transferase (GST)-KLF15 fusion proteins were generated by subcloning the KLF15 open...
Expression of KLF15 was decreased significantly more in the hypertrophied hearts that later progressed toward failure (Fig. 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 with those from sedentary rats. Training resulted in hypertrophy of individual cardiomyocytes as shown by an increased length, but not width, of these cells (supplemental Fig. 1, a–d). Despite this pronounced cardiac remodeling, no difference in KLF15 gene expression between the sedentary group and the trained rats was noted (Fig. 1b).

KLF15 Expression Is Regulated by the TGFβ-P38 MAPK Pathway—KLF15 is specifically down-regulated in pathological hypertrophy, which 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 (ET-1), and 10% fetal calf serum (FCS) on KLF15 expression. All stimuli were detected using quantitative real time PCR. (*, p < 0.01 compared with control cells, n = 3 per group). d, KLF15 mRNA levels do not decrease on stimulation of cultured neonatal rat cardiomyocytes with TGFβ-1 (100 nm), TGFβ-2 (100 nm), or insulin (100 nm) for 24 h after 24 h of serum starvation (n = 3 per group).

Statistical Analyses—Data are shown as mean ± S.E. 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 (15), 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 compensated for a prolonged period. 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 (20). This revealed that KLF15 expression was decreased significantly more in the hypertrophied hearts from the rats that remained compensated after the biopsy (n = 4–6 rats per group; *, p < 0.05 compared with control group; †, p < 0.02 compared with the compensated (Comp) hypertrophy group), b, in hypertrophic myocardium from rats that had undergone exercise training for 10 weeks. 5 days per week (n = 5), KLF15 expression was not altered compared with control rat hearts (n = 7). c, KLF15 mRNA expression is decreased in cultured neonatal rat cardiomyocytes in response to several hypertrophic stimuli. Cardiomyocytes were serum-starved overnight and then stimulated with endothelin 1 (ET-1) (100 nm), phenylephrine (PE) (50 μM), TGFβ (10 ng/ml), and 10% fetal calf serum (FCS) for 24 h. Ctrl, control. KLF15 levels were detected using quantitative real time PCR. (*, p < 0.01 compared with control cells, n = 3 per group). d, KLF15 mRNA levels do not decrease on stimulation of cultured neonatal rat cardiomyocytes with TGFβ-1 (100 nm), TGFβ-2 (100 nm), or insulin (100 nm) for 24 h after 24 h of serum starvation (n = 3 per group).

Results of KLF15 expression in pathological hypertrophy are shown in Fig. 1, a–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 (Fig. 2e). This resulted in an almost 80% decrease in KLF15 mRNA levels and induced expression of the hypertrophy marker BNP in cultured cardiomyocytes (Fig. 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 Coactivator of SRF—Earlier studies have shown that loss of KLF15 aggravates cardiac hypertrophy and dysfunction (15). We confirmed these observations, after transfecting cultured neonatal rat and mouse cardiomyocytes with two independent SMARTpool siRNAs against KLF15 or a nontargeting control...
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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 Fig. 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 Fig. 4). It remains unknown how KLF15 inhibits LVH. 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 we did not observe a direct interaction of KLF15 to either SRF or MEF2A (supplemental Fig. 5a). This suggested to us that the previously reported repression of MEF2 activity by KLF15 is indirect (15, 19). 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 (6, 25, 27). Indeed, GST-pulldown assays showed that KLF15 directly binds to myocardin (Fig. 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 (3×CArG) in COS7 cells. Repression of these promoters by KLF15 only took place in the presence (Fig. 3, a–c) and not in the absence of myocardin (supplemental Fig. 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 Fig. 3, d 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 (4). 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 show that repression of myocardin activity by KLF15 is relieved in a dose-dependent manner by addition of SRF (Fig. 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 (Fig. 5).

TGFβ Regulates the Expression of Myocardin Targets in Cardiomyocytes—The observations that TGFβ represses KLF15 expression and that KLF15 inhibits myocardin activity predict that TGFβ increases the expression of myocardin target genes. To evaluate this, we stimulated cultured cardiomyocytes under serum-free conditions for 24 h with 5 ng/ml TGFβ and measured the expression of ANF, SM22, and αSKA (i.e. ACTC1), three bona fide myocardin/SRF target genes (4, 6, 27). As shown in Fig. 4, b–d, these SRF targets were significantly up-regulated in response to TGFβ stimulation, whereas myocardin levels were unchanged (data not shown). Regulation of myocardin target genes by TGFβ was fully prevented by titrating in p38 inhibitors (Fig. 4). These results provide further evidence that TGFβ regulates myocardin activity in cardiomyocytes via activation of p38 MAPK as depicted in the model in Fig. 5.

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). This 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 histone deacetylases and the transcriptional repressor NAB1 (12, 14). Although NAB1 is activated during LVH, class II histone deacetylases 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 following findings: (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 with the healthy adult myocardin. 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 (15). This underlines the protective role of constitutive expression of KLF15. Because 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 (30). Although there is some debate about the role of p38 in the development of LVH (30, 31), published findings are in agreement 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 markers like ANF, BNP, and αSKA (32). In vivo studies using transgenic overexpression mice of the p38 MAPK upstream kinases, MKK3 and MKK6, do not show myo-
cyte hypertrophy, but instead they rapidly progress toward heart failure and have increased levels of embryonic genes like ANF, β-MHC, and αSKA (33), which is in line with the loss of repression of myocardin as we suggest here. Finally, it has been shown that activation of p38 MAPK via the TAK1 axis induces hypertrophy, which is in line with our findings (26). Furthermore, very recently it has been shown that KLF15 is not the only Krüppel-like factor that is regulated in cardiomyocytes. In cul-

![Figure 3](image-url)
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MAPK in the regulation of 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 (6, 27). Myocardin levels are reported to be increased upon induction of hypertrophy in cultured cardiomyocytes and in patients with LVH (27). Overexpression of myocardin in cardiomyocytes induces hypertrophy and increases ANF, BNP, and β-MHC expression (27). 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 (6). 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 GArG-boxes (Fig. 2).

In addition, binding studies revealed 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 up-regulated. Moreover, this up-regulation 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 underscore 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 (27). 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 toward heart failure. We 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 aska. 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 (Fig. 4).

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.

Acknowledgment—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