RNA binding proteins represent an emerging class of proteins with a role in cardiac dysfunction. We show that activation of the RNA binding protein Human antigen R (HuR) is increased in the failing human heart. To determine the functional role of HuR in pathological cardiac hypertrophy, we created an inducible cardiomyocyte-specific HuR deletion mouse, and showed that HuR deletion reduces left ventricular hypertrophy, dilation, and fibrosis while preserving cardiac function in a transverse aortic constriction (TAC) model of pressure-overload-induced hypertrophy. Assessment of HuR-dependent changes in global gene expression suggests that the mechanistic basis for this protection occurs through a reduction in fibrotic signaling, specifically through a reduction in transforming growth factor beta (Tgfb) expression. Finally, pharmacological inhibition of HuR at a clinically relevant time point following the initial development of pathological hypertrophy post-TAC also yielded a significant reduction in pathological progression, as marked by a reduction in hypertrophy, dilation, and fibrosis, and preserved function. In summary, this study demonstrates a functional role for HuR in the progression of pressure overload-induced cardiac hypertrophy and establishes HuR inhibition as a viable therapeutic approach for pathological cardiac hypertrophy and heart failure.
Human antigen R as a therapeutic target in pathological cardiac hypertrophy

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Abstract

RNA binding proteins represent an emerging class of proteins with a role in cardiac dysfunction. We show that activation of the RNA binding protein Human antigen R (HuR) is increased in the failing human heart. To determine the functional role of HuR in pathological cardiac hypertrophy, we created an inducible cardiomyocyte-specific HuR deletion mouse, and showed that HuR deletion reduces left ventricular-specific hypertrophy, dilation, and fibrosis while preserving cardiac function in a transverse aortic constriction (TAC) model of pressure-overload-induced hypertrophy. Assessment of HuR-dependent changes in global gene expression suggests that the mechanistic basis for this protection occurs through a reduction in fibrotic signaling, specifically through a reduction in transforming growth factor beta (Tgfβ) expression. Finally, pharmacological inhibition of HuR at a clinically relevant time point following the initial development of pathological hypertrophy post-TAC also yielded a significant reduction in pathological progression, as marked by a reduction in hypertrophy, dilation, and fibrosis, and preserved function. In summary, this study demonstrates a functional role for HuR in the progression of pressure overload-induced cardiac hypertrophy and establishes HuR inhibition as a viable therapeutic approach for pathological cardiac hypertrophy and heart failure.

Introduction

Heart failure is an increasing health burden that results from many common underlying factors including hypertension, coronary artery disease, and myocardial infarction, and is typically preceded by the enlargement, or hypertrophy, of the cardiac muscle, an effect that is initially beneficial and helps the heart maintain cardiac output in the face of hemodynamic stress.(1) However, the development of left ventricular hypertrophy (LVH) in response to such a pathological stimulus is not sustainable, and is associated with increased cardiac fibrosis, risk of arrhythmias, and development of heart failure (HF). The current standard-of-care treatments for HF mostly aim to relieve the underlying pressure overload and/or reduce the workload on the heart through inhibition of overstimulated neurohumoral pathways (ACE inhibitors, angiotensin receptor blockers, diuretics, and beta blockers).(2, 3) However, no current therapies directly target the molecular signaling pathways in cardiac myocytes and fibroblasts that mediate LVH and fibrosis to prevent or reverse this pathological cardiac remodeling.

Human antigen R (HuR; encoded by the Elav1 gene) is an RNA binding protein that binds to AU rich regions in the 3'-untranslated region of many different mRNAs, including many involved in inflammation, cell growth, and fibrosis, and it directly regulates the expression of target mRNA through modulation of its stability and/or translation.(4, 5) While relatively little is known about the role of HuR in the myocardium, RNA binding proteins such as HuR are becoming recognized as potentially central regulators of cardiac physiology and pathology.(6, 7) We have recently shown that HuR
is both necessary and sufficient for hypertrophic growth in cultured primary rat myocytes in response to hypertrophic stimuli in vitro.\(^{(8)}\)

In this work, we show that HuR activation is increased in failing human hearts. We then use a mouse model of transverse aortic constriction (TAC) to induce LV pressure overload, a well-established model of aortic stenosis, to demonstrate that cardiac myocyte specific deletion of HuR protects against pathological remodeling and functional decline in this model. Importantly, we also utilize a novel small molecular inhibitor of HuR to show that pharmacological inhibition of HuR at a clinically relevant time point following the onset of initial pathology improves survival and significantly slows the decline of cardiac function and progression of LV remodeling. Furthermore, HuR activity in the hypertrophic heart co-localizes with regions of fibrosis, and the development of fibrosis is blunted following either HuR deletion or pharmacological inhibition. Lastly, RNA-seq analysis also suggests modulation of fibrotic signaling as a key mechanism to HuR-mediated cardiac pathology.

This work demonstrates a functional role for HuR in the development and progression of pathological LVH and heart failure. Importantly, we not only establish the benefit of HuR targeting using either inducible, tissue-specific HuR deletion or pharmacological inhibition, but we also begin to decipher the underlying mechanisms of this effect. As there are currently no pharmacological inhibitors of HuR that have been demonstrated for in vivo applications, this work is also critical in demonstrating that HuR represents a viable therapeutic target for the treatment of pathological LVH and heart failure.

