Cardiac renewal

https://doi.org/10.1038/s44161-024-00483-3

Cardiac *ACTN2* enhancer regulates cardiometabolism and maturation

Francisco X. Galdos, Carissa Lee & Sean M. Wu

A study describes the role of the *ACTN2* enhancer in myocardial maturation, highlighting its relevance in regulating structural, functional and metabolic dynamics in the heart. These findings offer insights that may advance our understanding of cardiovascular disease.

As the first organ to form in the developing embryo, the heart undergoes coordinated structural, functional and metabolic changes throughout embryonic and postnatal development that enable it to circulate blood effectively to the growing body[1]. The maturation of cardiomyocytes — the contractile, conductive muscle cells of the heart — is essential to this dynamic process, and disruption of maturation may promote adult cardiovascular disease[2]. Current in vitro human pluripotent stem cell-derived cardiomyocytes (hPS-CMs) lack the full spectrum of elements necessary for adult maturation, limiting disease modeling capabilities and therapeutic studies[3]. Moreover, the published literature has so far focused extensively on the effect of protein-coding mutations in the development of cardiomyopathy; however, the contributions of non-coding genetic elements remain unknown[4]. Although genome-wide association studies (GWAS) have identified non-coding variants associated with heart failure, investigating the functional consequences of these variants remains challenging given the pleiotropic effects of non-coding genetic regions in the regulation of gene expression programs and our incomplete understanding of the specific target genes of putative enhancers[4,5].

Alpha-actinin 2 (ACTN2), encoded by the *ACTN2* gene, is a crucial protein involved in the formation of sarcomeres, the main structural units of striated and cardiac muscle tissue[6]. ACTN2 truncations have been shown to disrupt sarcomere generation and metabolic function in vivo[6]. Previously identified genetic variants located near the *ACTN2* promoter strongly correlated with heart failure independent of other cardiovascular risk factors[4]. Additional chromatin accessibility assays have suggested that one of these genetic variants falls within a transcriptionally active and highly evolutionarily conserved *ACTN2* enhancer[4].

In this issue *Nature Cardiovascular Research*, Htet et al.[7] build on their previous findings to show that deletion of a crucial *ACTN2* enhancer suppresses cardiac maturation both in vitro and in vivo. Furthermore, they uncover a mechanistic role for the HSP90A chaperone and the mTOR signaling pathway, providing insights into the function of enhancers in cardiomyocyte development and physiology.

To investigate the role of a previously identified putative *ACTN2* enhancer on human cardiomyocyte structure and function, the authors used the CRISPR–Cas9 system to generate a human induced pluripotent stem (hiPS) cell line with heterozygous deletion of the *ACTN2* enhancer. Although the efficiency of cardiomyocyte differentiation of these *ACTN2* enhancer-deleted hiPS cells was comparable to isogenic wild-type controls, the expression of ACTN2 at both the gene and protein levels was reduced, with immunostaining showing considerable hypertrophy, thinning of individual sarcomeres, and abnormal morphology of sarcomeric structure. Consistent with these observations, *ACTN2* enhancer-deleted hPS-CMs exhibited reduced calcium transients, beat rate and force generation, which indicates functional impairment. To characterize the global changes in gene expression among *ACTN2* enhancer-deleted hPS-CMs, the authors performed single-cell RNA sequencing, which showed consistent downregulation of sarcomeric and calcium-handling genes involved in cardiomyocyte contraction. The data also suggested metabolic disruption and persistent cellular senescence, as several aerobic metabolism pathways were downregulated whereas p53-mediated apoptosis and protein synthesis pathways were upregulated. Hypothesizing that these changes resulted in a shift towards a less mature, anabolic state, Htet et al.[7] performed a bioenergetic Seahorse analysis in *ACTN2* enhancer-deleted hPS-CMs, and found increased glycolysis and decreases in oxidative phosphorylation.

