Three fingers on the brake: Kruppel-like factor 15, a repressor of cardiac gene expression
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AAV9-mediated Gene Transfer of KLF15 Inhibits Hypertrophic Growth of the Heart

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Chapter 7

SUMMARY

As for many diseases also patients that suffer from cardiac failure would greatly benefit when more personalized treatment would be available. To treat a complex disease like heart failure the molecular etiology of the disease should be studied in depth. This could provide a basis for therapeutical interventions. Diseases like a myocardial infarction, pressure overload induced left ventricular hypertrophy or genetic forms of hypertrophic cardiomyopathy are well known causes of heart failure. Current therapeutic strategies often aim at relieving the stress on the weakened failing heart. Medication like ACE inhibitors and beta-blockers) do prolong the survival of heart failure patients, but the mortality rate remains high. Novel and efficient treatment strategies rely on fundamental knowledge of the molecular pathways that are activated during cardiac remodeling and heart failure. We and others identified the transcriptional regulator KLF15 as a repressor of cardiac hypertrophy and we hypothesized that overexpression of KLF15 could prevent the development of hypertrophy and subsequent heart failure. We made use of recombinant adeno-associated virus serotype 9 (AAV9) to overexpress KLF15. The cardiac troponin T promoter was used to guarantee cardiomyocyte-specific expression. Overexpression of KLF15 resulted in a reduced angiotensin II-induced hypertrophic response as measured by heart weight, cell size and hypertrophic gene expression. Even though the effect was mild, our findings indicate that KLF15 could be potentially used as a therapeutic target, but further research is warranted.
Despite ongoing research, heart failure remains a major and growing health problem. Heart failure is strongly correlated with age and because of improved medical treatment for a large range of diseases the life expectancy of people has increased significantly. Therefore the overall incidence of heart failure has strongly increased, especially among the elderly. Heart failure is characterized by the inability of the heart to meet the body's demand for nutrients and oxygen. There are several risk factors for developing heart failure of which left ventricular hypertrophy (LVH) is one of the most dominant. A myocardial infarction (MI), an occlusion of the aorta (aortic valve stenosis) or an increase in blood pressure are major risk factors for the development of LVH. LVH occurs because the heart has to adapt to an altered environment, which usually requires an increase in work force to maintain cardiac output, which subsequently results in an increased cardiac mass. In the initial phase this form of hypertrophy is called compensated hypertrophy. Without medical treatment to relieve the cause, the hypertrophic response will enter a negative spiral until the hypertrophic hearts reaches a point of no return (maladaptive hypertrophy) and the heart is prone to fail. Current therapies primarily focus on relieving the failing heart from stress. Despite the attempts of controlling heart failure using ACE inhibitors and beta-blockers, life expectancy has increased, but survival is still poor.

One approach to prevent LVH and the subsequent development of heart failure is to interfere in the intracellular signaling cascades that are activated in the hypertrophic heart and that promote hypertrophic growth. As a consequence of this interference, pro-hypertrophic signals from outside the cell cannot reach and activate the transcriptional machinery that is necessary for the expression of genes involved in hypertrophic growth. To manipulate prohypertrophic gene expression one can inhibit the activity of specific cardiac transcription factors (e.g. MEF2, GATA, NFAT) or enhance the activity of transcriptional inhibitors, such as histone deacetylases and kruppel-like factors (KLFs). The advantage of interference at the transcription level is that a more robust and specific effect can be expected, since many signaling cascades converge on a limited set of transcription factors.

We and others recently identified the transcriptional regulator kruppel-like factor 15 (KLF15) as an important inhibitor of cardiac gene expression and hypertrophy. KLF15 acts as a transcriptional repressor of the transcriptional regulators GATA4, MEF2A and myocardin. Interestingly, the expression of KLF15 decreases during hypertrophic growth of the heart and thereby its repressive action is lost, which results in increased cardiac gene expression and hypertrophy. In vivo mouse studies show that somatic
loss of KLF15 results in an increased susceptibility to pressure overload induced LVH and heart failure. Together these reports show that KLF15 acts as a repressor of LVH by inhibiting the activity of several cardiac transcription factors, which indicates KLF15 as a putative candidate for anti-hypertrophic therapy.

We set out this study to investigate whether forced overexpression of KLF15 in the mouse heart, using recombinant adeno-associated virusses (rAAV), can prevent the development of LVH. In recent years there has been a lot of focus on cardiac gene transfer using the adeno-associated virus serotype 9 (AAV9). This virus serotype has a natural preference for the heart and does not evoke an immune response, making it a potential specific and safe method to introduce foreign DNA in the human heart. The first, in vivo studies using rAAV1 and rAAV9 in mice and humans have been shown to be successful and safe.

