Abnormal Accumulation of Reactive Oxygen Species from Pitx2 Mutation Could Cause HLHS

Anant Naik[1], Vikram Puram[1], Jack Hedberg[2]

[1]Department of Biomedical Engineering, [2]Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota-Twin Cities

Abstract

Hypoplastic Left Heart Syndrome (HLHS) is a highly rare congenital heart defect in which the left side of the heart is critically underdeveloped. At the cellular level, changes in ventricular structure include myocyte growth and myocyte apoptosis. Ultimately, this progressive remodeling can result in heart failure and, in HLHS, premature death. Recent progress in understanding these mechanisms of myocardial remodeling has led to evidence that reactive oxygen species (ROS) and oxidative stress play a central role in regulating the phenotype of cardiac myocytes.

We hypothesize that ROS-mediated developmental destabilization due to *pitx2* dysfunction in the left ventricle, coupled with the low and high strain placed on the interventricular septum (IV) and left ventricular (LV) wall during cardiac contraction, respectively, causes HLHS. Genetic tests of mouse models and *in vitro* studies of cardiomyocytes confirm this proposed progression. Further investigation could open the door to curative gene therapy and prevent many years of suffering and complications with surgical procedures for affected infants.

Background

Hypoplastic Left Heart Syndrome (HLHS) is a complex heart disease with a largely elusive underlying etiology. It is characterized by severe underdevelopment of left heart structures, particularly the left ventricle and the mitral and aortic valves, which are either completely atretic or abnormally small. The prevalence of HLHS is known to be around 0.02% of the national population (7), and since it is one of the most difficult congenital heart diseases to manage, 30% of affected newborns do not survive through adulthood (13).

In utero, prenatal diagnosis is often superior to post-birth electrocardiography, electrocardiograms, MRI, and cardiac catheterization due to the potential ability to attempt fetal cardiac intervention and plan in advance for effective delivery and immediate stabilization.

Since an underdeveloped left ventricle results in the breakdown of systemic blood circulation, newborns affected with HLHS require immediate surgical intervention for survival (1). The current standard of care involves the Norwood Procedure, a set of three surgical procedures, which aims to increase cardiac output and bypass the poorly functioning left heart by connecting the right ventricle with the ascending aorta.

Although genetics seem to play a large role, because the underlying cause remains elusive, interventions are not curative but rather only reduce the harmful impacts of the disease (14). Better understanding of the mechanistic elements that contribute to HLHS would allow for interventions with more promising objectives.

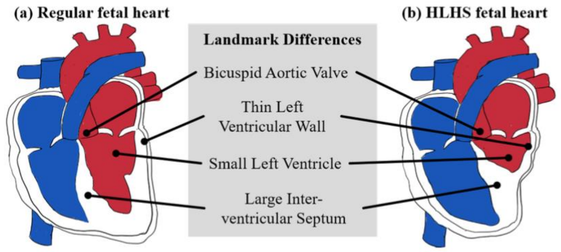

Figure 1. A juxtaposition of a (a) normal fetal heart to (b) an HLHS fetal heart. The landmark differences include a high comorbidity of a bicuspid aortic valve, thin left ventricular wall, small left ventricle, and large interventricular septum.

Hypothesis

The differential mechanical strain placed on tissues in the interventricular septum and in the left ventricular wall is potentially an important component necessary for ROS-mediated developmental destabilization to cause HLHS. In the presence of ROS, since cells in low strain undergo apoptosis while cells in high strain experience hyperplasia, we hypothesize that Pitx2 mutation-mediated ROS buildup in the left ventricle causes the simultaneous thickening of the IV septum and thinning of the LV wall observed in HLHS.

Rationale

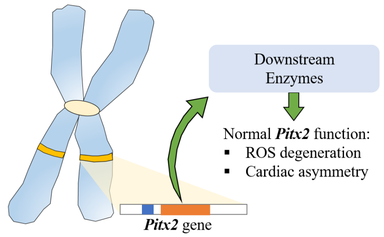

Figure 2: Our hypothesis regarding a decline in *Pitx2* functionality and ROS accumulation. The gene location of *Pitx2* on the long arm of human chromosome 4 encodes for Pitx2, a protein which acts as a transcription factor for enzymes that afford ROS degeneration and aid in cardiac asymmetry.

