In-Vivo model of endocardial fibroelastosis

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Introduction

Hypoplastic left heart syndrome

Congenital heart defects stand as the most prevalent anomalies observed during birth, afflicting approximately 8 out of every 1000 live births and standing as the foremost cause of birth defect-related fatalities. Among these congenital heart defects, hypoplastic left heart syndrome (HLHS) claims the majority of infant deaths marked by heart defects. Hypoplastic left heart syndrome manifests as a condition marked by profound underdevelopment of the left-sided structures of the heart during birth. This encompasses the left ventricle, mitral valve, and aortic valve. The International Society has provided a precise definition of hypoplastic left heart syndrome within the Nomenclature of Paediatric and Congenital Heart Disease, characterizing it as "*a spectrum of cardiac malformations with normally aligned great arteries without a common atrioventricular junction, characterized by underdevelopment of the left heart with significant hypoplasia of the left ventricle including atresia, stenosis, or hypoplasia of the aortic or mitral valve, or both valves, and hypoplasia of the ascending aorta and aortic arch*" (1).

It is noteworthy that these heart defects often occur in isolation, with no concurrent impact on other organ systems. However, the long-term ramifications, and frequently, lifelong consequences of these cardiac anomalies are substantial. Intriguingly, the root causes of these congenital heart diseases remain elusive, which significantly constrains therapeutic interventions. In practice, treatment options predominantly revolve around addressing the consequences and sequelae of the disease rather than targeting the elusive origins. Many of these congenital heart diseases persist without a definitive cure, and patients must rely on palliative surgeries performed after birth to manage their condition. However, there is cause for optimism, as clinical advancements in the management of hypoplastic left heart syndrome have evolved from a purely palliative approach to one that envisions a curative goal. This transformative shift has been achieved through innovative approaches, such as fetal catheter intervention involving the dilation of the aortic valve in conjunction with post-natal rehabilitation surgery (2–4).

Historically, individuals diagnosed with hypoplastic left heart syndrome faced the grim prospect of only univentricular circulation palliation as a means of survival. This univentricular palliation is achieved through a complex and staged surgical procedure, comprising three distinct operations. The initial operation is performed within the first few days following birth

and encompasses an atrioseptectomy, separating the pulmonary trunk from the ventricle, and creating a neo-aorta. Additionally, establishing a connection between the systemic and pulmonary circulations is paramount. The Norwood operation, combined with a modified Blalock-Taussig (BT) shunt, creates a connection using the brachiocephalic trunk or the right subclavian artery to link to the pulmonary artery. A Sano shunt has become the more common choice, offering a secure connection between the systemic and pulmonary circulations. The second stage unfolds as a Glenn or Hemi-Fontan procedure, typically performed between 4 to 6 months of age. During this stage, the shunt (BT or Sano) that links the systemic and pulmonary circulations is removed, and an anastomosis is forged between the superior vena cava and the pulmonary artery. The culmination of these stages adheres to the Fontan principle, resulting in the total cavopulmonary connection (TCPC) around the age of 2 to 3 years. The total cavopulmonary connection can be established through two approaches for anastomosing the inferior vena cava to the pulmonary artery. One method involves creating an intracardiac tunnel, while the other employs an extracardiac conduit for the connection. Both approaches can incorporate fenestration to the right atrium, mitigating arterial pressure increases in the pulmonary circulation. This fenestration can be subsequently closed through interventional procedures at a later stage (5).

While survival rates following surgical repair have witnessed continuous improvement, the cumulative mortality rate for patients subjected to the three-stage surgical palliation remains around 35% at the 5-year mark and further escalates to 45% at the 10-year juncture (6,7). Additionally, patients in this cohort must contend with a univentricular circulation primarily supported by the anatomically right ventricle. This reliance on the right ventricle presents a litany of long-term issues, including thromboembolic events, arrhythmias, plastic bronchitis, and protein-loss enteropathy, as extensively documented (8).

In the modern landscape of pediatric cardiology and cardiac surgery, there has been a remarkable emergence of pioneering and curative approaches aimed at addressing complex congenital heart conditions. Among these groundbreaking advancements, one particularly noteworthy initiative has been spearheaded by the highly esteemed team at Boston Children's Hospital. This innovative strategy represents a significant paradigm shift in the management of a select group of patients afflicted with hypoplastic left heart syndrome, a challenging congenital heart defect. The revolutionary approach developed by the Boston Children's Hospital team lies in the preservation and nurturing of the left ventricle's growth potential. This is achieved through a thoughtfully designed and multifaceted, multi-step process. The multi-

step process is a highly coordinated and precisely executed sequence of interventions. It involves a combination of surgical procedures, interventional techniques, and medical management that are customized to each patient's unique condition. The overarching goal of this process is to gradually expand the underdeveloped left ventricle, stimulate its growth, and ultimately enable it to take on a more substantial role in the circulation of oxygen-rich blood. The ultimate objective of this pioneering approach is nothing short of transformative: to establish a biventricular circulation. In simpler terms, it aims to reconfigure the heart's anatomy and function so that both the left and right ventricles work in concert to pump blood effectively, mimicking the natural state of a healthy heart. This represents a profound departure from traditional palliative approaches and holds the potential to offer these select hypoplastic left heart syndrome patients a chance at a more normal and fulfilling life (9–12).

Endocardial fibroelastosis

One crucial factor that has been identified as limiting the growth potential of the left ventricle (LV) is the presence of a thick layer of cellular fibroelastic tissue that envelops the myocardium of the left ventricle. This specific tissue has been designated as endocardial fibroelastosis (EFE) (13). The initial description of endocardial fibroelastosis dates back to 1943 when it was characterized as the diffuse thickening of the endocardium (14).

Endocardial fibroelastosis manifests as a substantial subendocardial tissue conglomerate, primarily composed of collagen and elastic fibers, albeit devoid of microvasculature. This tissue layer exhibits a notable thickness exceeding 20 micrometers (15). It's important to note that endocardial fibroelastosis can be categorized into two main forms: a primary form, which occurs independently of any heart malformations, and a secondary form that is associated with congenital heart malformations (14).

Until recently, there was limited knowledge regarding the origins of endocardial fibroelastosis or the specific stimuli that trigger the excessive growth of this tissue within the heart. The understanding of the etiology and pathogenesis of endocardial fibroelastosis has remained a subject of active research and investigation, with ongoing efforts to elucidate the factors and mechanisms that contribute to its development.