In the current study, we found that AAV9-mediated overexpression of KLF15 in the murine heart represses the AngII-mediated induction of hypertrophy. However, further research is warranted to establish the therapeutic potential of overexpression of KLF15.

MATERIALS AND METHODS

AAV9 construction and production
pAAV9-cTnT-GFP and pAAV9-cTnT-FLAG-KLF15 were derived by subcloning the open reading frame of GFP and mouse KLF15 into pAAV-MCS (Stratagene). Subsequently, the CMV promoter was removed by restriction and replaced by the -374+38 chicken troponin T promoter. Virus was produced in 293 cells and purified as previously described.

AAV9 injections and induction of hypertrophy
10^10 vector genomic copies (vg) of AAV9-KLF15 and AAV9-GFP were injected into the tail vein of eight week old male C57BL/6 mice. After one week, mini-pumps (ALZET model 2004; ALZACorp., Palo Alto, California, USA) filled with either AngII (H-1705, Bachem) or saline and a pumping rate of 1.5 ug/g/day were placed subcutaneously as described earlier. After 28 days, mice were sacrificed under 2.5% isofluorane. KLF15-FLAG protein levels were measured by Western Blotting using an anti-FLAG M2 antibody and anti-GAPDH antibody. AAV9 experiments are performed at the University of Leuven, Belgium, according to the guidelines of the institutional animal care facility committee.

Histochemical Studies
Mouse hearts were fixed overnight in 4% paraformaldehyde, dehydrated, and
embedded in paraffin using standard techniques. Consecutive 7-µm serial sections were cut and stained with hematoxylin and azophloxine. To determine the collagen content, deparaffinized sections were processed for Picrosirius red stain.

**Cell size measurements**

Cell size was determined in hematoxylin and azophloxine-stained sections using 20x magnification. To determine cell size, we measured the cross-sectional area of ~150 individual transversely cut cardiomyocytes using the image processing software Scion Image.

**Sirius Red Quantification.**

Sirius red staining was quantified by using an in-house made quantification macro. Per heart/section, 160 fields were recorded and of those 160 fields, 20 fields were randomly chosen for quantification. Perivascular fibrosis was manually omitted from the sections. The percentage of Sirius red positive areas in each section was automatically calculated as a percentage of the total tissue area.

**RESULTS**

In vitro studies using cultured rat neonatal cardiomyocytes recently showed that adenoviral-mediated overexpression of KLF15 prevents phenylepherine (PE)-induced cardiomyocyte hypertrophy. This prompted us to further investigate whether in vivo overexpression of KLF15 can also prevent AngII-induced LVH.

To increase cardiac levels of KLF15 we designed an rAAV9 for overexpression of KLF15 under the control of the -374 chicken cardiac troponin T promoter. This promoter has been shown to efficiently drive the expression of SERCA in cultured rat cardiomyocytes. To generate a rAAV9-KLF15 construct that is only active in the heart we subcloned the chicken cTNT promoter upstream of FLAG-tagged KLF15 open reading frame. As a control, we generated a cTnT-eGFP construct. Both sequences were subcloned into a rAAV-9 vector and virus was produced as previously described. To establish AAV-9 mediated gene transfer, eight week old male C57Bl6 mice were intravenously (i.v.)

![Western blot analysis of left ventricular samples revealed expression of FLAG-KLF15 protein.](image)

**Figure 1.** In vivo overexpression of FLAG-KLF15 using AAV9. Western blot analysis of left ventricular samples revealed expression of FLAG-KLF15 protein.
injected with 1*10^10 vector genomic copies of rAAV9-KLF15 and rAAV9-GFP. Five weeks after injection we assessed whether we could detect FLAG-tagged KLF15 in the hearts of animals that were transduced with AAV9-KLF15. Western blot analysis on cardiac lysates with an antibody directed against the FLAG epitope revealed that recombinant KLF15 protein was efficiently produced in the hearts of AAV9-KLF15 injected mice (Figure 1).

To determine whether overexpression of KLF15 also represses pressure overload-induced hypertrophy in vivo, we induced hypertrophy a week after injecting mice with the rAAV9-KLF15 and rAAV9–GFP virus. Hypertrophy was induced via continuous infusion of AngII (1.5 μg/g/day) via subcutaneously placed osmotic minipumps (Figure 2), which has previously been shown to result in hypertrophic growth of the heart. As can be appreciated from Figure 3a, rAAV9-GFP infected mice develop a mild but statistically significant increase of 25% in left ventricular weight (LVw) when corrected for body weight (Bw) after 4 weeks of AngII infusion. The body weights between groups are not significantly different (Figure 3b). AngII treatment of the rAAV9-KLF15 mice resulted in a blunted increase in LVw/Bw ratio as compared to the mice infected with AAV9-GFP. This indicates that forced expression of KLF15 can inhibit the hypertrophic response of the heart.