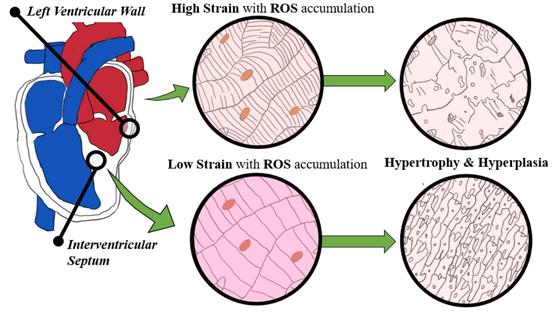

Figure 3: High strain coupled with ROS accumulation, like in that of the left ventricular wall, results in apoptosis and a thinning of the ventricular lining. Low strain coupled with ROS accumulation, like that in the IV septum, results in hypertrophy and hyperplasia, resulting in a thickening of the septal lining.

Pitx2 Regulates ROS Damage Protection

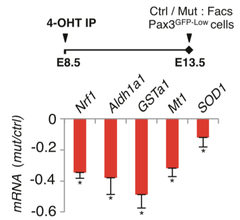

Figure 4. Mutant Pitx2/3 and control mouse embryos at E13.5 were obtained by Cre-Lox Recombination and 4-OHT intraperitoneal injection of pregnant mothers at E8.5 and used for isolation by flow cytometry of the $Pax3^{GFP\text{-}Low}$ population. qPCR was used to analyze antioxidant enzyme transcripts relative to GAPDH transcripts in control/mutant (Ctrl/Mut) cells, expressed as a log ratio, (n=2 embryos; mean ± SD, *p < 0.05) (L'honoré et al. 2014).

High Strain Conditions Afford Apoptosis

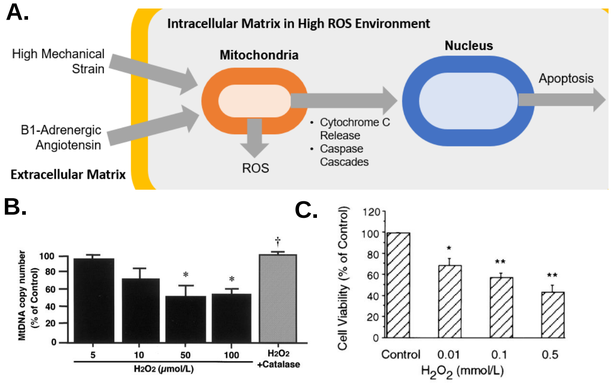

Figure 5. High strain conditions afford apoptosis. (a) Apoptotic stimuli are associated with an accumulation of ROS and appear to act via the mitochondrial pathway of cytochrome c release and caspase's cysteine protease activity, resulting in apoptotic cell death (Sawyer et. al. 2002). (b) Cardiomyocytes were exposed to increasing levels of H_2O_2, and mitochondrial RNA was monitored via qPCR. H_2O_2 exposure decreases the copy number of MtDNA (Sumatseu et al. 2002). (c) Effect of the H_2O_2 ROS on cardiac cells. Exposure leads to cell death through apoptosis as determined by cell-death detection ELISA (van Harsdorf et al. 1999).

Low Strain Conditions Afford Hypertrophy

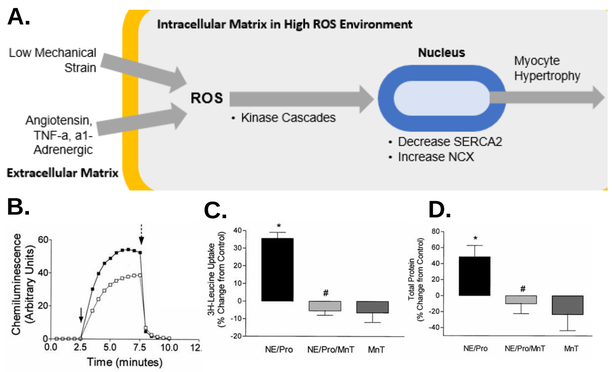

Figure 6. Low strain conditions afford hypertrophy. (a) Low mechanical strain and Angiotensin, TNF-a, or a1-Adrenergic stimulus affords ROS accumulation in the intracellular matrix. This stimulates kinase pathways that cause differential expression of regulatory factors, like SERCA2 and NCX, resulting in hypertrophy (Sawyer et. al. 2002). (b) Myocytes were exposed to norepinephrine without mechanical strain. A Chemiluminescence assay shows the significant increase in ROS in the cells. (c) An increase in ROS affords an increase in 3H-Leucine intake. (d) An increase in ROS affords an increase in total protein intake, suggesting hypertrophy. (Amin et. al. 2001).

Significance & Innovation

A pitx2 mutant cardiomyocyte cell line would be expected to accumulate ROS. Then, modalities like expansion microscopy and atomic force microscopy (AFM) could be used to apply a mechanical force to the differentiated cardiomyocytes.