Endothelial -to- mesenchymal transition

Through extensive investigations of pathological specimens, it has come to light that endocardial fibroelastosis is a remarkably prevalent occurrence, being present in a striking 70% of all individuals diagnosed with hypoplastic left heart syndrome. In the most severe instances, this enigmatic condition engulfs the entire left ventricle in a dense, white layer of fibroelastic tissue. A prevailing hypothesis suggests that this conspicuous layer exerts a constraining influence on the growth of the ventricular chamber. However, at a point in time when the origins and causative factors underlying endocardial fibroelastosis formation remained enigmatic, Dr. Friehs and her exceptional team at Boston Children's Hospital embarked on an ambitious and pioneering investigative journey. Their exploration led them to examine endocardial fibroelastosis tissue samples procured through resections performed during what was termed "ventricular rehabilitation surgery." This innovative surgical procedure had been pioneered at Boston Children's Hospital with the fundamental premise that the removal of endocardial fibroelastosis would serve to alleviate the constrictive physiological effects and, in doing so, promote the growth of the left ventricle. The ability to access endocardial fibroelastosis tissue through this surgical intervention opened up a realm of possibilities for the scientific community. It enabled researchers to delve deep into the cellular origin of endocardial fibroelastosis, identify potential triggers for its formation, and contemplate novel therapeutic interventions.

Crucially, the research endeavors unveiled that while endocardial fibroelastosis resection yielded favorable outcomes in terms of left ventricular growth and the achievement of successful biventricular repair in certain patients, it did not prove equally effective for all cases. In some instances, endocardial fibroelastosis regrowth emerged as a formidable and recurring obstacle. The precise determinants of this variability still elude complete elucidation, serving as a compelling enigma that ongoing investigations seek to decipher. These studies are geared towards illuminating the hitherto concealed triggers responsible for the formation of endocardial fibroelastosis, thereby bringing us closer to a comprehensive understanding of this perplexing cardiac condition. Subsequent investigations led to a pivotal revelation: endocardial fibroelastosis originates from endocardial endothelial cells undergoing a profound phenotypic transformation, transitioning into mesenchymal cells. This intricate process is well-known in scientific parlance as endothelial -to- mesenchymal transition (EndMT) (16). The diagnosis of endothelial -to- mesenchymal transition hinges on meticulous immunohistological staining techniques targeting specific cellular markers. Specifically, scientists employ endothelial cell

markers such as CD31 or vascular endothelial cadherin (VE-cadherin), in conjunction with mesenchymal markers like alpha smooth muscle actin (αSMA). When individual endothelial cells simultaneously manifest staining for both these marker types, it serves as a telltale sign that endothelial -to- mesenchymal transition has taken place (16).

Endothelial -to- mesenchymal transition is a well-established biological mechanism, and it plays a pivotal role during the embryonic development of critical cardiac structures such as heart valves and the non-muscular septum. This intricate process falls primarily under the regulation of the transforming growth factor beta/bone morphogenic protein (TGFβ/BMP) growth factor family (17). However, it is important to note that endothelial -to- mesenchymal transition is not limited to embryonic development but also extends its influence to various pathological processes, including cardiac fibrosis and hypertrophy (18). The multifaceted nature of endothelial -to- mesenchymal transition underscores its significance in both normal cardiac development and the context of cardiac diseases.

The transition of endothelial cells to mesenchymal cells, characterized by the loss of endothelial cell markers and the acquisition of mesenchymal properties, is orchestrated by transcription factors like SNAIL, TWIST, or SLUG. These transcription factors actively suppress the expression of vascular endothelial cadherin (VE-cadherin), a key molecule responsible for cell-cell adhesion among endothelial cells. As a result of this suppression, endothelial cells undergo structural changes, including the loss of cell-cell contacts, leading to a shift toward a more migratory behavior, which is a defining characteristic of mesenchymal cells. In the context of the heart, this process is regulated by the transforming growth factor beta/bone morphogenic protein (TGFβ/BMP) family of growth factors, which also plays a crucial role in driving endothelial -to- mesenchymal transition.

In their research, Dr. Friehs and her team uncovered a unique regulatory mechanism related to endothelial -to- mesenchymal transition and endocardial fibroelastosis formation. They found that endocardial fibroelastosis tissue samples from hypoplastic left heart syndrome patients exhibited hypermethylation of the bone morphogenic protein (BMP) promoter. This epigenetic modification results in an imbalance in signaling pathways, favoring increased activity of the transforming growth factor beta (TGFβ) signaling pathway (13). This discovery sheds light on the intricate molecular processes involved in endocardial fibroelastosis development and provides a potential target for future therapeutic interventions to mitigate or prevent endocardial fibroelastosis formation in individuals with hypoplastic left heart syndrome.

In addition to the primary form of endocardial fibroelastosis not associated with congenital heart defects, it has been established that a secondary form of endocardial fibroelastosis can develop in response to various external stimuli. These stimuli include inflammation, hypoxia (insufficient oxygen supply), ischemia (restriction of blood flow), and mechanical factors. Among these potential triggers, mechanical factors, particularly those associated with hypoplastic left heart syndrome, have been proposed by the research group at Boston Children's Hospital and are supported by clinical observations (19). The mechanical factors in hypoplastic left heart syndrome, which can contribute to the development of secondary endocardial fibroelastosis, are likely associated with the abnormal hemodynamic conditions characteristic of this congenital heart defect. These conditions involve altered blood flow patterns and increased pressure on specific areas of the heart. These mechanical stresses on the endocardium, the inner lining of the heart chambers, may induce the formation of endocardial fibroelastosis as a response to these unfavorable conditions. Collectively, these research findings and clinical observations deepen our understanding of the intricate mechanisms involved in endocardial fibroelastosis formation. They underscore the multifactorial nature of this condition, where both congenital heart defects and external stimuli can contribute to its development. This growing knowledge base holds promise for the development of targeted interventions and therapies in the future, aimed at preventing or mitigating endocardial fibroelastosis in individuals, particularly those with hypoplastic left heart syndrome.

To investigate the intricate mechanism underlying the formation of endocardial fibroelastosis, a specialized animal model was developed, carefully designed to encompass the critical factors observed in endocardial fibroelastosis formation in human cases. These factors include both the developmental immaturity of the cardiovascular system and alterations in blood flow patterns, particularly relevant to the hemodynamic conditions seen in hypoplastic left heart syndrome. Previous attempts to directly replicate the hemodynamic conditions of hypoplastic left heart syndrome had proven unsuccessful, prompting the creation of this animal model specifically tailored to investigate endocardial fibroelastosis formation. The two main aspects addressed in this model were immaturity, achieved by utilizing newborn rats that are still developmentally akin to the fetal stage, and the manipulation of blood flow through modifications to the heterotopic heart transplantation model (20–22).

