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Se Hyeon Park April 12, 2022

Encapsulation of Pediatric C-kit+ Cells in Left Ventricular Myocardial Extracellular Matrix Hydrogels for Enhanced Cardiac Function

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An abstract of
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of Emory University in partial fulfillment
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Biology

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Abstract

Encapsulation of Pediatric C-kit+ Cells in Left Ventricular Myocardial Extracellular Matrix Hydrogels for Enhanced Cardiac Function

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Affecting one in 5,000 births, Hypoplastic Left Heart Syndrome (HLHS) is a congenital heart defect in which the left side of a newborn's heart is underdeveloped.[1] Despite surgical palliation to redirect blood flow to right ventricle (RV), HLHS patients are at risk of RV hypertrophy, inefficient contractile force, and eventually RV failure.[2] Current treatments for HLHS include transplantation for RV failure; however, this is challenging due to limited donors and risk of rejections.[2] Recently, cell therapies, such as pediatric cardiac derived c-kit$^+$ progenitor cells (CPCs), have been extensively investigated as a promising therapy for RV repair.[3] However, complications regarding low cell retention and transient paracrine effects after injection require a more efficient mechanism of cell delivery.[4] To improve the reparative effects of CPCs, this study aims to investigate a minimally invasive therapy that combines CPCs and porcine cardiac-derived extracellular matrix (LV cECM) hydrogel. LV cECM hydrogel has been proven by recent clinical trials to promote cardiac repair by mimicking the native extracellular matrix.[5] In this study, CPCs were encapsulated in LV cECM hydrogels to be used as a platform of cell delivery. Cellular function post encapsulation and improvements in cardiac reparative function were investigated through both in vitro and in vivo studies. Tube formation assays were performed to evaluate paracrine release and pro-angiogenic capacities of conditioned media from the 3D encapsulated CPC cultures. To evaluate the improvements in cardiac functional outcomes, these treatment groups were injected into RV failure rat model, and cardiac outcomes such as tricuspid annular plane systolic excursion (TAPSE), vessel development, and myocyte hypertrophy were analyzed.

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Acknowledgements

I would like to thank my honors committee members and Dr. Davis, Ph.D., Dr. Hyun-Ji Park, Ph.D., Milton Brown, and Preety Shakya of the Davis Lab for assisting me throughout my Honors Program.

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1. Introduction

Hypoplastic Left Heart Syndrome (HLHS) is one of the most severe cases of congenital heart defects that affects systemic circulation of a newborn's heart.[2] Accounting for about 25% of deaths in infants with congenital heart defects, HLHS leads to structural defects in the left heart, including an underdeveloped left ventricle, mitral valve, and aortic valve[17]. Initially, during the first few days after birth, newborns with HLHS can still have oxygenated blood pumped to their body due to the patent ductus arteriosus and the patent foramen ovale.[19] These are two small openings connecting the left and the right sides of the heart that are present in babies growing in womb during pregnancy.[18] These openings do not close until a few days after birth, so newborns with HLHS are still able to get oxygenated blood to bypass the underdeveloped left heart into the right heart, which will then perform systemic circulation.[19] However, once the patent ductus arteriosus and the patent foramen ovale close, the right heart can no longer support the inadequate systemic circulation of the underdeveloped left heart.[19] Within the first few days of birth, HLHS hinders the flow of oxygenated blood from the lungs and is fatal if untreated.

Current treatments for HLHS involve a series of palliative surgeries starting from the neonatal period for survival. Composed of three stages - the Norwood Procedure done within the first few days of life, the Glenn Procedure done 4 to 6 months after birth, and the Fontan Procedure done 18 to 36 months after birth - these procedures are designed to redirect blood flow to the right ventricle, allowing survival.[20] At the end of these three procedures, the HLHS patients have a completely univentricular heart, relying entirely on the RV for systemic blood flow. However, having a single ventricle causes volume and pressure overload on the RV as it has to perform

both pulmonary and systemic blood flow.[6] Eventually, this may lead to RV failure, which causes one-third of HLHS patients to die before the age of 25.[7] Another treatment option currently available for HLHS is heart transplantation for RV failure. Transplantation for RV failure is also challenging due to limited donors, risk of transplant rejection, and other associated risks. There is a 17% to 55% operative mortality of patients undergoing transplantation, and the average transplant lasts less than 17 years, meaning subsequent heart transplantations will be required for survival.[8]