**Results**

*HuR activation is increased in human heart failure*

HuR resides predominately in the nucleus in an inactive form and translocates to the cytoplasm upon activation where it exerts its post-transcriptional regulation via target mRNA binding.\(^{(4, 9)}\) We have previously shown that HuR cytoplasmic translocation is increased in primary neonatal rat ventricular myocytes (NRVMs) following a hypertrophic stimulus.\(^{(8)}\) To determine HuR activity in failing human myocardium, we performed HuR immunofluorescence staining on healthy donor hearts as well as explanted tissue from left ventricular assist device (LVAD) implantation. Representative images show an increase in HuR cytoplasmic translocation in failing human myocardium (LVAD) vs. healthy donor tissue (Fig. 1). In addition, HuR staining also shows a similar pattern of increased HuR activation in a mouse model of transverse aortic constriction (TAC)-induced pathological left ventricular hypertrophy (LVH) (Fig. S1).

*Cardiac Myocyte-specific deletion of HuR reduces development of pathological LVH*

To achieve inducible cardiomyocyte-specific HuR deletion (iCM-HuR\(^{-/}\)), we crossed HuR-floxed mice (HuR\(^{fl/fl}\))\(^{(10)}\) with mice expressing a cardiac myocyte specific inducible Cre recombinase (\(\alpha MHC-mER-Cre-mER\))\(^{(11)}\), and induced Cre recombinase
activity through a daily i.p. injection of 4-hydroxy-tamoxifen (4-OHT) (60 mg/kg/day) for 5 days as previously described (Fig. S2). (11) Importantly, deletion of HuR did not affect basal cardiac function (Fig. S3). The absence of a basal cardiac phenotype in iCM-HuR^-/- mice is not surprising given our data that HuR appears mostly inactive in adult myocardium under resting conditions (Fig. 1 and S1).

To determine the role of HuR in pathological cardiac hypertrophy, iCM-HuR^-/- mice and tamoxifen-treated Cre^+/HuR^+/+ littermate controls underwent transverse aortic constriction (TAC), a model of LV pressure overload that results in a predictable and reproducible progression from compensated LVH to decompensated LVH to heart failure. Sham procedure groups were included as surgical/manipulation control groups. At eight weeks post-TAC, iCM-HuR^-/- hearts show a preserved cardiac architecture and reduced hypertrophy (LV weight/body weight ratio) compared to Cre^+/HuR^+/+ control hearts (Fig. 2A-B). Interestingly, while myocyte-specific deletion of HuR does not completely inhibit the development of LVH, it does appear to completely inhibit the induced expression of the hypertrophic marker genes ANF (atrial natriuretic factor; Nppa) and BNP (brain natriuretic protein; Nppb) at eight weeks following TAC (Fig. 2C-D).

**HuR deletion delays the transition from compensated to decompensated LVH**

Longitudinal assessment of LV mass (normalized to body weight) via echocardiography shows a significant increase in LV mass in control mice at two weeks following aortic constriction, whereas the LV mass of iCM-HuR^-/- mice increases in a much more gradual manner (Fig. 3A) with a significant blunting in total LV mass gain from baseline compared to control at eight weeks post-TAC (Fig. 3B). Moreover, LV posterior wall thickness was significantly increased at two weeks post-TAC in the control mice, followed by a gradual thinning of the LV wall as expected with a progression to decompensated hypertrophy and LV dilation (Fig. 3C). However, much like the gradual progression of LV mass, the LV posterior wall thickness in iCM-HuR^-/- mice shows a gradual hypertrophy, suggesting a delayed progression of the hypertrophic response in the absence of HuR (Fig. 3C).

LV dilation and wall thinning are both hallmarks of the maladaptive cardiac remodeling that occurs as pathological LVH progresses to decompensation and heart failure. (12) Weekly assessment of both LV end diastolic (Fig. 3D) and LV end systolic (Fig. 3E) volume shows a significant reduction in LV dilation starting at 4 weeks post-TAC in the iCM-HuR^-/- mice. Accordingly, iCM-HuR^-/- mice display significantly less LV dilation at 8 weeks post-TAC than their littermate controls (Fig. 3F).

**HuR deletion preserves cardiac function following LV pressure overload**

The initial development of hypertrophy is thought to be a compensatory response to maintain cardiac output in the face of hemodynamic stress. Because of this, there is concern by some that inhibition of the hypertrophic process may result in a rapid loss of systolic function in pressure overload. A modest decline in cardiac function, as measured via LV ejection fraction, is observed in both control and iCM-HuR^-/- mice in
the first few weeks following TAC (Fig. 3G). However, as the control mice continue to decline from 6-8 weeks post-TAC, LV ejection fraction in the iCM-HuR\(^{-}\) mice remains stable and significantly improved compared to control (Fig. 3G). At 8 weeks following TAC, total decline of LV ejection fraction was significantly less in the iCM-HuR\(^{-}\) mice compared to control (Fig. 3H).

*In vivo*, invasive pressure catheterization was performed to confirm equal pressure gradients between control and iCM-HuR\(^{-}\) mice (Fig. S4) and determine the rate of LV pressure changes in the two groups. Results show that iCM-HuR\(^{-}\) mice had a preserved rate of both LV developed pressure (+dP/dt; Fig. 3I) and LV relaxation (-dP/dt; Fig. 3J).