While there is a strong suspicion that hemodynamic alterations play a pivotal role in triggering endocardial fibroelastosis formation by inducing endothelial -to- mesenchymal transition, a direct causative relationship between these factors and endocardial fibroelastosis development

remains elusive. With the establishment of this animal model, researchers can now delve into a more comprehensive examination of flow alterations in a live, in vivo setting. This entails the implementation of essential imaging techniques, such as, to more accurately scrutinize localized flow disturbances. Furthermore, researchers aim to measure the associated induction of endothelial -to- mesenchymal transition and delve into the regulatory pathways governing this transition. Ultimately, the animal model will serve as a valuable tool for testing potential treatments aimed at directly targeting flow-induced pathways and intermediaries. These potential treatments are currently being identified in the research laboratory led by Dr. Friehs at Boston Children's Hospital, paving the way for innovative approaches to mitigate or prevent endocardial fibroelastosis formation in individuals with hypoplastic left heart syndrome.

Material and Methods

In this particular study, our focus was exclusively on utilizing the unloaded animal model, although it's worth noting that various flow conditions have been previously documented for this particular model. Our primary objective was to establish the ideal conditions for inducing the formation of endocardial fibroelastosis and to achieve this, we specifically opted for the unloaded condition, a well-documented approach in our previous studies (17,18,20,23).

The ethical treatment and care of all animals involved in our research were of paramount importance. All animals received the highest standard of humane care and were provided for by the dedicated Animal Resources team at Boston Children's Hospital. To ensure that our research adhered to rigorous ethical and regulatory standards, all protocols were meticulously reviewed and subsequently approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital. Our IACUC-approved protocol bore the identifier #00001695, serving as a testament to our commitment to upholding the highest ethical standards in animal research.

In our pursuit of maintaining the utmost sterility and safety during surgical procedures, all surgical equipment underwent rigorous sterilization. This involved subjecting the surgical equipment to thorough steam autoclaving before each and every surgical intervention. This rigorous sterilization process was an essential component of our efforts to ensure the health and well-being of the experimental animals and the integrity of our research outcomes.

Donors heart explantation

In our experimental approach, neonatal Lewis rat hearts were chosen as the donor organs due to their unique characteristics. These hearts were sourced from rats aged 1 to 3 days, with a corresponding weight range of 8 to 12 grams. The rationale behind this selection was the immaturity of these hearts, which rendered them particularly susceptible to the development of endocardial fibroelastosis, the condition of interest in our research.

To ensure optimal conditions during the procedure, neonatal rats were administered a dose of 300 U/kg of heparin at least 15 minutes prior to the heart extraction. This preemptive heparin administration was essential to prevent any undesired blood clotting during the subsequent steps of the surgical process.

For anesthesia induction, the rats were placed within an isoflurane chamber containing a 2% concentration of isoflurane, initiating a state of anesthesia. Additionally, intraperitoneal injections of ketamine (75 mg/kg) and xylazine (5 mg/kg) were administered. The adequacy of anesthesia was assessed through the absence of the inter-toe reflex, serving as a reliable indicator of sufficient anesthetic depth.

The surgical preparation began with meticulous attention to aseptic techniques. The rat was positioned in a supine manner, and the surgical field underwent a rigorous disinfection process. Alternating applications of betadine and 70% ethanol were meticulously applied three times to ensure a sterile environment for the upcoming procedure.

Subsequently, the surgical procedure was initiated by excising the frontal thoracic wall. This was achieved through a horizontal incision made at the plane of the diaphragm, along with two vertical incisions at the anterior axillary line. Finally, a second horizontal incision was made at the superior thoracic opening to entirely remove the frontal rib cage. This surgical approach exposed the heart and its associated vessels for further manipulation.

To prepare for heart explantation, several 7-0 silk sutures were strategically placed around the vessels without immediate closure. Two silk sutures were encircled around the inferior vena cava, the left and right superior vena cava, inclusive of the vena azygos. Additionally, one 7-0 silk suture each was placed around the right and left pulmonary hiatuses.

Following this preparation, all sutures were closed except for the proximal sutures surrounding the inferior vena cava.

In preparation for the delicate transplantation process, an iced-cold modified high-potassium Krebs-Henseleit solution was meticulously prepared a day prior to the procedure. This specialized cardioplegic solution consisted of specific concentrations of sodium chloride (NaCl, 118 mmol/l), potassium chloride (KCl, 25 mmol/l), potassium dihydrogen phosphate (KH2PO4 1.2 mmol/l), magnesium sulfate (MgSO4 1.2 mmol/l), hydrogen bicarbonate (NaHCO3, 25 mmol/l), glucose (11 mmol/l, and calcium chloride (CaCl2, 2.5 mmol/l). The iced-cold cardioplegic solution was then introduced into the inferior vena cava using a 30G needle. This solution served a dual purpose: it effectively flushed the heart and the arresting its natural beating rhythm to preserve its function during the subsequent phases of the procedure. Once this was accomplished, the last remaining proximal suture around the inferior vena cava was securely closed.

Continuing with the procedure, the inferior vena cava, along with the left and right superior vena cava and the vena azygos, was meticulously sectioned. Subsequently, the right and left lungs were carefully removed using scissors, while positioned adjacent to the closed silk suture. The ascending aorta and aortic arch were isolated from the surrounding tissue and the aorta was carefully excised, ensuring precision at the level just beyond the first outgoing branch, known as the truncus brachiocephalicus. Finally, the pulmonary trunk was dissected free from the surrounding tissue, extending all the way to both pulmonary arteries, providing ample tissue for the upcoming anastomosis.

The donor heart, hereafter referred to simply as the "Htx heart," was then immersed in the ice-cold high-potassium Krebs-Henseleit solution to maintain its viability and functionality until the implantation phase of the procedure. This meticulous series of steps and preparations laid the groundwork for the subsequent transplantation and research objectives of our study.

Recipient heart implantation

In our study, young adult male Lewis rats, weighing approximately 150 grams and aged around 5 to 6 weeks, were selected as the recipients for the heart transplantation procedure. Just as with the neonatal rats, meticulous attention was given to ensuring appropriate anesthesia and surgical preparation.

Anesthesia Procedure: The anesthesia protocol mirrored that employed for the neonatal rats. Initially, the recipient rat was placed within an anesthesia chamber containing 2% isoflurane to induce anesthesia. Additionally, the rat received intraperitoneal injections of heparin (300 U/kg), ketamine (75 mg/kg), and xylazine (5 mg/kg) to maintain the anesthetic state. An essential indicator of sufficient anesthesia was the absence of the inter-toe reflex, confirming the depth of anesthesia.

