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Calcium signaling regulates ventricular hypertrophy during development independent of contraction or blood flow☆

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ABSTRACT

In utero interventions aimed at restoring left ventricular hemodynamic forces in fetuses with prenatally diagnosed hypoplastic left heart syndrome failed to stimulate ventricular myocardial growth during gestation, suggesting chamber growth during development may not rely upon fluid forces. We therefore hypothesized that ventricular hypertrophy during development may depend upon fundamental Ca^{2+}-dependent growth pathways that function independent of hemodynamic forces. To test this hypothesis, zebrafish embryos were treated with inhibitors or activators of Ca^{2+} signaling in the presence or absence of contraction during the period of chamber development. Abolishment of contractile function alone in the setting of preserved Ca^{2+} signaling did not impair ventricular hypertrophy. In contrast, inhibition of L-type voltage-gated Ca^{2+} influx abolished contraction and led to reduced ventricular hypertrophy, whereas increasing L-type voltage-gated Ca^{2+} influx led to enhanced ventricular hypertrophy in either the presence or absence of contraction. Similarly, inhibition of the downstream Ca^{2+}-sensitive phosphatase calcineurin, a known regulator of adult cardiac hypertrophy, led to reduced ventricular hypertrophy in the presence or absence of contraction, whereas hypertrophy was rescued in the absence of L-type voltage-gated Ca^{2+} influx and contraction by expression of a constitutively active calcineurin. These data suggest that ventricular cardiomyocyte hypertrophy during chamber formation is dependent upon Ca^{2+} signaling pathways that are unaffected by heart function or hemodynamic forces. Disruption of Ca^{2+}-dependent hypertrophy during heart development may therefore represent one mechanism for impaired chamber formation that is not related to impaired blood flow.

1. Introduction

Hypoplastic left heart syndrome (HLHS) is a devastating congenital heart malformation that accounts for approximately 25% of cardiac deaths within the first year of life [1]. The cardinal feature of HLHS is a small, underdeveloped left ventricle that is unable to support the systemic circulation. In addition, restrictive flow defects of the aorta, aortic valve, and/or mitral valve are also commonly present, leading to reduced blood flow through the developing ventricle [2]. The combination of restricted ventricular blood flow and impaired ventricular growth led to the classic belief that the primary embryologic insult in HLHS was the formation of a left-sided flow limiting lesion, and ventricular underdevelopment was thought to be a secondary consequence of reduced ventricular blood flow [3,4]. This pathogenetic model spawned the design of fetal interventions for HLHS, based on the hypothesis that correction of left ventricular flow dynamics during development would alleviate the harmful effects of altered blood flow on the maturing myocardium and rescue left ventricular growth [4,5]. However, human fetal interventions aimed at relieving outflow obstruction in select fetuses with aortic stenosis and evolving HLHS failed to improve left ventricular growth during gestation, suggesting the myocardial growth defect in HLHS may not depend upon altered blood flow or hemodynamic forces [5]. Alternative explanations for the ventricular chamber defect in HLHS are therefore warranted.

The morphologic period of chamber formation occurs between 22 and 28 days after fertilization in the human when the primordial heart tube loops and balloons into a structure with expanded precursor

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chambers. This transformation is initiated by regional increases in the volume of outer curvature ventricular cardiomyocytes, followed by increases in cellular proliferation [6]. Thus, coordinated increases in cell size, termed 'hypertrophy' represent the first necessary prerequisite for chamber expansion. At the cellular level, ventricular cardiomyocytes from HLHS hearts appear disorganized with scant cytoplasm and are reduced in size and number, suggesting that they suffer from developmental defects in both hypertrophy and proliferation [7]. Given that hypertrophy precedes proliferation during chamber expansion, the chamber defect observed in HLHS may be caused by a primary error in developmental hypertrophy which then disrupts the downstream sequence of cellular proliferation and chamber growth.

The mechanisms of normal cardiomyocyte hypertrophy during development remain poorly understood. However, the mechanisms of pathologic pressure overload hypertrophy have been carefully examined in adults and have been found to result from alterations in Ca^{2+} signaling pathways that lead to activation of quiescent growth pathways [8]. The dominant growth pathway identified in the adult heart is the Ca^{2+}/calcineurin pathway which culminates in an increase in cardiac mass through re-expression of genes associated with fetal hearts [9]. Despite the emphasis placed on the re-expression of the fetal gene program by hypertrophic adult myocardium [10], little is known about the cellular and molecular biology of normal myocardial growth during embryonic development. However, in support of conservation of growth pathways between adult and fetal hearts, prior studies have shown reduction in components of the Ca^{2+}/calcineurin pathway in tissue from HLHS hearts [11], as well as impaired chamber formation in hearts lacking L-type voltage-gated Ca^{2+} entry [12]. We therefore hypothesized that the dominant Ca^{2+} signaling pathways active during pathological hypertrophy in adult cardiomyocytes also regulate developmental hypertrophy in embryonic cardiomyocytes. We further predicted that these growth pathways were not directly modulated by hemodynamic forces or blood flow given the apparent unresponsiveness of HLHS myocardium to these stimuli.