Next, we assessed whether KLF15 overexpression affects hypertrophic growth of individual cardiomyocytes. We measured cross-sectional areas of ~150 individual cardiomyocytes in LV tissue on H&E stained sections. As can be appreciated from Figure 3c-d, the cardiomyocyte cross sectional area is significantly increased (11%) in response to AngII, in controls (rAAV9-GFP). However, in the rAAV9-KLF15 mice, the hypertrophic growth is in response to AngII. These results are in line with the blunted increase in LV mass that we observed in response to AngII in the rAAV9-KLF15 mice, and provides further evidence that KLF15 represses cardiac hypertrophy.

Another hallmark of cardiac remodeling and hypertrophy is the increased expression of a specific subset of genes of which atrial natriuretic factor (ANF) and alpha skeletal actin (αSKA) are typical examples. To study the expression of these genes in
Figure 3. Development of hypertrophy in control and KLF15 overexpressing mice. 

a) Body weight is evenly distributed in both sham and AngII treated animals. 
b) AngII induces hypertrophy in GFP expressing mice, as measured by correcting the left ventricular weight (LVw) for body weight (Bw). This effect is blunted in mice with cardiac specific overexpression of KLF15. 
c) and d) The hypertrophic response was also measured by analysis of individual cardiomyocyte size. Mice overexpressing KLF15 showed a blunted hypertrophic response to angiotensin. 
e) quantitative real time PCR on left ventricular tissue showed an increased expression of the hypertrophic markers ANF and alpha skeletal actin (aSKA) in GFP mice treated with angiotensin. In mice that overexpress KLF15, there is a reduction in expression of these genes, but this is not significant. * p<0.05 compared to GFP control. # p<0.05 compared to GFP ANGII. Group size: n=4 (GFP) or n=5 (KLF15).
our AAV9 transduced mice we performed quantitative real-time PCR on left ventricular tissue and this revealed that in AngII treated AAV9-GFP animals, the expression of ANF and αSKA is increased as expected. In rAAV9-KLF15 mice, this increase is attenuated, however this difference did not reach statistical significance (Figure 3e).

The presence of fibrosis is another characteristic of LVH, and KLF15 has been implicated in this process\(^6\). We performed sirius red stainings on cardiac sections to determine the amount of interstitial fibrosis in rAAV9-GFP and rAAV9-KLF15 mice. As can be appreciated from Figure 4a and b AngII infusion results in an increased collagen content in AngII treated hearts. In hearts of AAV9-KLF15 mice treated with AngII, the increase in fibrosis is attenuated, but this difference did not reach statistical significance.

**DISCUSSION**

Despite years of ongoing research, the therapeutic strategies to treat or prevent heart failure are inadequate, even though our knowledge on the molecular basis of hypertrophy has grown tremendously. In this study we investigated the putative beneficial effects of overexpression of the transcriptional repressor, KLF15 on the development of cardiac hypertrophy and fibrosis. Previous studies already indicated that KLF15 acts as a repressor of hypertrophy, but these studies primarily focused on the effects of loss of KLF15. Even though in vitro studies show a beneficial effect of over expression of KLF15 on cell growth, in vivo studies are lacking\(^6\). In this study we were able to partially prevent the AngII-induced hypertrophic response of the heart through overexpressing of KLF15 in the cardiac myocyte.