Intubation: Following the induction of anesthesia, intubation was performed by inserting an 18G cannula into the rat's trache. The intubation process was carefully guided from outside the neck, utilizing the front teeth as reference points and skillfully maneuvering the tongue to facilitate the procedure. Once intubation was successful, the rat was transferred to the surgical table, and the intubation cannula was connected to a small animal ventilator. The ventilator provided a controlled oxygen supply, supplemented with 0.5-1% isoflurane. Ventilation settings were adjusted to ensure optimal respiratory parameters, including a respiratory rate of 55 to 60 breaths per minute, an inspiratory to expiratory ratio of 40%, and a tidal volume tailored to the individual weight of the rat.

Surgical Preparation: With the rat in a supine position on a heating mat to maintain body temperature, the abdominal surgical field was meticulously shaved and sterilized. A triple sterilization process was employed, alternating between betadine and 70% ethanol applications three times to create a sterile surgical environment. Subsequently, a midline laparotomy was performed to access the abdominal cavity, and the intestine was gently retracted from the surgical field and covered in warm saline-soaked gauze.

Vessel Dissection and Clamping: The infrarenal recipient's inferior vena cava and aorta were carefully dissected to enhance accessibility for the subsequent vascular anastomosis. When necessary, side branches were ligated with 7-0 silk sutures to ensure a controlled surgical field. Following the precise surgical procedure, the next crucial step involved the careful placement of clamps on either side of the designated anastomosis area within the blood vessels. These clamps served as instrumental tools to temporarily occlude the vessels, effectively halting the flow of blood in preparation for the upcoming anastomosis. The clamps were strategically chosen and sized to match the diameter of the vessels, ensuring a snug and secure fit without causing any undue damage or compression. This delicate maneuver required utmost precision,

as the temporary cessation of blood flow was essential to create a controlled environment for the subsequent surgical tasks

Anastomosis Procedure: The transplantation procedure commenced with the veno-venous anastomosis. An incision was made in the recipient's inferior vena cava, and the vessel was meticulously flushed with either saline or heparinized saline solution. The donor's heart was then introduced into the recipient's body, and the pulmonary trunk was adjusted to ensure a seamless anastomosis with the inferior vena cava. The venous anastomosis was executed using two running 11-0 nylon sutures, initiated from the proximal to distal ends on each side.

For the aorta-aortal anastomosis, the donor's aorta was appropriately shortened to match the length required for the venous anastomosis. A similar incision was made in the recipient's aorta, followed by flushing with saline or heparinized saline. The anastomosis was accomplished using 10-0 nylon single stitches, approximately 8 to 10 stitches in total.

Clamp Removal and Assessment: After completing all anastomoses, the distal clamps were carefully removed, allowing observation of the filling of the coronary vessels within the donor's heart. Ideally, the heart would resume beating at this point. The anastomoses were scrutinized for any signs of inadequate suturing. If any deficiencies were detected, the distal clamps were reinstalled, additional single sutures were applied, and the anastomoses were inspected again. Once confidence in the anastomoses' integrity was established, the proximal clamps were removed as well. With an increase in blood pressure, the anastomoses were monitored for several seconds to ensure their stability.

Closure and Recovery: To conclude the surgical procedure, the intestines were gently placed back into the abdominal cavity, ensuring they did not interfere with the implanted heart and were free from any twisting. The abdominal wall was closed using a 5-0 vicryl running suture, and the skin was meticulously sutured with an intradermal 6-0 vicryl running suture.

Awakening and Postoperative Care: The isoflurane was gradually turned off to facilitate the rat's awakening. After observing initial movements, ventilation was suspended, and the rat's breathing was closely monitored. In cases where spontaneous breathing was insufficient, ventilation was briefly reinitiated. Subsequently, the intubation tube was removed, and the rat was carefully transferred to an individual housing cage. Adequate postoperative pain management was administered, involving buprenorphine and meloxicam to ensure the rat's comfort and well-being during the recovery period.

This comprehensive protocol outlined the surgical steps and considerations undertaken to successfully perform heart transplantation in young adult male Lewis rats (24). The stringent attention to detail and postoperative care ensured the best possible outcome for both the research objectives and the well-being of the experimental animals.

Echocardiographic assessment

The assessment of flow dynamics, left ventricular morphology, and contractile function in hearts subjected to heterotopic transplantation (Htx heart), as well as the diagnosis of localized endocardial fibroelastosis, was carried out through meticulous echocardiographic examinations. These echocardiographs were conducted following established protocols (22,25) to ensure accuracy and consistency. To provide a brief overview of the procedure, rats were gently anesthetized using 1-2% isoflurane and positioned in a supine manner on a temperature-controlled heating platform, meticulously maintaining their body temperature at a precise 37°C. Electrocardiogram leads were thoughtfully connected through the animal's paws to monitor cardiac electrical activity throughout the assessment.

The transabdominal echocardiography images were acquired postoperatively using the state-of-the-art Vevo 3100 Imaging system developed by VisualSonics Inc. in Canada. This advanced system was equipped with a high-frequency 40 MHz linear array transducer (MS550D, VisualSonics Inc., Canada), ensuring exceptional image resolution and clarity.

The evaluation of blood flow within the cardiac chambers was performed using Doppler echocardiography, which encompassed spectral Doppler time-velocity measurements and color flow Doppler imaging. Spectral Doppler allowed for the precise quantification of blood flow velocities, offering valuable insights into the hemodynamic performance of the transplanted hearts. Meanwhile, color flow Doppler provided a visual representation of blood flow patterns, enabling the semiquantitative assessment of blood flow characteristics at specific regions of interest within the cardiac chambers. This comprehensive echocardiographic approach facilitated a thorough examination of both structural and functional aspects of the Htx hearts, ultimately contributing to a comprehensive understanding of their cardiovascular health and performance.

Modified Stanford Score for Graft Viability

The modified Stanford Score for graft viability, as described by Blanchard and Pollak (26), serves as a crucial postoperative evaluation tool for assessing the health and performance of a transplanted heart. This scale, ranging from 0 to 4, provides a comprehensive framework for researcher to gauge the condition of the transplanted organ. Here's an elaboration of the score's categories and their respective meanings:

0 – No Organ Function Detectable: In this category, there is no discernible organ function either visually or through palpation. The heart does not exhibit any signs of activity, and its function is completely absent.

1 – Minimal Organ Function Observable: At this stage, there is some residual organ function, albeit not palpable through touch. These subtle signs of function are detectable only under the scrutiny of a microscope or echocardiographically, emphasizing the delicate nature of the recovery process.