Delving more deeply into the mechanisms that drive *ACTN2* regulation, Htet et al.[7] targeted a 258-bp evolutionarily conserved region within the enhancer locus that contains the rs535411 variant associated with heart failure[4]. Using an inhibitory CRISPR approach targeted to this evolutionarily conserved region in day 20 hPS-CMs, the authors showed by quantitative PCR (qPCR) that *ACTN2* expression was significantly decreased among hPS-CMs transfected with guide RNAs targeting the evolutionarily conserved region or positive controls targeting the *ACTN2* promoter, thus corroborating the role of the region as a regulator of *ACTN2*. This was further supported by a luciferase reporter assay for ACTN2 expression showing that the presence of the evolutionarily conserved region increases luciferase activity among hPS-CMs. Interestingly, no increase in luciferase activity was observed in the presence of an identical region containing the rs535411 variant, which suggests that the genetic variant itself may also regulate the expression of *ACTN2*.

To determine potential transcription factors that bind to the inhibitory CRISPR-identified *ACTN2* enhancer, the authors used the JASPAR and ENCODE databases to identify all binding motifs present in the conserved region. From this list, they focused on the transcription factors MEF2C, MEF2A, GATA4 and TEAD1 owing to their established roles in cardiac development[8]. Using chromatin immunoprecipitation together with qPCR, the authors identified an MEF2C-binding site approximately 30 bp proximal to the rs535411 variant. Overexpression of MEF2A- and MEF2C-modified mRNA in hPS-CMs transfected with the *ACTN2* enhancer–luciferase reporter resulted in increased luciferase activity, which suggests that members of the MEF2 transcription factor family bind to the *ACTN2* enhancer to influence transcription.