Recently, cell therapies have been under investigation for RV treatment of RV dysfunction. Pediatric cardiac derived c-kit$^+$ progenitor cells (CPCs) are currently being tested in clinical trials after showing regenerative effects in preclinical trials (NCT03406884).[9] A recent study done by the Davis laboratory demonstrated the capability of c-kit$^+$ CPCs in contributing to cardiac repair, especially using CPCs of younger age.[9] Both CPCs isolated from neonate patients (1 day to 1 month) and CPCs isolated from infant patients (1 to 12 months) showed improved TAPSE outcomes when treated to RV failure rat models.[9] TAPSE measures the displacement of the lateral tricuspid annulus during apex-to-base shortening and serves as a global parameter for RV function. Neonate CPCs also showed significant improvements in the right ventricular ejection fraction (RVEF), which is a measure of amount of blood pumped by the RV, and decrease in RV wall thickness, which indicates decreased right ventricular hypertrophy.[9] Analyzing the age-dependent effect of CPCs in RV failure models, the Davis lab was able to conclude that CPCs isolated from neonate patients show higher reparative potential compared to CPCs isolated from child patients.[9] However, complications regarding low cell retention and transient paracrine effects after injection of CPCs of both ages require a more efficient mechanism of cell delivery.

Porcine left ventricular cardiac-derived extracellular matrix (LV cECM) hydrogels are biomaterials naturally derived from heart tissues that are being used in ongoing clinical trials for cardiac repair in myocardial infarction (MI) patients (NCT02305602).[5] LV cECM hydrogel provides the necessary biochemical cues of the native tissue microenvironment by degrading into products that are angiogenic and chemoattractant, and promoting cell migration and proliferation.[5] A previous study has also shown that when growth factors were delivered in LV cECM hydrogels to MI models, the combination led to increased arteriogenesis compared to injecting LV cECM hydrogels or the growth factors alone.[5] Given recent studies showing cardiac reparative potential of c-kit^{+} CPCs and LV cECM hydrogels, this study aims to develop a minimally invasive therapy, combining both cell and biomaterial therapeutics. As using standalone treatments of CPCs and LV cECM hydrogels have been shown to promote cardiac repair in preclinical studies, combining these two methods may contribute to better cardiac outcomes compared to standalone techniques

2. Materials and Methods

2.1. Cardiac derived c-kit$^+$ progenitor cells (CPCs) Isolation and Culture:

Cardiac derived c-kit$^+$ progenitor cells (CPCs) were isolated from atrial appendage tissue biopsies of pediatric patients undergoing cardiopulmonary bypass surgery at Children's Healthcare of Atlanta. Tissues underwent magnetic bead sorting to isolate c-kit$^+$ cells, and cells were cryopreserved with 1-2 million cells per vial. Neonate CPCs were obtained from donors less than 1 month old, and child CPCs were obtained from donors more than 1 year old. For CPC culture, previously isolated and cryopreserved cells were thawed and cultured in a T-75 flask with 10mL of warm media containing Ham's F-12, 1x P/S/G, 10% fetal bovine serum, 10ng/mL basic fibroblast growth factor. Cell culture media was changed after 24 hours of initial seeding and every 2 days subsequently, at 80% cell confluency level. Cell culture flasks were incubated at 37°C and 5% CO_2.

2.2 LV cECM Fabrication:

Porcine left ventricular myocardium was decellularized and processed by the Christman laboratory in UC San Diego to form acellular left ventricular cardiac-derived extracellular matrix (LV cECM) hydrogels. The fabricated gels were delivered to the Davis lab in lyophilized form that were reconstituted to form hydrogels. For reconstitution of LV cECM hydrogels, lyophilized LV cECM were diluted to a concentration of 8mg/μL in sterile water and carefully sheared with a 25G needle syringe to a final homogenous solution.

2.3. 3-D Encapsulation of CPC:

Neonatal and child CPCs were 3D encapsulated either in collagen, as a control or in LV cECM hydrogel. Collagen gels were prepared using PureCol® EZ Gel diluted 3.33mg/ml in 1X sterile PBS and incubated in 37°C. Prior to encapsulation, 2D culture of CPCs in T-175 flask was treated with trypsin and centrifuged at 1000 rpm for 5 minutes to obtain a pellet of 1.5 million cells. Then, the cell pellet was mixed thoroughly with 125µL of either the reconstituted LV cECM hydrogels or collagen by gently pipetting up and down. 25µL of the cells in LV cECM hydrogel or collagen mixture was pipetted into each well of a 24 well plate and left in an incubator at 37°C and 5% CO_2. After 45mins - 1hour of incubation, 500µL of CPC culture media was gently added to each well from the side of the well. Media was changed every 2 days.