Efficient and long-term delivery of potential therapeutic genes to the heart remains a major challenge for clinical implementation. One of the most promising tools in this context are the AAVs, which are able to take up large DNA fragments and enable stable gene transfer to the heart\(^14\). Furthermore, rAAVs are non-pathogenic, as they are replication-deficient and do not evoke an immune response. AAV vectors have been studied for the past 30 years and these parvovirus-related viruses currently encompasses 9 serotypes\(^17, 18\). With the discovery of different AAV serotypes and variants it became evident that serotypes can be used to establish an organ specific expression of the transgene\(^9\). Two recent studies showed that specifically the AAV9 serotype can very efficiently transduce the heart after intravenous delivery\(^19, 20\). This in contrast to rAAV-1, rAAV-2 and rAAV-6 that only slightly transduce the heart when high doses of viral genome are injected. rAAV-8 does efficiently transduce cardiac tissue, but relatively high virus doses are needed\(^16\).
Swinnen et al. showed that rAAV9 mediated gene transfer is not only very efficient, but it also has therapeutic potential for heart disease. When they overexpressed thrombospondin-2 (TSP-2) using AAV9-CMV-TSP vectors in the hearts of TSP-2 null mice they were able to rescue accelerated age-induced cardiomyopathy completely. In 2009, Jaski et al. performed a human phase 1/2 clinical trial using rAAV1 to overexpress SERCA2a in 9 patients with advanced heart failure. This study did not raise any safety concerns. Even though the study cohort was too small to conduct statistical analysis, some patients showed improvements in LV function (ejection fraction and end systolic volume), biomarkers (NT-proBNP) and function (walking test and VO2 max). The above described findings emphasize that AAV mediated gene therapy is a potentially efficient and successful way to overexpress therapeutic targets, however further clinical evaluation is warranted.

**Figure 4.** Fibrosis is slightly impaired by KLF15 overexpression. a) Upon induction of hypertrophy, fibrosis is increased in GFP mice. This effect is impaired in mice overexpressing KLF15, but the difference is not statistically significant. b) Sirius red sections. *p<0.05 compared to GFP control. Group size: n=4 (GFP) or n=5 (KLF15).
Specificity of gene transfer to cardiomyocytes can be increased by incorporating a cardiac promoter, instead of the CMV promoter into the AAV9 vector. In the current study, but also in the study of Prasad et al., the chicken cardiac troponin T (cTnT) -374+43 promoter was introduced in the rAAV9 vector. This promoter has been shown to efficiently drive transgene expression in cardiomyocytes. In this regard, Prasad et al. provided evidence that the cTnT-rAAV9-eGFP vector efficiently transduces the heart (up to 96% of cardiac cells) resulting in high eGFP expression levels. In the current study, we use western blot analysis to demonstrate that cTnT-rAAV9-KLF15 transduction results in the generation of KLF15 protein in the heart.

Another elegant approach to establish cardiac specific expression, or more specifically to reduce expression in the liver, was recently introduced by Qiao et al. They showed that miRNAs can be used to render an rAAV9 vector heart specific without making use of a cardiac specific promoter. miRNAs are small RNA molecules that can bind unique binding sites in the 3' untranslated region (3'UTR) of a gene, thereby inhibiting its translation. To reduce transgene expression in the liver, Qiao et al. took advantage of the liver-specific expression of miRNA-122 and introduced several artificial miR-122 bindingsites in the 3'UTR of the transgene. This results in translational repression of the transgene in the liver, but not the heart.

As mentioned above, we made use of the chicken cTnT promoter to ensure cardiac specific expression. However, this promoter is only active in cardiomyocytes and not in other cell types of the heart such as fibroblasts and endothelial cells. Previous work of Wang et al. showed that KLF15 also has a function in the cardiac fibroblast, where it represses the expression of the pro-fibrotic cytokine CTGF and inhibits ECM synthesis. Mice lacking KLF15 are not only sensitized to the development of hypertrophy and heart failure, but they also develop severe fibrosis. Despite the knockout study showing an involvement of KLF15 in the control of cardiac fibrosis, it was surprising to observe diminished fibrosis in the hearts of cTNT-AAV9-KLF15 treated mice, since we did not target the fibroblast. We postulate two possible explanations for the attenuated fibrotic response: 1) it may have been a secondary effect to the blunted hypertrophic response, as it was reported in both in vitro and in vivo experiments that mechanical strain or load on cardiac fibroblasts has an impact on ECM synthesis. 2) It is known that KLF15 inhibits CTGF expression in fibroblasts, but it is very well possible that this mechanism is shared by the myocytes of the heart. In this regard, cardiomyocytes also express and secrete CTGF, particularly in the stressed myocardium. cTnT driven KLF15 expression may therefore inhibit CTGF secretion from myocytes thereby affecting ECM synthesis in fibroblasts. Nevertheless, the formation of excessive extracellular matrix is a negative hallmark of cardiac remodeling since it stiffens the heart thereby hindering...
proper contraction. The fact that cardiomyocyte-specific KLF15 overexpression reduces fibrosis, directly or indirectly seems beneficial and suggests that KLF15 may be used as a therapeutic target for hypertrophy and heart failure. Future studies are warranted to firmly establish whether overexpression of KLF15 improves cardiac function and whether it can also prevent hypertrophy in more severe models of hypertrophy and heart failure, such as transverse aortic constriction.
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