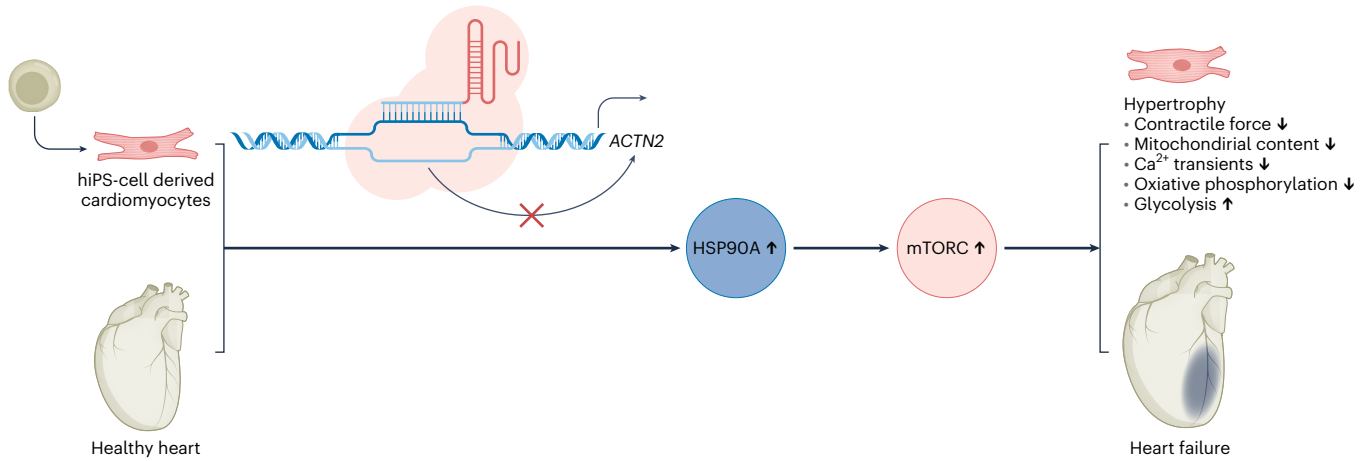

Fig. 1 | An enhancer of *ACTN2* regulates cardiometabolic function and maturation. Schematic overview of heterozygous in vitro and in vivo CRISPR-mediated deletion of the *ACTN2* enhancer. Both hiPS cell-derived cardiomyocytes and adult mutant mouse hearts exhibit upregulation of the mTOR pathway. In hPS-CMs, activation of the HSP90A chaperone protein leads to increased mTORC signaling. Furthermore, both models demonstrate impaired generation of contractile forces due to decreased calcium transients, mitochondrial content and cellular hypertrophy characterized by abnormal sarcomere morphology that leads to heart failure. In addition, a metabolic switch from oxidative phosphorylation to glycolytic metabolism is observed. These findings highlight the crucial role of the *ACTN2* enhancer in the structural, functional and metabolic maturation of the myocardium and highlight its implications in cardiovascular disease. CRISPRi denotes the CRISPR interference technique for inhibition of gene expression.

To further understand the role of the *ACTN2* enhancer in vivo, Htet et al.[7] mapped the human enhancer region to the mouse genome, identifying a region upstream of the mouse *Actn2* gene that is 90% orthologous. Using CRISPR–Cas9 genome editing, the authors generated a mouse line containing knockout of the orthologous mouse *Actn2* enhancer. Phenotypically, the mutant adult mice showed reduced left ventricular systolic function and dilation, indicative of heart failure. Consistent with the in vitro *ACTN2* hiPS cell data, the mouse mutants exhibited reduced expression of key sarcomeric, cytoskeletal and mitochondrial genes, demonstrating an overall disruption in the contractile and metabolic machinery required for normal cardiac function (Fig. 1). At the molecular level, the authors observed reduced calcium handling properties with decreased sarcomere shortening. In addition to the downregulation of these key cardiomyocyte genes and pathways, RNA-sequencing data from the *Actn2* enhancer mutant hearts showed evidence of a metabolic switch from oxidative phosphorylation to glycolysis in the *Actn2* enhancer mouse knockouts. Using the Seahorse assay, the authors confirm that this metabolic switch occurs in vivo, supporting the in vitro data in *ACTN2* enhancer-deleted hPS-CMs. Together, these data provide evidence for the evolutionary conservation of the *Actn2* enhancer as well as an in vivo demonstration of heart failure caused by the absence of this gene-regulatory region.

To explore the mechanism by which knockout the *Actn2* enhancer can lead to cardiometabolic changes, the authors conducted an analysis to identify jointly upregulated genetic pathways in RNA-seq data from both in vitro and in vivo *Actn2* enhancer knockouts. Using Gene Ontology analysis, they identified the PI3K–AKT–mTOR pathway as a commonly upregulated gene set.

we first performed western blotting in adult hearts to quantify the levels 331 of phosphorylated 70-kDa ribosomal protein S6 kinase (p70S6K) at Threonine 389, a 332 downstream effector of mTOR that is known to mediate increase protein synthesis and 333 cell growth. Consistent with our transcriptomic analysis, phospho-p70S6K levels were 334 upregulated in Actn2 enh del adult mouse hearts.

Western blot analysis of a downstream effector of mTOR signaling – levels of phosphorylated 70-kDa ribosomal protein S6 kinase (p70S6K) – showed a significant increase in p70S6K levels in *Actn2* enhancer-deletion adult mouse hearts, indicating increased mTOR pathway activation. Similarly, increased levels of phosphorylated 4EBP1, a translational repressor downstream of mTORC1 signaling, were observed in *ACTN2* enhancer knockout hPS-CMs. To test whether inhibition of mTORC1 could rescue the phenotype of *ACTN2* enhancer-deleted hPS-CMs, mutant cells were treated with the mTORC1 inhibitor everolimus. Everolimus treatment improved sarcomeric structure and the cardiometabolic deficits observed in the knockout cells, suggesting that mTORC1 could reverse the maturation defects.