To screen for potential Pitx2 interactions and transcriptional activities significant to Pitx2's role in the development of HLHS, immunoprecipitation assays would be performed on cells in ROS-abundant conditions using antibodies against Pitx2. After testing for potential binding candidates in Pitx2 pulldowns, we would utilize immunofluorescent microscopy to investigate the effect of ROS accumulation on the loci formation of Pitx2 and its potential ROS-dependent binding partners. Gene knockouts of these partners would then be performed in wild type backgrounds to investigate the effect of their interactions with Pitx2 on cellular growth and development.

The degree of Pitx2 mutations in cell lines could be varied such that a partial loss-of-function could be better modeled. ROS cultured in radioactive oxygen could also be artificially added to the mutated cells to measure the binding of the ROS to particular regions of the genome depending on the mechanical stress conditions.

The monitoring of birth complications of the offspring, examination of the heart of the offspring, and genomic analysis would verify the findings. This would give further credence to the idea that HLHS is linked to Pitx2 mutation. If the concentration of ROS are also found to be elevated in the left heart, along with the selective hyperplasia and apoptosis via immunostaining, it would support our hypothesis.

References

1. Barron DJ et al. Hypoplastic Left Heart Syndrome (2009). The Lancet, 374: 551-64.
2. Brandes, R., Maier, L. S., & Bers, D. M. (1998). Regulation of mitochondrial [NADH] by cytosolic [Ca2+] and work in trabeculae from hypertrophic and normal rat hearts. Circulation research, 82(11), 1189-1198.
3. Crespo, R., Wei, K., Rodenak-Kladniew, B., Mercola, M., Ruiz-Lozano, P., & Hurtado, C. (2017). Effect of geraniol on rat cardiomyocytes and its potential use as a cardioprotective natural compound. Life Sciences, 172, 8-12.
4. Heinzel, F. R., Luo, Y., Dodoni, G., Boengler, K., Petrat, F., Di Lisa, F., ... & Heusch, G. (2006). Formation of reactive oxygen species at increased contraction frequency in rat cardiomyocytes. Cardiovascular research, 71(2), 374-382.
5. L'honoré, A., Commère, P. H., Ouimette, J. F., Montarras, D., Drouin, J., & Buckingham, M. (2014). Redox regulation by Pitx2 and Pitx3 is critical for fetal myogenesis. Developmental cell, 29(4), 392-405.
6. Norwood, William I., Peter Lang, and Dolly D. Hansen. 1983. "Physiologic Repair of Aortic Atresia–Hypoplastic Left Heart Syndrome." New England Journal of Medicine 308 (1): 23–26. doi:10.1056/NEJM198301063080106.
7. Sawyer, D. B., & Colucci, W. S. (2000). Mitochondrial oxidative stress in heart failure.
8. Sawyer, D. B., Siwik, D. A., Xiao, L., Pimentel, D. R., Singh, K., & Colucci, W. S. (2002). Role of oxidative stress in myocardial hypertrophy and failure. Journal of molecular and cellular cardiology, 34(4), 379-388.
9. Suematsu, N., Tsutsui, H., Wen, J., Kang, D., Ikeuchi, M., Ide, T., ... & Takeshita, A. (2003). Oxidative stress mediates tumor necrosis factor-α–induced mitochondrial DNA damage and dysfunction in cardiac myocytes. Circulation, 107(10), 1418-1423.
10. Von Harsdorf, R., Li, P. F., & Dietz, R. (1999). Signaling pathways in reactive oxygen species–induced cardiomyocyte apoptosis. Circulation, 99(22), 2934-2941.
11. Von Harsdorf, Rüdiger, Pei-Feng Li, and Rainer Dietz. "Signaling pathways in reactive oxygen species–induced cardiomyocyte apoptosis." Circulation 99.22 (1999): 2934-2941.
12. Wen, J. J., Porter, C., & Garg, N. J. (2017). Inhibition of NFE2L2-ARE pathway by mitochondrial ROS contributes to development of cardiomyopathy and left ventricular dysfunction in Chagas disease. Antioxidants and Redox Signaling, (ja).
13. Xiao, Lei, et al. "Role of reactive oxygen species and NAD (P) H oxidase in α1-adrenoceptor signaling in adult rat cardiac myocytes." American Journal of Physiology-Cell Physiology 282.4 (2002): C926-C934.

Acknowledgments

We'd like to thank Dr. James V. Harmon for his mentorship. This work was motivated on the support from the IMPACT program at the Mayo Clinic, and from the lab of Dr. Tim Nelson at the Mayo Clinic.