2 – Partial Organ Function Visible and Palpable, but Weak: Category 2 signifies the presence of (partial) organ function that is macroscopically visible to the naked eye and can also be felt through palpation. However, this function remains notably feeble and lacks the robustness typically associated with a healthy organ.

3 – Clear Organ Function with Reduced Intensity or Frequency: In this category, there is a clear indication of organ function, characterized by regular and recognizable activity. However, this function exhibits reduced intensity or frequency compared to the expected norm. It represents a level of recovery but is not yet fully optimal.

4 – Optimal Contraction with Heart Rate Between 120 and 160 BPM**:** Category 4 represents an encouraging state of graft viability. At this stage, the transplanted heart displays optimal contraction, leading to a heart rate falling within the range of 120 to 160 beats per minute. This level of functionality suggests a robust and healthy cardiac performance, approaching the ideal state for a transplanted heart.

Histological and molecular biological analysis

Histological analysis of collagen and elastin content was conducted on cryo sections embedded in optimal cutting temperature compound (OCT). Two distinct staining techniques were employed to provide a comprehensive assessment of tissue composition: Mason's trichrome staining and external elastin van Giesson staining.

Mason's Trichrome Staining: This technique was applied externally to evaluate collagen distribution within the tissue. Mason's trichrome staining is a versatile method that enables the differentiation of collagen fibers from other tissue components, providing insights into the structural integrity and fibrotic changes within the examined samples.

Elastin van Giesson Staining: The assessment of elastin content was carried out externally, utilizing the elastin van Giesson staining method. This specialized staining procedure allows for the visualization of elastin fibers, which are critical components of the extracellular matrix and play a pivotal role in tissue elasticity.

To gain deeper molecular insights into the flow-induced activation of pathways governing endothelial -to- mesenchymal transition, quantitative real-time polymerase chain reaction (qRT-PCR) was employed. This molecular technique enables the precise identification and quantification of genetic markers associated with these regulatory pathways.

The qRT-PCR process involved several key steps:

1. RNA Extraction: Ribonucleic acid (RNA) was meticulously extracted from the tissue specimens using specialized kits from Qiagen designed for this purpose. This step is critical for obtaining high-quality RNA samples that accurately represent the genetic content of the tissue.
2. Primer Selection**:** Specific target sequences by Taq-Man primers were carefully chosen for the qRT-PCR analysis. These primers, including ThermoFisher Rn01463848_m1 and Rn00709370_m1, were tailored to detect crucial genetic markers, including collagen and SLUG/SNAIL, which are indicative of endothelial -to- mesenchymal transition processes.
3. Amplification and Detection: The qRT-PCR assay was performed to amplify and detect the target genetic sequences within the RNA samples. This step enables the

quantification of gene expression levels associated with collagen and SLUG/SNAIL, shedding light on the molecular changes occurring within the tissue.

4. Delta Delta Cycle Threshold (ΔΔCT) Calculation: To quantify the relative changes in gene expression, the delta delta cycle threshold (ΔΔCT) method was applied. This involved comparing the individual mean cycle threshold (CT) values obtained from the samples with the mean of native hearts, providing a normalized measure of gene expression alterations in response to flow-induced activation.

In summary, this comprehensive approach encompassed histological staining techniques for collagen and elastin assessment, as well as qRT-PCR analysis to explore the molecular pathways governing endothelial -to- mesenchymal transition. These methods collectively provided a multi-dimensional understanding of the tissue's composition and gene expression profile.

Results

The visual depiction in Figure 1 showcases the successful implantation of a donor heart, meticulously anastomosed to the infrarenal vessels within the recipient. This intricate surgical procedure was executed with precision to ensure optimal graft integration and vascular connection.

At the critical time point of 14 days postoperative, an extensive echocardiography evaluation was performed to scrutinize the outcomes of the transplantation. This assessment revealed two noteworthy findings. Doppler echocardiography, depicted in Figure 2A, demonstrated the presence of sufficient anastomosis and a measurable velocity in the connected vessel. This vital observation indicated that the vascular connections between the donor heart and the recipient's circulatory system were operating effectively, facilitating the crucial blood flow required for cardiac function. The parasternal long axis view, as presented in Figure 2B, revealed the existence of an intracavity endocardial fibroelastosis layer. This finding suggested that there was the development of fibrous and elastic tissue within the cardiac cavities.

The modified Stanford Score was diligently employed to assess the condition of the first four successfully transplanted hearts immediately post-operative. The initial evaluation yielded a score of 3.5, indicating a promising start. However, it's noteworthy that by the first postoperative day (POD), this score had escalated to 4 for all recipients. This score was consistently maintained over the subsequent postoperative days, including POD 7 and POD 14, as illustrated in Figure 3. This trend signaled the persistence of specific cardiac changes and adaptations in response to transplantation.

A representative image (Figure 4) from the histological analysis was included to visually elucidate the structural changes within the left ventricular cavity. This image, captured using Mason's trichrome staining, revealed a distinct endocardial layer characterized by collagen deposition. This collagen-rich layer was indicative of endocardial fibroelastosis, corroborating the findings from the echocardiography assessment.

Quantitative real-time polymerase chain reaction (qRT-PCR) was employed to probe deeper into the molecular alterations within the transplanted hearts. The qRT-PCR analysis focused on assessing the expression of collagen and SLUG/SNAIL genes. The results, as depicted in Figure 5, unveiled significant disparities between the native (untransplanted) rat hearts and the transplanted Htx hearts. These differences in gene expression highlighted the unique molecular signatures associated with the transplantation process, further enriching our understanding of the underlying mechanisms at play.

In summary, this comprehensive series of evaluations, spanning echocardiography assessments, modified Stanford Scores, histological analysis, and qRT-PCR, provided a holistic view of the transplanted hearts' condition and the changes occurring at both structural and molecular levels.