Given that the mTOR pathway exhibits several downstream signaling partners[9], Htet et al.[7] hypothesized that there may be a direct interaction between ACTN2 levels and mTOR signaling proteins. By analyzing a published ACTN2 biotinylation proximity labeling dataset in hPS-CMs, they identified the stress-induced HSP90A protein as a chaperone that could interact with *ACTN2* and mediate mTOR activation.

Co-immunoprecipitation experiments confirmed the interaction of HSP90A with ACTN2 in hPS-CMs, and levels of HSP90A were upregulated in *ACTN2* enhancer-deleted hPS-CMs, potentially suggesting that HSP90A levels change in response to *ACTN2* levels. Importantly, short interfering RNA (siRNA)-mediated knockdown of HSP90A could suppress mTORC1 signaling upregulation in the mutant cells, thus suggesting the mechanistic link between *ACTN2* expression and mTOR activation.

As mTORC signaling was upregulated in response to decreased *ACTN2* expression, the authors asked whether upregulation of *ACTN2* through the identified enhancer could drive the expression of hPS-CM maturation genes. Using an enhancer CRISPR activation system, activation of the *ACTN2* enhancer region resulted in a 1.7-fold upregulation in *ACTN2* expression. qPCR also showed a nearly twofold

upregulation of genes associated with cardiomyocyte maturation, including *MYH7* and *TNNI3*, as well as genes involved in mitochondrial biogenesis, consistent with an increase in genes associated with cardiomyocyte maturation.

In summary, the study by Htet et al.[7] validated the importance of an evolutionarily conserved *ACTN2* enhancer and delineated its requirement in systolic heart function and cardiomyocyte maturation. The authors provide a mechanistic explanation for the role of non-coding elements in the development of heart failure, and use both in vitro and in vivo models to show that disruption of an enhancer can trigger impaired cardiomyocyte maturation. The identification of the mTORC pathway as an effector of the heart failure phenotype raises important therapeutic implications given the availability of drugs that can potentially reverse detrimental effects of heart failure in patients with cardiomyopathies that may arise from non-coding genetic variants. Importantly, given the immaturity of hiPS cell-derived cardiomyocytes, the work by Htet et al.[7] highlights the need to further understand the role of enhancer regions upstream of genes involved in cardiomyocyte maturation to better drive stem cell-derived cardiomyocyte maturation and improve the fidelity of disease modeling and the function of engineered heart tissues.

Francisco X. Galdos[1,2], Carissa Lee[1] & Sean M. Wu[1,2,3]
[1]Stanford Cardiovascular Institute, Stanford University, Stanford, CA, USA. [2]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA, USA. [3]Division of Cardiovascular Medicine, Department of Medicine, Stanford University, Stanford, CA, USA.
e-mail: smwu@stanford.edu

Published online: 30 May 2024

References

1. Vincent, S. D. & Buckingham, M. E. *Curr. Top. Dev. Biol.* **90**, 1–41 (2010).
2. Guo, Y. & Pu, W. *Circ. Res.* **126**, 1086–1106 (2020).
3. Tu, C., Chao, B. S. & Wu, J. C. *Circ. Res.* **123**, 512–514 (2018).
4. Arvanitis, M. et al. *Nat. Commun.* **11**, 1122 (2020).
5. Wamstad, J. A., Wang, X., Demuren, O. O. & Boyer, L. A. *Trends Cell Biol.* **24**, 294–302 (2014).
6. Sjöblom, B., Salmazo, A. & Djinović-Carugo, K. *Cell. Mol. Life Sci.* **65**, 2688–2701 (2008).
7. Htet, M. et al. *Nat. Cardiovasc. Res.* https://doi.org/10.1038/s44161-024-00484-2 (2024).
8. Akazawa, H. & Komuro, I. *Circ. Res.* **92**, 1079–1088 (2003).
9. Saxton, R. A. & Sabatini, D. M. *Cell* **168**, 960–976 (2017).

Competing interests

The authors declare no competing interests.