2.4. Cardiac Endothelial Cell (CEC) Culture and Angiogenesis Assay in Conditioned Media:

To assess paracrine release of encapsulated CPCs, tube formation assay was performed to evaluate pro-angiogenic capability of conditioned media from different CPC encapsulations when added to cardiac endothelial cells (CECs). At day 6 post 3D encapsulation of CPC, media was replaced with ITS liquid media free of growth factors. At day 7 post encapsulation, the ITS media from each treatment group were collected and frozen at - 80°C to be used as conditioned media for angiogenesis assay.

Prior to angiogenesis tube formation assay, CECs were cultured in a T-25 flask using 3mL of EGM media at 37°C and 5% CO_2. One day prior to seeding and treatment of CECs with conditioned media (day 0), one of the T-25 flasks was fed again with EGM positive control media, while the other T-25 flask was replaced with EBM (serum free) media. On day 1, 10uL of thawed matrigel (Corning Matrigel Phenol Free VWR 356237) was pipetted into each well of Ibidi uSlide 84506 on ice and incubated at 37°C and 5% CO_2 for 30 minutes on a Petri Dish for the matrigel to be polymerized. To seed the CECs in matrigel on the uSlide, 200,000 cells/mL solution was prepared from the T-25 flask with EGM positive control media. Then, 50 uL of the cell solution was added into each well as the EGM positive control group. For negative control and the two experimental groups (LV cECM hydrogel and collagen), 400,000 cells/mL solution was prepared from the T-25 flask with EBM serum free media. 25uL of this cell solution was first added to each well for EBM negative control and the two experimental groups. Then, 25uL of ITS media was added to each negative control well, and 25uL of conditioned media (collected on Day 7 of CPC encapsulation) was added to respective experimental group wells. On day 2, 20 hours after CEC seeding, media from each well was replaced with Calcein AM in PBS solution and incubated for an additional 30 mins at 37°C and 5% CO_2. Using the FITC filter on the microscope, 4x images of each well were taken.