**Deletion of HuR reverses genome wide changes in TAC-induced gene expression**

To determine the mechanisms by which myocyte-specific HuR deletion mediates the hypertrophic response, we performed RNA sequencing to identify HuR-dependent changes in transcript expression. RNA-seq was performed on control sham, iCM-HuR\(^{-}\) sham, control TAC, and iCM-HuR\(^{-}\) TAC hearts at 8 weeks following surgical manipulation (Fig. 4A; Table S1). Comparison of the sham groups confirms that HuR plays little role in mature, non-stressed myocytes as HuR deletion had minimal impact on basal transcript levels; only 4 of 46,989 transcripts were found to be significantly dysregulated between control and iCM-HuR\(^{-}\) sham groups (Table S1). Applying a rather stringent statistical filter of a false detection rate at 0.01 we identified 2006 distinct mRNA transcripts whose expression was significantly altered by TAC (control sham vs. control TAC; Fig. 4B; Table S2). Applying the same strict statistical filter, we found that 59 of these genes are strongly regulated by HuR (Fig. 4B; Table S3).

Using a principal component analysis (PCA) plot to visualize broad level changes in TAC and HuR-dependent changes in transcript expression shows the two sham groups clustering together with a strong shift in TAC-dependent transcript changes in control mice that is reversed in HuR-deletion mice (Fig. 4C). A more in-depth look at the 2006 TAC-dependent transcript changes shows a recovery/reversal of TAC-induced changes in 1865 (92.9\%) of these 2006 transcripts, with 1 (0.1\%) being unaffected, and 140 (7.0\%) being exacerbated upon HuR deletion (Fig. 4D). Furthermore, all 59 of the transcripts identified as being strongly HuR-dependent show a reversal or recovery of TAC-mediated changes in iCM-HuR\(^{-}\) mice (Fig. 4D; inset).

Gene ontology clustering and enrichment analysis of the 59 HuR-dependent transcripts shows that HuR predominately regulates the expression of genes involved in cell growth and development (tissue development, system development) and fibrosis/ECM remodeling (fibril organization, collagen fibril organization) (Fig. 4E). Given that pathological LVH is known to induce both cell growth (hypertrophy) and fibrotic signaling pathways, a reversal in the expression changes of these genes in iCM-HuR\(^{-}\) mice is not unexpected.

*HuR mediates the development of cardiac fibrosis*
In accordance with the RNA-seq data suggesting a strong role for HuR in the development of cardiac fibrosis, we also observe a significant reduction in fibrosis in the iCM-HuR\textsuperscript{-/-} hearts compared to control at 8 weeks post-TAC (Fig. 5). RNA-seq results show a significant HuR-dependent blunting of TAC-induced expression of 16 pro-fibrotic genes (taken from the 59 identified HuR-dependent genes in Fig 4; Fig. 6A). Furthermore, we show a robust increase in protein expression of periostin, a marker for activated myofibroblasts (13), in the control mice that is nearly ablated in the iCM-HuR\textsuperscript{-/-} mice (Fig. 6B).

While compelling, this data alone does not allow us to conclude whether HuR deletion plays a direct role in the inhibition of fibrotic signaling in the heart, or whether the reduction in fibrosis is an indirect consequence due to the inhibition of pathological LVH. However, activation of HuR in hypertrophic myocytes at 8 weeks post-TAC co-localizes with regions of fibrosis (Fig. 6C), supporting a functional link between the two. We utilized isolated neonatal rat ventricular myocytes (NRVMs) to show that Tgfb mRNA expression in response to hypertrophic stimuli in cardiac myocytes is dependent on HuR (Fig. 6D). To demonstrate that HuR directly regulates Tgfb expression in myocytes, we performed an RNA-protein cross-linking immunoprecipitation (CLIP) to show that direct binding of HuR to the Tgfb mRNA transcript (Fig. 6E). Additionally, HuR appears to mediate Tgfb expression by stabilizing the transcript, as our results show that, following treatment with actinomycin D (2.5 ug/ml), HuR inhibition led to expedited degradation of Tgfb mRNA (Fig. 6F). Finally, a HuR-dependent role in Tgfb signaling in hypertrophy is also supported by our RNA-seq data showing a recovery of TAC-dependent gene expression changes for transcripts associated with positive regulation of Tgfb signaling (Fig. 6G and Table S4).

**Pharmacological inhibition of HuR reduces further progression of LVH and preserves cardiac function**

To determine the potential feasibility of inhibiting HuR as a therapeutic approach, we used KH-3, a novel small-molecule inhibitor of HuR, to achieve in vivo inhibition following the initial onset of pathological LVH. We have previously demonstrated the inhibition of hypertrophic growth in vitro using earlier generation precursors of KH-3.(8) In this experiment, we subjected 20 mice to TAC and allowed them to progress for 4 weeks prior to randomization into two groups with indistinguishable cardiac mass and function (Fig. S5). These groups were then treated with either vehicle or KH-3 (60 mg/kg injected i.p. 3x weekly) for 7 additional weeks.

Mice treated with KH-3 display increased survival (Fig. 7A) and maintain relatively normal sized hearts (Fig. 7B-C). Longitudinal assessment of LV mass confirms that all animals had a significant increase in LV mass prior to initiating KH-3 treatment (Fig. 7D). However, LV mass in vehicle treatment mice continued to increase, whereas KH-3 inhibited further increase in LV mass, and LV mass in KH-3 animals at 11 weeks post-TAC is not significantly different than immediately prior to randomization (Fig. 7D-E). In addition, KH-3 treated animals had significantly less cardiac mass when normalized to either body weight or tibia length (Fig. 7F-G) as well as a significant
reduction in ANF mRNA expression (Fig. 7H). Treatment with KH-3 also significantly reduced further LV dilation (Fig. 7I–J).