In cardiac muscle, variations in intracellular Ca^{2+} are involved in electromechanical coupling, leading to heart contraction and blood flow, and also serve as signaling mediators, leading to alterations in gene transcription. In most organisms, the functional and signaling roles of Ca^{2+} in the heart are unable to be dissociated given the requirement of heart function for survival. Zebrafish represent ideal organisms for studying the interaction between Ca^{2+}-dependent growth pathways and hemodynamic forces, as embryos can survive through all stages of heart development without the requirement for blood flow [13]. We therefore established a zebrafish model to define the role of Ca^{2+} signaling in developmental myocardial hypertrophy in the ventricle uncoupled from heart function and fluid forces.

2. Methods

2.1. Zebrafish strains

Zebrafish (*Danio rerio*) strains utilized included Tübingen wild-type, *Tg*(*cmlc::DsRed2-nuc*) [14] (kindly provided by Dr Kenneth Poss, Duke University, with permission from Dr C. Geoffrey Burns, Harvard Medical School), *Tg*(*cmlc2::GFP*) [15] (kindly provided by Dr Deborah Yelon, UC San Diego, with permission from Dr Huai-Jen Tsai, National Taiwan University), and *Tg*(*Flk1::EGFP*)^{s843} [16] (kindly provided by Dr Kenneth Poss with permission from Dr Suk-Won Jin, University of North Carolina). Zebrafish were maintained following published protocols [17]. All zebrafish experiments were approved by the Institutional Animal Care and Use Committee of Duke University.

2.2. Pharmacology and drugs

Blebbistatin (5 uM), nisoldipine (10 uM), cyclosporine A (CsA, 10 μg/ml), FK506 (1 μg/ml), and BayK8644 (20 uM) (all from Sigma-Aldrich, St. Louis, MO) were dissolved in DMSO and diluted to final working concentration in embryo media without antibiotics. Embryos (24 hpf) were chemically and manually dechorionated using pronase. Drug solutions were applied to embryos at 24 hpf and reapplied every 6 h during the period of drug treatment. Media containing an equivalent volume of DMSO was used as control. Embryos were incubated at 28.5 °C in the dark to prevent light degradation of drugs.

2.3. Antisense morpholino knockdown and RNA rescue analysis

Morpholino oligonucleotides (Gene Tools, Philomath, OR) were diluted and injected at 2–4 ng per embryo at the one cell stage. The *tnnt2* morpholino (CATGTTTGCTCTGATCTGACACGCA) was used as previously described [18]. A cocktail of two morpholino oligonucleotides against *cacna1c* ([CCCGTTCCTAGACAGACGAAACAGA] and [GGATCTTG CACTCACCTACGAACCA]) was used as previously described [19]. Gene Tools standard control morpholino (CCTCTTACCTCAGTTACAATTTATA) was used as negative control. cRNA rescue constructs were coinjected with the *cacna1c* morpholinos at 800 pg per embryo as previously described [19]. Rescue constructs used were wild type *cacna1c* ($Ca_V1.2^{WT}$ cRNA), Timothy Syndrome *cacna1c* ($Ca_V1.2^{TS}$ cRNA), and constitutively active calcineurin (caCN cRNA).

2.4. Video recording

Tg(*cmlc2::GFP*) embryos treated with drugs or morpholinos were anesthetized in 0.016% tricaine at 48 hpf and imaged using a Leica DM RAZ microscope equipped with a fluorescence imaging system. Videos were captured using a standard CCD camera at 20 frames/s.

2.5. Immunohistochemistry

For experiments to determine cardiomyocyte cell volume, *Tg*(*cmlc::DsRed2-nuc*) embryos were stained with anti-DsRed polyclonal antibody and anti-Zn5 monoclonal antibody as previously described [20]. This protocol allowed visualization of cardiomyocyte borders in green and nuclei in red. For experiments to determine endocardial development, *Tg*(*Flk1::EGFP*)^{s843} embryos were stained with anti-GFP polyclonal antibody and anti-MF20 antibody as previously described [20]. This protocol allowed visualization of myocardium in red and endocardium in green.

2.6. Cell volume measurements

Antibody stained embryos were embedded in 4% low melt agarose. A vibratome was used to create 50 μm floating sections taken perpendicular to the long-axis of the embryo. The section containing all or most of the heart was identified using fluorescence microscopy and mounted for confocal microscopy. Three dimensional Z-stacks of the hearts were generated using a Zeiss LSM510 confocal microscope with a 40× objective. ImageJ software (NIH) was then used to calculate the volume of select cardiomyocytes. Cells were chosen for measurement only when their outlines were clearly visible within the xy plane of view. For cell measurements at the 24 hpf stage, outflow tract cells that will contribute to the future ventricle were measured. Given that myocardial cells at the 24 hpf stage possess a rounded morphology [21,22], cell volume was estimated by measuring the radial length and width of the cell in the xy plane and the radial height of the cell in the z plane. The volume of an ellipsoid was then calculated using the three elliptic radii with the equation $4 / 3\pi Rx * Ry * Rz$. For cell measurements at the 48 hpf stage, outer curvature ventricular cardiomyocytes were measured following the period of volume expansion. Given that outer curvature ventricular cardiomyocytes at the 48 hpf stage possess a flattened and elongated morphology [21,22], cell volume was estimated by treating the cells as thin cuboidal sheets and multiplying the cross-sectional area of the cell by the thickness of the