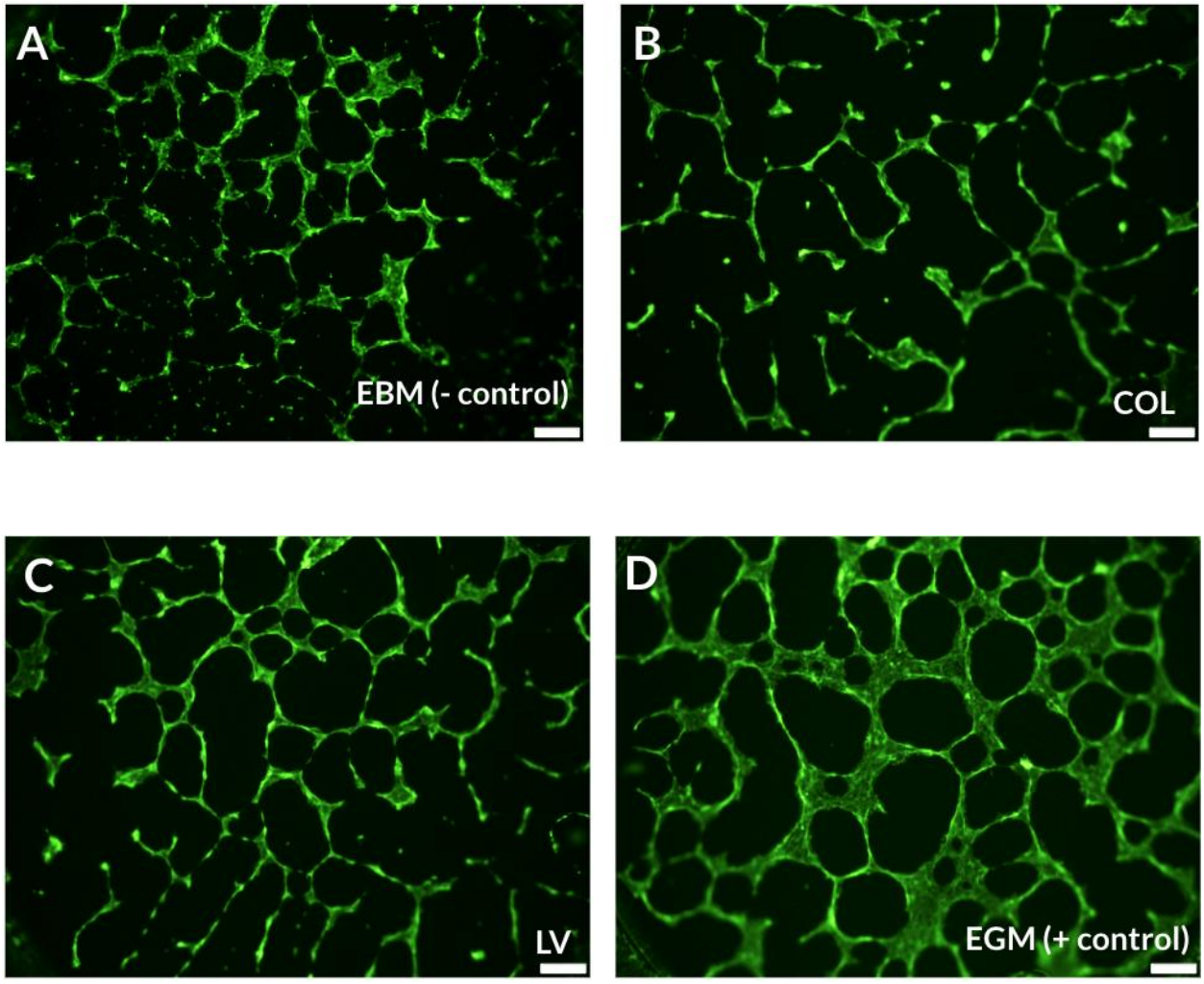

Figure 1. Fluorescent images of tubes at Day 2, formed by CECs treated with conditioned media collected from each labeled treatment group. Conditioned media collected from 3D encapsulated child CPCs. Scale bar = 100um.

2.5. Intramyocardial Injections for In-vivo Study:

Encapsulation of CPCs for intramyocardial injections were prepared in a similar manner as discussed as above, but CPCs were dyed with DiIC18(7); 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR dye) solution in dimethyl sulfoxide (DMSO) prior to encapsulation. This enabled retention of CPCs to be tracked post injection using IVIS Spectrum

in vivo imaging system. Athymic, immunocompromised rats underwent Pulmonary Artery Band (PAB) surgery to mimic right ventricular failure in HLHS. Rats with failing RV were randomized into either sham (positive control), PAB only (negative control) and five different treatment groups, including LV cECM hydrogel only, child CPCs only, neonate CPCs only, neonate CPCs in LV cECM hydrogel and child CPCs in LV cECM hydrogel. Two weeks after the PAB surgery, the therapeutics were injected directly into the RV wall as per treatment groups using the Vevo2100 ultrasound machine. A concentration of 1 million DiR-labeled cells/75uL saline or LV cECM hydrogel was used depending on the treatment group - saline for injections of CPCs only and LV cECM hydrogel for injections of CPCs encapsulated in hydrogels. For LV cECM hydrogel only injections, 75uL of LV cECM hydrogel were injected, and Echocardiography-guided injections were performed in a randomized and blinded manner, and all protocols were performed under the guidelines set by the *Institutional Animal Care and Use Committee* (IACUC).

2.6. IVIS Spectrum in vivo imaging:

To assess the retention of injected CPCs encapsulated in hydrogels, live rats were imaged using the IVIS Spectrum in vivo imaging system on days 0, 7, 14, 21, and 28. Day 0 being the day of injection, fluorescence intensity of injections on this day was set as 100% cell retention rate and further quantified over time as a percentage retention value of the initial intensity.

2.7. Transthoracic Echocardiography:

Echocardiography was performed on live rats prior to PAB surgery, prior to injection two weeks post PAB, two and four weeks post injection for longitudinal assessment of cardiac function. Tricuspid annular plane systolic excursion (TAPSE) values were quantified from echocardiographic images for all treatment groups as a measure of cardiac functional outcome.