KH-3 treatment was also able to significantly reduce the rate of decline in cardiac function. Echocardiography shows a significantly preserved LV ejection fraction in KH-3 treated mice compared to vehicle (Fig. 7K–L). KH-3 also shows a significant preservation in the rate of positive LV pressure generation (+dP/dt) (Fig. 7M–N). Importantly, we show via *in vivo* pressure catheterization that KH-3 and vehicle treated groups had similar LV pressure gradients (Fig. S6) and that KH-3 treatment had no effect on peripheral blood pressure (Fig. S7).

**Inhibition of HuR via KH-3 reduces cardiac fibrosis**

Vehicle treated hearts show a significant degree of cardiac fibrosis at 11 weeks post-TAC (Fig. 7O, upper panel), while treatment with KH-3 significantly reduced the amount of cardiac fibrosis (Fig. 7O, lower panel). Interestingly, the overall reduction in fibrosis appears to be most strongly attributed to a reduction in interstitial fibrosis (Fig. 7O, right panels). Western blotting also shows a significant reduction in the protein expression of fibronectin and periostin in mice given KH-3 (Fig. 7P).

**Discussion**

Despite being highly expressed in the heart, relatively little is known about the role of HuR in the myocardium. However, there is growing interest in the functional role that HuR and other RNA binding proteins may play as central regulators of post-transcriptional gene expression in cardiac physiology and pathology.(6, 7) To assess the functional role of HuR in the development and progression of pathological cardiac hypertrophy, we created the first cardiac-specific HuR deletion mouse (*iCM-HuR*–/–). Characterization of these mice did not identify any basal differences in phenotype between *iCM-HuR*–/– and control mice, and HuR deletion had minimal impact on basal (sham) gene expression. This was expected given our prior *in vitro* work (8), as well as results presented here, showing minimal cytoplasmic localization (activation) of HuR in non-stressed myocytes. However, following TAC, the *iCM-HuR*–/– mice are significantly protected from pathology, as evidenced by reduced LV hypertrophy, preserved cardiac function, reduced LV chamber dilation, and lessened cardiac fibrosis.

It is interesting to note that some degree of compensated LV hypertrophy still occurs in the *iCM-HuR*–/– mice. These results demonstrate that myocyte-specific deletion of HuR only partially blunts or slows the development of hypertrophy, but more strongly reduces the pathological LV remodeling and fibrosis associated with progression to a decompensated phase and functional decline. It has been shown that load-induced compensated LV hypertrophy is not a strict requirement to maintain contractile performance in a mouse heart(14), and it has been argued that cardiac hypertrophy in response to pathological stimuli is not in fact a beneficial compensatory response.(15) Thus, the protection observed from the deletion of HuR could be due to an inhibition of hypertrophic growth, or a more direct inhibition of one or more underlying mechanisms.
that drive the transition from a ‘compensated’ hypertrophy to a state of pathological hypertrophy.

Prior work has suggested that HuR plays a central role in the cardiac response to stress following ischemia/reperfusion injury (myocardial infarction). (16, 17) Krishnamurthy, et al showed that HuR expression increases following ischemic injury and plays a role in mediating post-infarct remodeling and Tgfb expression. However, it was unclear from this work in which cell types HuR plays a central role, and the authors postulated a key role for HuR expression in infiltrating leukocytes. While HuR is certainly highly expressed in these cells, it is also expressed in many resident cell types within the myocardium, and our results are the first to demonstrate a clear role for HuR signaling specifically in myocytes. In this work, we show that HuR activation is increased in the myocytes of hypertrophic hearts, and the mechanistic basis of the protection observed in iCM-HuR<sup>−/−</sup> mice following TAC is potentially through an inhibition of Tgfb expression and subsequent development of cardiac fibrosis. This repressed level of Tgfb expression is confirmed in our RNA-seq studies, where additional positive regulators of this pathway are reduced and suppressive genes show little to no changes.

TGFβ is produced and secreted by myocytes in response to hypertrophic stimuli, and can initiate myofibroblast activation (and subsequent cardiac fibrosis) in a paracrine fashion. (18-20) TGFβ is increased in hypertrophic myocardium and is a primary initiator of myofibroblast activation and fibrotic signaling, which is a critical step that leads to extracellular matrix (ECM) remodeling and cardiac fibrosis. (18, 19) However, the mechanistic details of how TGFβ expression is regulated in hypertrophic cardiomyocytes remain unclear. HuR has been shown to mediate TGFβ expression and pro-fibrotic functions in fibroblasts as well as macrophages (17, 21), and our data shows for the first time that HuR may play a similar role in the myocytes, as HuR knockdown dramatically reduced NRVM expression of TGFβ in response to phenylephrine. Our data also shows that Tgfb is a directly bound target of HuR and that HuR stabilizes Tgfb mRNA. Moreover, a central role for HuR in controlling myocyte-mediated paracrine activation of fibrotic signaling would explain our RNA-seq results demonstrating that many of the HuR-dependent genes were identified as having pro-fibrotic roles. Interestingly, Tgfb itself was not among the genes that we found to be differentially regulated by TAC or HuR, but is supported by seminal work by Villarreal and Dillman showing that Tgfb mRNA expression was increased early (within 12 hours) following aortic banding, but returned to baseline within two weeks. (22) At least a few of the genes identified as having changed in a HuR-dependent manner are not thought to be expressed by myocytes, which would suggest an indirect or much farther upstream regulation by HuR (e.g. loss of HuR-dependent TGFβ expression in myocytes and a subsequent decrease in paracrine activation of myofibroblasts). However, 50 of the 59 HuR-dependent genes are predicted to have conserved HuR binding sites, representing a significant enrichment compared to predicted transcriptome-wide HuR binding sites (Fishers Exact Test, P < 0.05; Table S5). On the other hand, in our data showing a co-localization of HuR activation and fibrosis, HuR appears to be activated in additional cell
types than just myocytes. Thus, future work will explore the role of HuR in additional cell types in the heart as well, specifically myofibroblasts.