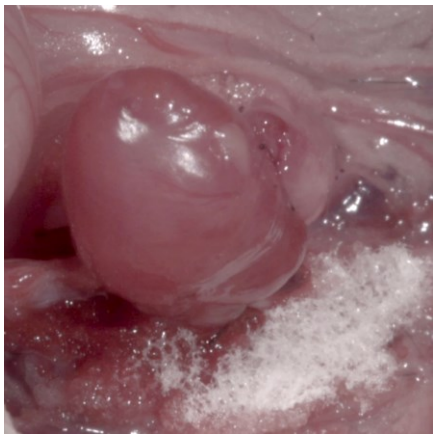

Figure 1: Infrarenal anastomosed neonatal heart

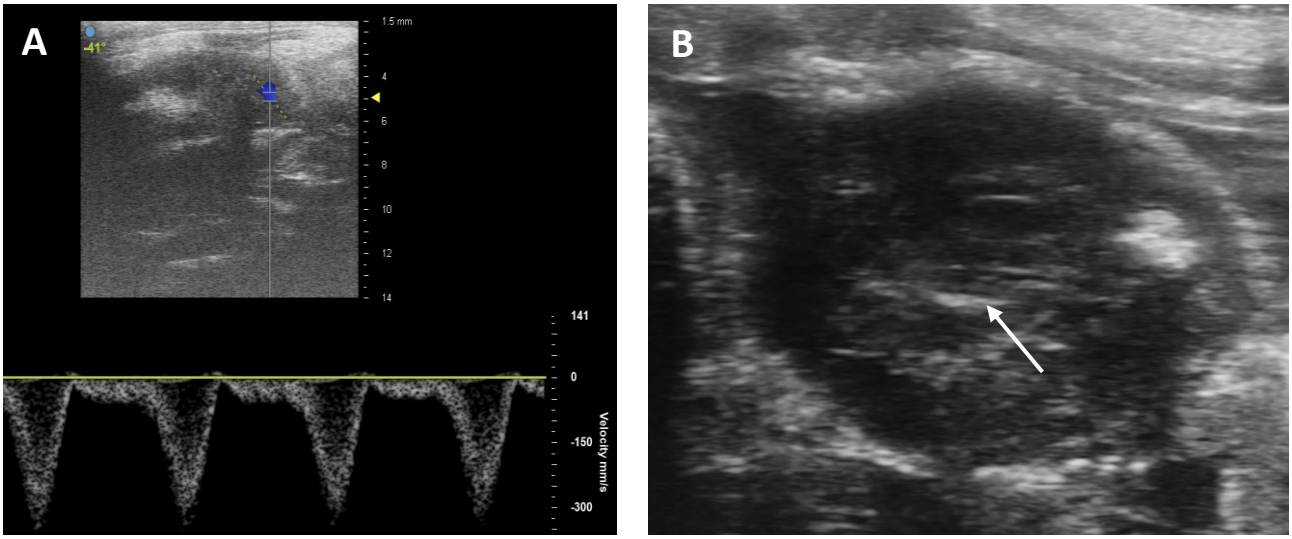

Figure 2: Representative echocardiographical pictures, A: Doppler with velocity assessment, B: parasternal long axis, arrow marks endocardial fibroelastosis layer

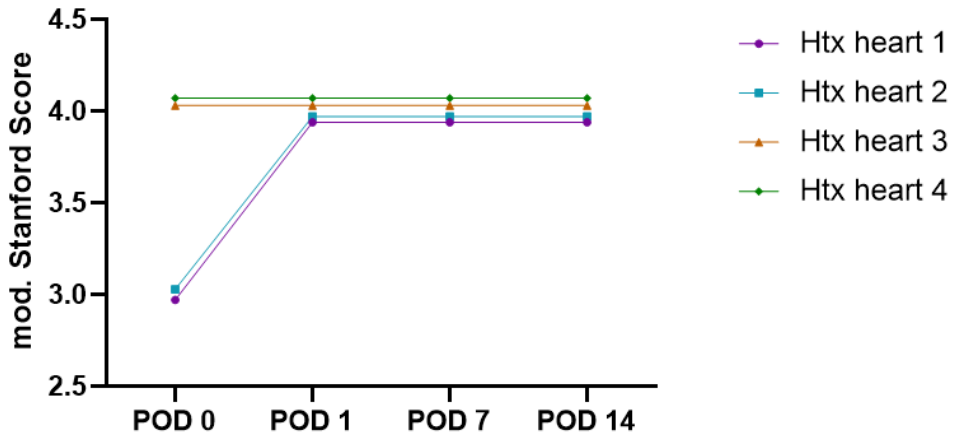

Figure 3: Individuel modified Stanford score on post operative days (POD) 0, 1, 7 and 14

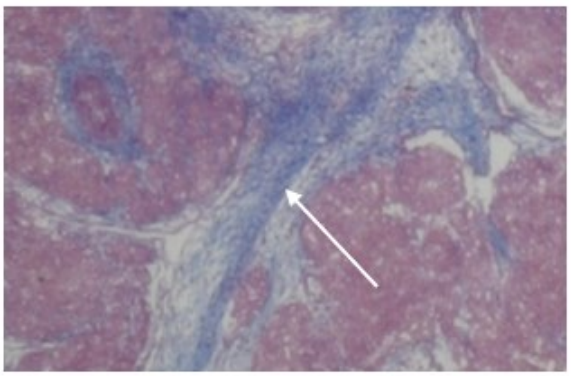

Figure 4: Representative picture of MTC staining, arrow marks collagen deposit

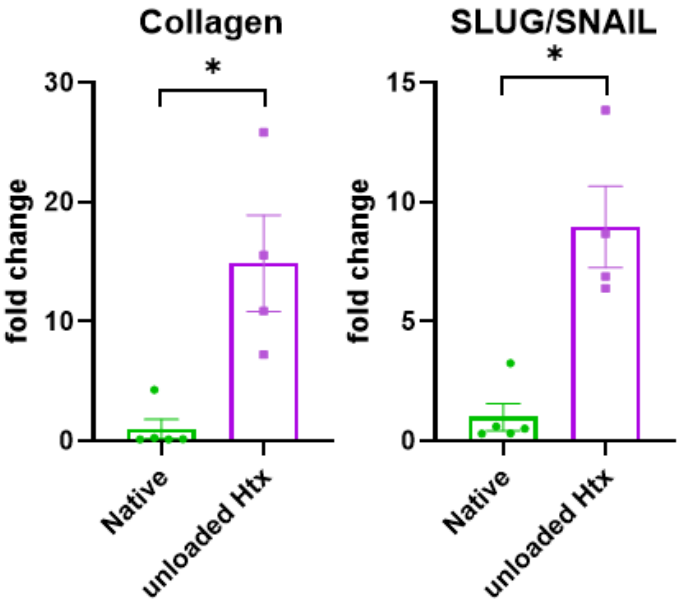

*Figure 5: Graph bar qRT-PCR analysis Collagen and SLUG/SNAIL, mean ± SEM, * p<0.05*

Discussion

The technique described here is undoubtedly intricate and demands meticulous attention to detail, as numerous potential challenges and pitfalls may arise throughout the procedure (24). This includes (24):

1. Intubation and Anesthesia Management: The procedure commences with intubation, a crucial step where utmost caution is necessary to prevent inadvertently placing the tube into the esophagus instead of the trachea. Additionally, managing the depth of anesthesia, particularly with inhalation agents like isoflurane, plays a pivotal role. The choice of anesthesia depth significantly impacts heart rate and arterial pressure. Striking the right balance is essential, as excessive anesthesia can lead to systemic pressure suppression and reduced cardiac contractility. This can complicate the preparation and anastomosis of the infrarenal vessels and the provision of adequate flow to the intestine during the procedure. Conversely, insufficient anesthesia poses the risk of the rat waking up prematurely, which must be avoided at all costs.