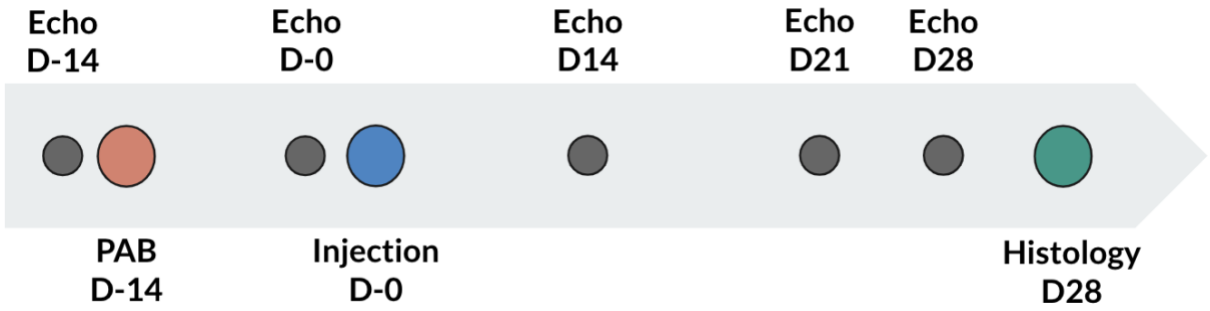

Figure 2. Timeline of in-vivo study. Grey points indicate time-points echocardiography was performed on live rats. Blue point represents time point therapeutics were injected into rats and is set to Day 0. Red point indicates time point PAB surgery was done, 2 weeks prior to injections. Green point represents when rats were euthanized and histological analysis were done on isolated hearts, 4 weeks after injections.

2.8. Heart Isolation and Cryosectioning:

The rats were euthanized in a carbon dioxide chamber four weeks after injection, and their hearts were isolated under the protocol set by IACUC. Isolated hearts were frozen in O.C.T. compound in a block and stored in a cryo-freezer box at -80°C. The frozen hearts were cryosectioned into 8μm sections using a cryostat. About 50 heart sections were made from each animal heart.

2.9. Immunohistochemistry Staining:

Heart sections were stained for histological analysis using wheat germ agglutinin (WGA) for quantifying myocyte hypertrophy in RV. Heart sections were hydrated in PBS prior to staining as it is necessary to avoid light exposure and drying of slides. After 5 minutes of hydration, slides were incubated in 0.1% Triton X-100 for 15 mins to allow for permeabilization. Then, 1X Na-Citrate antigen retrieval buffer was used to unmask antigens in formalin-fixed tissue sections. Slides were incubated in citrate buffer for 15 mins on a hot plate at 95°C - 100°C. After washing slides with PBS thoroughly again, 100uL of blocking buffer (4% goat serum in PBS) was added to each tissue section and incubated at room temperature in a humidified dark chamber for 1 hour. WGA antibody solution was made to an appropriate dilution of 1:200 in the blocking buffer. 100 uL of antibody solution was added per tissue section and incubated overnight at 4°C in a dark chamber. For staining of the nuclei of heart tissues, 100uL of DAPI solution was added to each tissue and incubated for 10 mins at room temperature. Finally, slides were washed with PBS thoroughly again, and mounted with coverslips using VECTASHIELD HardSet Antifade Mounting Medium (H-1400, Vector Labs). Slides were stored in -20°C and imaged using the Olympus IX81 FluoView FV1000 confocal microscope with TRITC filter at 20x magnification.