One of the more significant aspects of this work is that it establishes HuR inhibition as a potentially viable therapeutic approach to the clinical treatment of pathological LVH and heart failure. In demonstrating the efficacy of pharmacological targeting of HuR, we took a more clinically relevant approach of allowing the mice to progress out to four weeks post-TAC when pathological LVH could already be observed prior to randomization to drug or vehicle. We show that a chronic (7 week) treatment with a small molecule inhibitor of HuR, following the initial development of a pathological LV hypertrophy, improved survival and stunted pathological progression of LVH while eliciting no observable deleterious off-target effects to other tissues.

In summary, our results demonstrate that HuR activation in hypertrophic myocytes contributes to pathological LV remodeling in response to pressure overload. This is demonstrated through a preservation of LV function and inhibition of LV dilation following TAC in cardiac-specific HuR deletion mice. Furthermore, we show that TGFβ expression and the development of cardiac fibrosis are inhibited in the absence of HuR, suggesting these pathways as a central mechanistic target of HuR activation in the myocyte. Finally, these results identify HuR as a novel therapeutic target to prevent or reduce pathological progression of left ventricular hypertrophy in response to pressure overload.

Methods

Mouse models. HuR floxed mice were described by Ghosh et al(10) and obtained from Jackson Labs (stock # 021431). To generate a cardiac-specific inducible HuR deletion model, HuR-floxed mice were crossed with mice expressing a cardiac myocyte specific inducible Cre recombinase (αMHC-mER-Cre-mER) (Jackson Labs, stock # 005657). (11) HuR deletion was achieved through a daily i.p. injection of 4-hydroxy-tamoxifen (4-OHT) (60 mg/kg/day) for 5 days as previously described. (11) Mice were then allowed to recover for a minimum of 10 days following 4-OHT dosing prior to being incorporated into studies. Mice for KH-3 drug studies and RNA immunoprecipitation were wild-type C57Bl/6 mice obtained from Jackson Labs (stock # 000664). All mice were male, 10-16 weeks old at time of surgery. For tissue collection, tissues were removed and either flash frozen or fixed in 4% paraformaldehyde in PBS for further analysis.

Pharmacological HuR inhibition. HuR was pharmacologically inhibited using the small molecule KH-3, a second generation of HuR inhibitor compounds previously described by Wu et.al 2015. (23) For HuR inhibition, the KH-3 was i.p. injected 3x weekly at a dose of 60 mg/kg.

Transverse aortic constriction. Pressure overload of the LV was induced by transverse aortic constriction (TAC) surgery, as previously described. (24, 25) Briefly, animals were anesthetized under isoflurane, intubated, and ventilated. Following sternotomy, a 7-0 silk suture was used to tie down the aorta (between the innominate and left carotid arteries) around a 27-gauge needle (which was subsequently removed)
to produce a constriction of uniform and reproducible diameter. Sham operated animals were subjected to the identical surgical procedures, except the suture was not tied to produce an occlusion.

**In vivo hemodynamic measurements.** Pressure gradients across the aortic constriction site were determined in all animals by simultaneous pressure recording in the left and right carotid arteries as previously described.(24) As a terminal procedure, mice were weighed and anesthetized using an i.p. injection of ketamine (50 μg/g) and inactin (thiobutabarbital, 100 μg/g). A 1.4-French Mikro-Tip Millar catheter pressure transducer was used to determine carotid pressure and subsequently advanced into the LV to determine dynamic pressure response and function as previously described.(26, 27) Following pressure recording, hearts were removed and atria were dissected away prior to weighing and either fixing or flash freezing for further analysis as described.

**Echocardiography.** All echocardiographic studies were performed as previously described.(28) Briefly, mice were anesthetized with isoflurane and body temperature was maintained at 37°C during imaging. Using a Vevo 2100, parasternal images were obtained in short and long axes in two-dimensional mode and motion (M)-mode for quantification. These were then analyzed using VevoStrain software (Vevo 2100, v1.1.1 B1455, Visualsonic, Toronto, Canada). *iCM-HuR*− mice had baseline echocardiographic measurements pre- and post-tamoxifen treatment, and all mice had measurements weekly following TAC until euthanasia.

**NRVM isolation and culture.** Neonatal Rat Ventricular Myocytes (NRVMs) were isolated using collagenase digestion and adhesion differential from fibroblasts. Sprague Dawley neonatal rats (1-2 days old) were decapitated and the hearts were isolated. Following removal of the atria, the ventricles were cut into small pieces and digested first in .05% trypsin/EDTA (Corning) overnight, then in collagenase II (Gibco) for 30 minutes. Cells were then spun at 100 x g followed by a 40 minute pre-plating process on non-treated plates to allow the fibroblasts to adhere. The non-adherent NRVMs were then transferred to cell culture-treated dishes in MEM alpha media (Gibco) with 10% FBS.