2. Receipient's Vessel Preparation: The meticulous preparation of vessels is of paramount importance, especially when dealing with the fragile and thin-walled inferior vena cava. Anatomical variations can further complicate access to the infrarenal vessels, necessitating careful consideration when implementing permanent occlusion.

3. Clot Formation: Despite pre-heparinization, clot formation at the anastomosis site can occur. Flushing the opened vessels with a heparinized saline solution is a helpful strategy to mitigate this risk. However, it's essential to be aware of the potential downside, as excessive anticoagulation can impede clotting after the final suture to close the anastomosis. To address this issue, absorbable gelatine sponges have proven effective, as illustrated in Figure 1. These sponges can be strategically placed around the anastomoses to prevent clot formation while facilitating the closure of the vessels. Ileus development in some rats postoperatively can be attributed to complications associated with the use of sponges. In some cases, the intestines may adhere to the sponge, causing a kink and obstructing the normal passage of contents. To prevent such complications, it's advisable to use small pieces of sponge and exercise extreme care when returning the intestine back into the body.

4. Heart Handling and Vessel Fragility: The handling of donor hearts requires a delicate touch. These hearts are still in the developmental stage, and their vessels are fragile. Excessive manipulation with forceps or overly tight sutures can inflict irreversible damage to the vessel

walls. Such damage may not always be immediately apparent but can lead to significant bleeding once the rat regains consciousness, potentially resulting in death within hours.

5. Adequate Heart Function: Despite the provision of sufficient blood flow, there remains the possibility that the transplanted hearts may not exhibit adequate contractile function. One potential factor contributing to this issue is prolonged ischemic time. To ensure a successful procedure, it is imperative to limit the ischemic time to a duration of less than 100 minutes.

Despite the myriad challenges and potential pitfalls, once this model is successfully established, it opens up exciting opportunities for investigating endocardial fibroelastosis. Both histological and molecular biological analyses have indicated the development of this condition in the transplanted hearts. When consistently reproducible in the laboratory, this animal model becomes a valuable foundation for conducting research studies aimed at unraveling the intricacies of endocardial fibroelastosis formation.

In this experimental setup, we employed a model known as the "unloaded condition" to investigate the formation of endocardial fibroelastosis. This model represents one of three distinct flow conditions that can be achieved through heterotopic heart transplantation. The other two conditions are the "loaded condition" and the "turbulent flow condition," each offering unique insights into endocardial fibroelastosis development (27).

The turbulent flow condition represents a more realistic model for studying endocardial fibroelastosis development, as it introduces some flow into the left ventricle, albeit in a non-laminar fashion. In this setup, the donor’s inferior vena cava is still anatomised to the recipient’s infrarenal inferior vena cava and the donor's aorta is still connected to the recipient's aorta, similar to the unloaded condition. However, there is a key difference: the donor's aortic valve is intentionally compromised by a wire, preventing it from closing sufficiently. This compromise allows for regurgitated blood flow into the left ventricle, resulting in turbulent flow patterns. endocardial fibroelastosis can develop in this condition, although it may not be as pronounced as in the unloaded condition.

In contrast to the unloaded condition, the loaded condition serves as an internal control in the research. Previous studies have shown that this condition does not lead to the development of endocardial fibroelastosis. Therefore, it is considered a reference point for understanding the absence of endocardial fibroelastosis under normal flow conditions. In this specific

experimental setup, was established within the heterotopic heart transplantation (Htx) model to closely mimic normal blood flow patterns through the donor's heart. This control condition was meticulously designed to replicate physiological circulation and provided the left ventricle with an approximately normal flow of blood. The connection between the donor's aorta and the recipient's aorta was retained, maintaining a direct link between the two aortas. Unlike the unloaded or turbulent flow conditions, where the venous connection configurations differed, in this loaded condition, the venous connection was established using the donor's superior vena cava and the recipient's inferior vena cava. This arrangement allowed venous blood from the recipient to flow into the donor's heart through the superior vena cava, replicating the typical venous return observed in normal circulation. One distinctive feature of this loaded condition was the creation of a loop connecting the donor's pulmonary artery directly to the donor's left atrium. This loop enabled blood to circulate through the donor's heart as in a more physiological way. It entered the right atrium, proceeded into the right ventricle, passed through the pulmonary valve into the pulmonary artery, and then returned to the left atrium. Finally, it entered the left ventricle and flowed through the aortic valve into the aorta. The primary objective of this negative control was to provide the left ventricle with a flow pattern closely resembling that of a healthy heart. Unlike the unloaded condition, where left ventricular flow was significantly reduced, and the turbulent condition, which introduced irregular flow patterns, this control aimed to replicate normal hemodynamics in the left ventricle. By establishing this negative control, researchers aimed to create a reference point for comparing the impact of altered blood flow conditions, such as those observed in the unloaded and turbulent models, on the development of endocardial fibroelastosis.

It's important to note that these three distinct flow conditions serve as essential tools for researchers to investigate the role of hemodynamics in endocardial fibroelastosis development. By comparing outcomes across these conditions, researchers can gain valuable insights into the complex interplay between blood flow patterns and the formation of endocardial fibroelastosis. This knowledge contributes to a deeper understanding of the disease mechanisms and potential avenues for therapeutic intervention.

In conclusion, while navigating the complexities and pitfalls of this technique can be demanding, the insights and knowledge gained from its successful implementation hold immense promise for advancing our understanding of endocardial fibroelastosis and related cardiac research. Careful attention to each step, continuous refinement of the procedure, and

strict adherence to best practices are essential for achieving consistent and reproducible outcomes in this valuable research model.