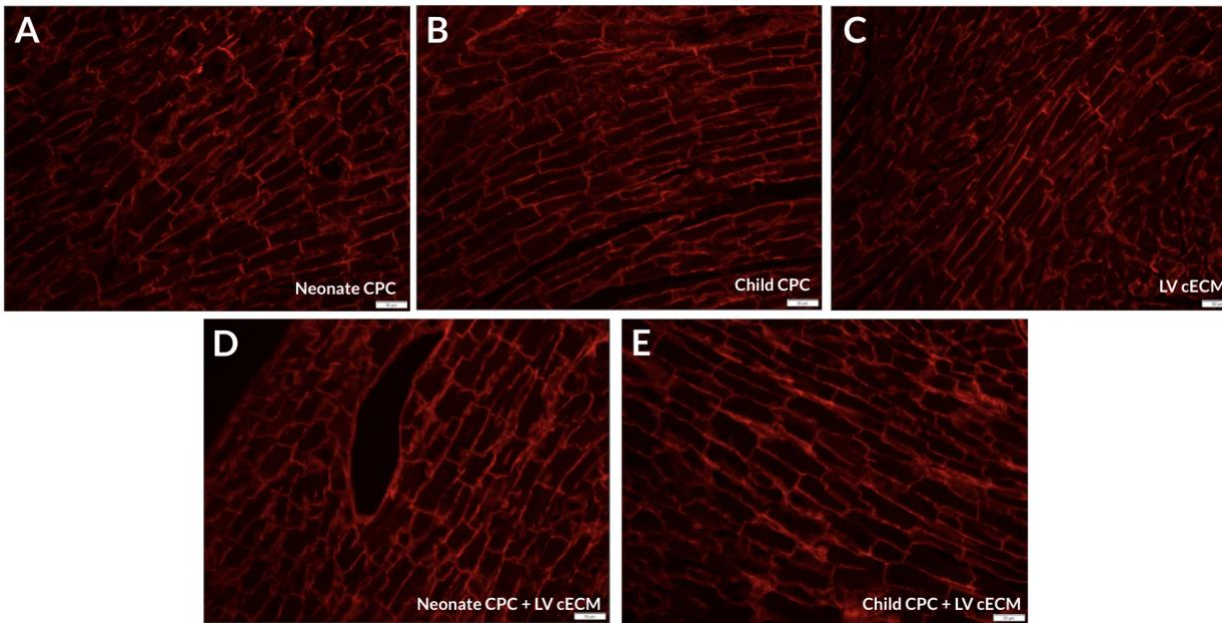

Figure 3. Fluorescent images of cryosectioned hearts after WGA staining. Labeled treatment group corresponds to injection RV failure model received. Scale bar = 50um.

3. Results

3.1. Assessment of paracrine release by encapsulated CPCs using conditioned media

To evaluate cellular function after 3D encapsulation of CPCs in LV cECM hydrogels, the paracrine release by encapsulated CPCs were assessed for angiogenic potential using tube formation assay. By adding the conditioned media used to culture 3D encapsulated CPCs to cardiac endothelial cells (CECs), different parameters of angiogenic capabilities of these conditioned media were analyzed. Results of tube formation assays using conditioned media from 3D cultures of child CPCs and neonate CPCs in different treatment groups are shown below. Angiogenic data were normalized to the negative control group, which used EBM serum free media, and data for conditioned media from LV cECM hydrogels and collagen are represented as fold change.

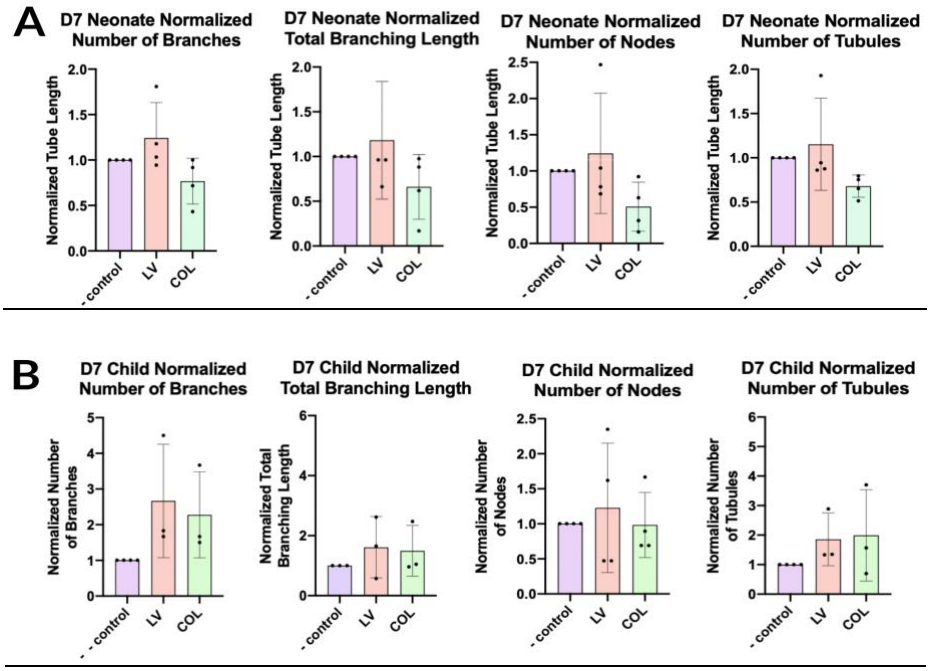

Figure 4. Angiogenesis Analysis. Different parameters of angiogenesis are measured from tube formation assay using respective conditioned media from **(A)** 3D encapsulated neonate CPCs

and **(B)** 3D encapsulated child CPCs. Data normalized against negative control using EBM serum free media and represented as fold change. All data analyzed using One-way ANOVA with Tukey's Test for Post Hoc Analysis across all groups.