**Histological analysis.** Fixed hearts were paraffin embedded and sectioned at 6 μm thickness by the Cincinnati Children’s Hospital Medical Center Department of Pathology Research Core (Cincinnati, OH, USA), and were subsequently stained with hematoxylin and eosin (H&E) and/or Masson’s trichrome (MT). Immunofluorescence (IF) of HuR was performed on human and mouse failing heart samples. Samples were deparaffinized with xylene and rehydrated in serial ethanol dilutions. The samples were then heated in a sodium citrate solution (10mM Sodium citrate, 0.5% Tween20, pH=6) for 20 minutes for antigen retrieval. Triton-X 100 (0.06% solution) was used to permeabilize the samples for 30 minutes. The tissues were then incubated in blocking solution (5% BSA, Sigma Aldrich) for 1 hour, followed by primary HuR antibody (Abcam ab200342) overnight, and AlexaFluor secondary antibody (Thermo Fisher Scientific A11008) for 1 hour. For DAB staining, following antigen retrieval, samples were incubated in 1% hydrogen peroxide to block endogenous peroxidase activity, followed by blocking in 5% BSA. They were then incubated overnight in primary HuR antibody
(Santa Cruz sc5261), followed by HRP secondary antibody (BioRad 172-1011) for 1 hour, and DAB substrate for 5 minutes. Images were taken using a BioTek Cytation 5.

**Protein isolation and Western blotting.** Total protein was isolated from crushed tissue in RIPA buffer with 0.5 mM DTT, 0.2 mM sodium-orthovanadate, and a protease inhibitor mixture tablet (Complete mini; Roche Applied Science). 25 µg of protein extract per lane was separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. Blocking was performed for 1 h at room temperature using 5% dry milk in 0.1% Tween 20, tris-buffered saline (T-TBS). Primary antibodies for Periostin (Novus Biologicals NBP1-30042), Fibronectin (Abcam ab2413), and GAPDH (Santa Cruz Biotechnology sc25778) were incubated overnight at 4°C, and secondary antibodies were incubated for 1–2 h at room temperature in T-TBS.

**RNA isolation and quantitative RT-PCR.** RNA was isolated using a Macherey-Nagel NucleoSpin RNA kit and cDNA was synthesized using a BioScript All-in-One cDNA Synthesis SuperMix (Biotool). Samples were run on a Stratagene Mx3005P (Agilent Technologies) using ABSolute Blue SYBR Green ROX (ThermoFisher) to assess levels of ANF, BNP, and 18S. Results were analyzed using the ΔΔCt method, normalized to 18S. Primers are as listed: ANF, F, 5'-AGGAGAAGATGCCGCTAG-3', R, 5'-GCTTTTCAAGAGGGCAGA-3'; BNP, F, 5'-AAATCCCTAGCCAGTCCAGA-3', R, 5'-GAGCTTGCTCTGGGCCATTT-3'; 18S, F, 5'-AGTCCCTGCGCCCTTTGAACA-3', R, 5'-CGAGGGCCCTCATAAAC-3'; TGFβ, F, 5'-CAATTCCTGGCGTTACCTTG-3', R, 5'-CCCTGTATTGCCTTCTGG-3'; GAPDH, F, 5'-ACCACAGTCCATGCGCATC-3', R, 5'-TCCACCACTGCTGGCTGTA-3'.

**RNA immunoprecipitation.** Protein G magnetic beads (Pierce; 88847) were washed three times with wash buffer (50mM Tris, 150mM NaCl, 1mM MgCl₂, 0.05% NP-40). The beads were then incubated with either 2µg HuR antibody (abcam; ab28660) or a control secondary antibody, goat anti-rabbit (Invitrogen; 65-6120). Cardiac tissue from C57Bl/6 mice treated with either vehicle (DMSO) or HuR inhibitor (10mg/kg DHTS; Sigma D0947) was minced in PBS and triturated using the Bead Bug microtube homogenizer (Benchmark Scientific) to achieve a single cell suspension. The cells were then crosslinked using the Stratalinker® UV crosslinker (400 µJ X 3). The cells were then spun down (4°C, 3000 rpm, 5 min) and the pellet was resuspended in lysis buffer (100mM KCl, 5mM MgCl₂, 10mM Heps, 0.5% NP-40, protease inhibitor cocktail (Pierce)) and lysed with a dounce homogenizer. The lysed cells were added to the prepared protein G beads and incubated for one hour at room temperature using a Hula Mixer (Invitrogen). After one hour, the cells were washed five times with lysis buffer. RNA was eluted using 100µL of lysis buffer with 2.5µL of Proteinase K (20mg/mL) and incubating at 55°C for 30 min with mixing. RNA was purified using phenol/chloroform isolation and a Macherey-Nagel NucleoSpin RNA kit (as described above).

**RNA stability.** NRVMs were treated with 2mM Actinomycin D (Sigma; A1410), DMSO vehicle, or Actinomycin D + HuR inhibitors CMLD1/2 (used previously in Slone et al (8)). RNA was collected at 0, 3, 8, and 24 hour time points and analyzed for Tgfb expression.
RNA-seq analyses. RNA was isolated as described in Slone et al. (8) and poly(A) library and sequencing were conducted at the DNA sequencing and Genotyping Core at the Cincinnati Children’s Hospital Medical Center (CCHMC). RNA was quantified with a Qubit 3.0 Fluorometer (Life Technologies). Total RNA (150-300 ng) was poly(A) selected and converted to cDNA with a TruSeq Stranded mRNA Library Preparation Kit (Illumina). Different 8-base molecular barcodes were added to allow for high-level multiplexing. After 15 cycles of PCR amplification, libraries were sequences on a HiSeq 2500 sequencing system (Illumina) in Rapid Mode.