Reference

1. Tchervenkov CI, Jacobs JP, Weinberg PM, Aiello VD, Béland MJ, Colan SD, u. a. The nomenclature, definition and classification of hypoplastic left heart syndrome [Internet]. Bd. 16, Cardiology in the Young. Cambridge University Press; 2006. S. 339–68. Verfügbar unter: https://doi.org/10.1017/S1047951106000291
2. Guseh SH, Friedman KG, Wilkins-Haug LE. Fetal cardiac intervention—Perspectives from a single center. Prenat Diagn. März 2020;40(4):415–23.
3. Friedman KG, Tworetzky W. Fetal cardiac interventions: Where do we stand? Arch Cardiovasc Dis. Februar 2020;113(2):121–8.
4. Pickard SS, Wong JB, Bucholz EM, Newburger JW, Tworetzky W, Lafranchi T, u. a. Fetal Aortic Valvuloplasty for Evolving Hypoplastic Left Heart Syndrome: A Decision Analysis. Circ Cardiovasc Qual Outcomes. April 2020;13(4):e006127.
5. Haas NA, Jux Ch, Photiadis J, Kramer HH. Guidelines German Society of Pediatric Cardiology: Hypoplastic Left Heart Syndrome [Internet]. 2013. Verfügbar unter: http://www.kinderkardiologie.org/fileadmin/user_upload/Leitlinien/29 LL Hypoplastisches Linksherzsyndrom.pdf
6. Murdison KA, Baffa JM, Farrell PE, Chang AC, Barber G, Norwood WI, u. a. Hypoplastic left heart syndrome. Outcome after initial reconstruction and before modified Fontan procedure. Circulation. 1990;82(5 SUPPL.).
7. Barron DJ, Kilby MD, Davies B, Wright JG, Jones TJ, Brawn WJ. Hypoplastic left heart syndrome. The Lancet. August 2009;374(9689):551–64.
8. Khairy P, Fernandes SM, Mayer JE, Triedman JK, Walsh EP, Lock JE, u. a. Long-term survival, modes of death, and predictors of mortality in patients with Fontan surgery. Circulation. Januar 2008;117(1):85–92.
9. Tchervenkov CI, Tahta SA, Jutras LC, Béland MJ. Biventricular repair in neonates with hypoplastic left heart complex. Ann Thorac Surg. Oktober 1998;66(4):1350–6.
10. Emani SM, McElhinney DB, Tworetzky W, Myers PO, Schroeder B, Zurakowski D, u. a. Staged Left Ventricular Recruitment After Single-Ventricle Palliation in Patients With Borderline Left Heart Hypoplasia. J Am Coll Cardiol. November 2012;60(19):1966–74.
11. Emani SM. Staged Left Ventricular Recruitment and Biventricular Conversion for Patients With Borderline Left Heart. Oper Tech Thorac Cardiovasc Surg. 2016;21(2):112–23.
12. Emani SM, Bacha EA, McElhinney DB, Marx GR, Tworetzky W, Pigula FA, u. a. Primary left ventricular rehabilitation is effective in maintaining two-ventricle physiology in the borderline left heart. J Thorac Cardiovasc Surg. Dezember 2009;138(6):1276–82.
13. Emani SM. Biventricular Repair in Patients With Borderline Left Heart-The „Growing“ Experience. World J Pediatr Congenit Heart Surg. 2019;10(1):18–9.
14. Lurie PR. Endocardial fibroelastosis is not a disease [Internet]. Bd. 62, The American Journal of Cardiology. 1988. S. 468–70. Verfügbar unter: https://linkinghub.elsevier.com/retrieve/pii/0002914988909812

15. Cole CR, Eghtesady P. The myocardial and coronary histopathology and pathogenesis of hypoplastic left heart syndrome. Cardiol Young. Januar 2016;26(1):19–29.

16. Weixler V, Marx GR, Hammer PE, Emani SM, del Nido PJ, Friehs I. Flow disturbances and the development of endocardial fibroelastosis. J Thorac Cardiovasc Surg. Februar 2020;159(2):637–46.

17. Xu X, Friehs I, Hu TZ, Melnychenko I, Tampe B, Alnour F, u. a. Endocardial fibroelastosis is caused by aberrant endothelial to mesenchymal transition. Circ Res. Februar 2015;116(5):857–66.

18. Eisenberg LM, Markwald RR. Molecular Regulation of Atrioventricular Valvuloseptal Morphogenesis. Circ Res. Juli 1995;77(1):1–6.

19. Diaz-Gil D, Carreon CK, Silva-Gomez N, Benheim AE, Emani SM, Del Nido PJ, u. a. Case report: Active clinical manifestation of endocardial fibroelastosis in adolescence in a patient with mitral and aortic obstruction–histologic presence of endothelial-to-mesenchymal transformation. Front Cardiovasc Med. 30. November 2022;9:1041039.

20. Weixler V, Hammer PE, Marx GR, Emani SM, del Nido PJ, Friehs I. Flow disturbances and progression of endocardial fibroelastosis — a case report. Cardiovasc Pathol. September 2019;42:1–3.

21. Friehs I, Illigens B, Melnychenko I, Zhong-Hu T, Zeisberg E, del Nido PJ. An animal model of endocardial fibroelastosis. J Surg Res. Juni 2013;182(1):94–100.

22. Shimada S, Robles C, Illigens BMW, Casar Berazaluce AM, del Nido PJ, Friehs I. Distention of the Immature Left Ventricle Triggers Development of Endocardial Fibroelastosis: An Animal Model of Endocardial Fibroelastosis Introducing Morphopathological Features of Evolving Fetal Hypoplastic Left Heart Syndrome. BioMed Res Int. 2015;2015:462469.

23. Illigens BMW, Casar Berazaluce A, Poutias D, Gasser R, Del Nido PJ, Friehs I. Vascular Endothelial Growth Factor Prevents Endothelial-to-Mesenchymal Transition in Hypertrophy. Ann Thorac Surg. September 2017;104(3):932–9.

24. Gierlinger G, Rech L, Emani SM, Del Nido PJ, Friehs I. A Neonatal Heterotopic Rat Heart Transplantation Model for the Study of Endothelial-to-Mesenchymal Transition. J Vis Exp. 21. Juli 2023;(197):65426.

25. Price AN, Cheung KK, Cleary JO, Campbell AE, Riegler J, Lythgoe MF. Cardiovascular magnetic resonance imaging in experimental models. Open Cardiovasc Med J. November 2010;4:278–92.

26. Blanchard JM, Pollak R. Techniques for perfusion and storage of heterotopic heart transplants in mice. Microsurgery. 1985;6(3):169–74.

27. Oh NA, Hong X, Doulamis IP, Meibalan E, Peiseler T, Melero-Martin J, u. a. Abnormal Flow Conditions Promote Endocardial Fibroelastosis Via Endothelial-to-Mesenchymal Transition, Which Is Responsive to Losartan Treatment. JACC Basic Transl Sci. 1. Dezember 2021;6(12):984–99.