Sequenced reads were trimmed and filtered for quality with Trimmomatic as previously described.(29) Libraries were examined for quality at each step with the use of FastQC. Reads were mapped to the mouse genome (GRCm38) with the use of CLC Genomics Workbench (v. 9.5.1, Qiagen Co.). Each RNA-seq read was matched allowing 50% overlap at 95% matching to each transcript. The maximum number of mismatches allowed for each mapped read was set at 2. Gene expression was set as RPKM. Data analyses were conducted through the use of CLC Genomics with the Advanced RNA-seq plugin (1.5). Statistical tests for assessment of differential expression were conducted with a negative binominal generalized linear model with edgeR(30) with a false discovery rate of 0.01. Principal component analysis was based upon the normalized expression levels of each transcript with the use of the PCA package of R (version 3.3.2). Gene ontology of differential regulated transcripts was conducted with the use of g:Profiler.(31) Complete lists of transcripts with differential expression in relation to TAC are provided in Table S1. RNA-seq data has been deposited to NCBI Sequence Read Archive and is available under the Bioproject PRJNA434656.

Prediction of HuR binding sites was done using RBPMap (http://rbpmap.technion.ac.il).(32) Statistical significance of enrichment of HuR binding sites was determined using a Fishers exact test.

Statistics. Statistical analysis was performed using GraphPad PRISM software. Primary statistical tests include 2-tailed Student’s t test for single comparisons and 2-way or repeated measures ANOVAs for multiple comparisons. A p value of less than 0.05 was considered significant.

Study Approval. All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee of the University of Cincinnati and in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Author contributions: L.C.G. and S.R.A contributed equally to the writing and preparation of this manuscript as well as experimental design, execution, and data analysis. L.C.G. performed all HuR IHC staining and Western blotting and contributed to experimental design, data collection, and analysis. S.R.A designed, executed, and oversaw the KH-3 in vivo experiments, and provided general oversight and coordination of all animal experiments. S.S. performed all NRVM isolation, culture, and experiments. L.L. assisted with RNA isolation and qPCR experimental execution and analysis. M.L.N. performed all TAC surgeries and in vivo pressure catheterizations. X.W. assisted with
experimental design for KH-3 studies as well as synthesis of KH-3. N.R. performed the weekly echocardiograms and echocardiography analysis. S.M.J. performed all H&E and Masson’s trichome staining. A.P.O assisted with experimental design for histological analyses. J.A. and S.R. were responsible for the synthesis of KH-3. B.C.B assisted with general experimental design and provided human cardiac tissue. J.N.L. provided expertise with regard to experimental design, execution, and analysis of TAC and *in vivo* catheterization experiments. J.B.B. provided all analysis and interpretation of RNA-Seq results. L.X. assisted with experimental design for KH-3 studies as well as collaboratively providing KH-3 for use in this study. J.R. assisted with interpretation of echocardiography data. M.T. oversaw all experimental design, execution, analysis, interpretation, and communication of results. All authors contributed to the final editing and approval of the final version of this manuscript.

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Figures

**Fig 1. HuR activation is increased in failing human hearts.** HuR immunofluorescence staining from healthy donor hearts as well as explanted tissue from left ventricular assist device (LVAD). HuR immunofluorescence staining from control sham and TAC mice. Scale bars: 1000μm (4X), 100 μm (20X). Images are representative of n=3/group.
Fig. 2: Cardiac-specific deletion of HuR reduces hypertrophy in response to TAC-induced LV pressure overload. (A) Hearts from control and iCM-HuR<sup>−/−</sup> mice were isolated eight weeks post-TAC or sham surgery. These hearts were then embedded in paraffin, sectioned, and H&E stained. Scale bar: 3000μm. (B) Ventricular weight to body weight ratio of control and iCM-HuR<sup>−/−</sup> mice 8 weeks post-TAC or sham surgery. qRT-PCR for ANF (C) or BNP (D) mRNA levels from mice 8 weeks post sham or TAC surgery relative to 18S. For (B) through (D), a two way ANOVA was used to compare sham and TAC groups and the *<i>P</i> < 0.05, **<i>P</i> < 0.01 for indicated comparison. Data are shown as means ± SEM. N ≥ 5 per group.
Fig. 3 Cardiac-specific deletion of HuR delays progression from compensated to decompensated hypertrophy. (A) Left ventricular mass to body weight ratio of control and iCM-HuR−/− mice pre-TAC (baseline) and weekly until 8 weeks post-TAC. (B) Quantification of the total LV mass/body weight ratio change from baseline to 8 weeks post-TAC/sham surgeries. (C) Left ventricular posterior wall thickness, (D) left ventricular end diastolic volume, and (E) left ventricular end systolic volume quantified by echocardiography and baseline and weekly until 8 weeks post-TAC or sham surgery. (F) Quantification of total LV End Diastolic and Systolic volume change from baseline to 8 weeks post TAC or sham surgeries. (G) Left ventricular Ejection Fraction of control and iCM-HuR−/− mice from baseline to 8 weeks post-TAC or sham surgeries. (H) Quantification of total change in LV ejection fraction from baseline to 8 weeks post TAC or sham surgeries. (I) Positive and (J) negative ventricular contractility assessment (dP/dt) of control and iCM-HuR−/− mice 8 weeks post-TAC or sham surgeries. For (A) through (H), ANOVA and student’s T tests were performed. *P <0.05, **P <0.01, ***P <0.001 for indicated comparison. Data are shown as means ± SEM. N ≥ 6 per group.
Fig. 4. Identification of HuR-dependent changes in transcript levels. (A) Schematic for experimental groups compared denoted by connecting line. (B) Venn diagram showing overlap in the number of transcripts with significant differences in control and iCM-HuR^−/− mice following TAC. (C) Principal component (PC) analyses based on normalized expression values for RNA-seq data. (D) Proportion of expression change recovery in iCM-HuR^−/− vs. control mice for all significant TAC-dependent gene expression changes (from Table S1) between Control Sham and Control TAC. Blue represents recovery following HuR deletion. Inset, overlapping set of significant HuR-dependent genes displayed in B. (E) Specific gene ontology categories shifted during HuR dependent recovery that underlie changes in heart hypertrophy. N ≥ 3 per group.
Fig. 5: HuR blunts the development of cardiac fibrosis. Masson’s trichrome images of sectioned hearts from control and iCM-HuR$^{-}$ mice post-TAC or sham surgery. Scale bar: 3000μm, 400μm (4X and 10X)
Fig. 6: HuR mediates the development of cardiac fibrosis. (A) Differential expression of genes associated with tissue development and are differential regulated in iCM-HuR−/− mice based upon those identified in Figure 4E. Percent following bars in expressional recovery in iCM-HuR−/− compared to control during TAC. (B) Western blot for periostin protein expression from cardiac tissue isolated from control or iCM-HuR+/− mice 8 weeks post-TAC or sham surgery. GAPDH protein expression serves as the loading control. (C) DAB staining of HuR (brown) alongside Masson’s trichrome staining (blue indicative of fibrotic regions) of serial sections of the heart of a wild type mouse 8 weeks post-TAC. (D) TGFβ mRNA expression levels in response to a hypertrophic stimulus (Phenylepherine; PE) in the presence/absence of HuR determined by performing qRT-PCR on RNA isolated from cultured neonatal rat ventricular myocytes with HuR siRNA/control siRNA, N ≥ 3 replicates per treatment. (E) qPCR qualitative measure of Tgfb RNA eluted using a HuR antibody or a goat, anti-rabbit IgG control in the presence and absence of a HuR inhibitor; representative N=1. (F) qPCR quantification of Tgfb mRNA after treatment with vehicle, Actinomycin D (Act D), or Actinomycin D + HuR inhibitor; N=6. (G) TAC-dependent transcripts associated with positive regulation of TGFβ (Gene ontology: 0071560) signaling based on Table S2. Blue represents recovery following HuR deletion. For (D), a two way ANOVA was performed. *P <0.05, **P <0.01 for indicated comparison. Data are shown as means ± SEM.
**Fig. 7.** Pharmacological inhibition of HuR following the initial onset of pathological LVH reduces progression to heart failure. (A) Kaplan Meier survival curve of mice treated with KH-3 or vehicle post randomization (randomization occurred 4 weeks post-TAC surgery). (B) Whole hearts isolated 11 weeks post-TAC from mice treated with vehicle or KH-3. Scale: mm (C) H&E stains of heart sections from mice treated with vehicle or KH-3. Scale: 3000 μm. (D) Left ventricular mass determined by weekly echocardiogram from baseline to 11 weeks post-TAC (with KH-3 or vehicle treatment beginning at 4 weeks post-TAC). (E) Total change in LV mass of mice treated with KH-3 or vehicle from randomization (4 weeks post-TAC) to 11 weeks post-TAC. (F) Ventricular weight to body weight ratio and (G) ventricular weight to tibia length of mice treated with KH-3 or vehicle 11 weeks post-TAC. (H) qRT-PCR of ANF mRNA levels from cardiac tissue of mice treated with KH-3 or vehicle 11 weeks post-TAC. (I) Left ventricular diastolic volume determined by weekly echocardiogram from baseline to 11 weeks post-TAC (with KH-3 or vehicle treatment beginning 4 weeks post-TAC). (J) Total change in left ventricular systolic and diastolic volume of mice treated with KH-3 or vehicle from randomization (4 weeks post-TAC) to 11 weeks post-TAC. (K) Left ventricular ejection fraction determined by weekly echocardiogram from baseline to 11 weeks post-TAC (with KH-3 or vehicle treatment beginning 4 weeks post-TAC). (L) Total change in LV ejection fraction of mice treated with KH-3 or vehicle from randomization (4 weeks post-TAC) to 11 weeks post-TAC. (M) Positive and (N) negative ventricular contractility (dP/dt) of KH-3 and vehicle treated mice 11 weeks post-TAC. (O) Masson’s trichrome images of sectioned KH-3 and vehicle treated hearts isolated 11 weeks post-TAC. Scale bar: 100 μm. (P) Western blot of periostin and fibronectin (GAPDH loading control) in cardiac tissue isolated from mice treated with KH-3 or vehicle. Where appropriate, ANOVA and Student’s T tests were performed. *P <0.05, **P <0.01, ***P <0.001 for indicated comparison. Data shown as means ± SEM. N ≥ 5 per group.
REFERENCES