Although no significance of data was found, tube formation assay using conditioned media collected from to encapsulated child and neonate CPCs showed a general trend that the number of tubes is lower when grown in conditioned media from CPCs encapsulated in collagen compared to those from LV cECM hydrogel. In-terms of age dependent effects, there was a trend that the difference in number of tubes generated using conditioned media of LV cECM hydrogel and collagen groups were more noticeable for the neonate CPC group than those from the child CPC group.

3.2. Assessment of cardiac function outcome in RV failure models using Transthoracic Echocardiography:

To evaluate the effects of CPC encapsulations in LV cECM hydrogels in cardiac functional outcome, treatment groups were injected into RV failure rat models and TAPSE scores were recorded over time. PAB surgeries have been extensively used in previous studies for generating rat models with RV failure and dysfunction. By putting a band over the rat's pulmonary artery, PAB surgeries are known to cause reduced blood flow and pressure overload in the RV and, therefore, lead to a gradual development of RV failure. All analyses were performed after confirming that the PAB surgery successfully decreased RV functionality. Prior to injections, measuring the TAPSE values before and after PAB surgery were necessary, as decrease in TAPSE values from D-14 (prior to PAB) to D0 (2 weeks after PAB) ensured that the PAB surgery successfully mimicked RV dysfunction This provided us with measurements of cardiac

function over time before and after the PAB surgery and injection of therapeutics. TAPSE values recorded at Day 28 of injecting RV failure rat models with respective treatment methods are shown below.

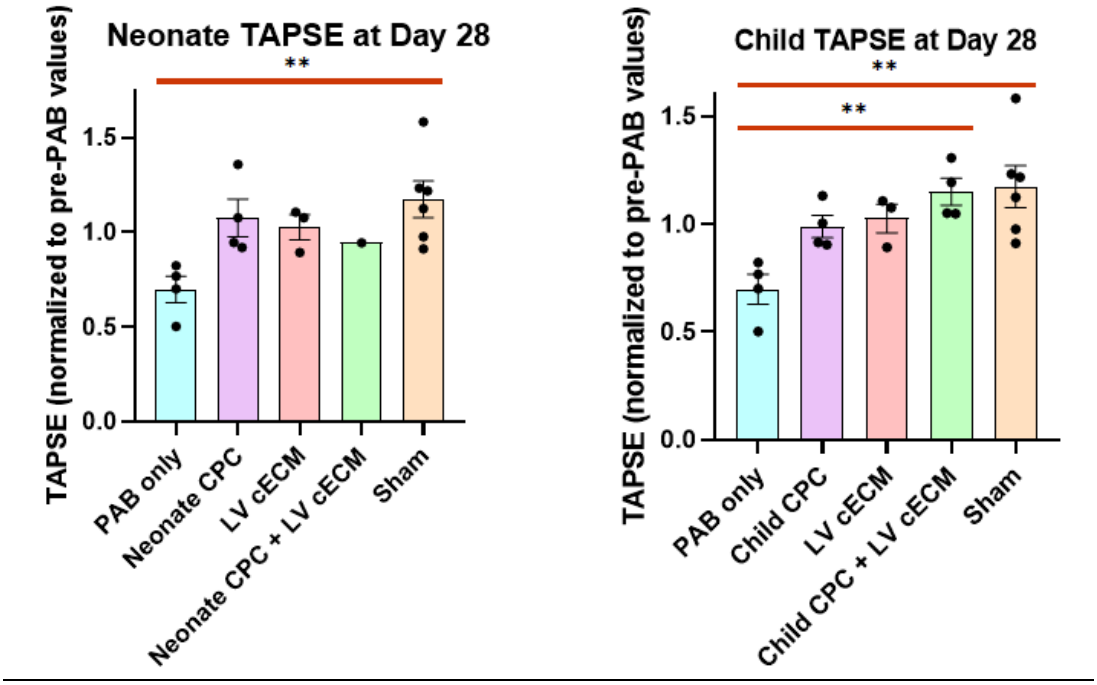

Figure 5. Cardiac functional outcome analysis. TAPSE values recorded at Day 28 of injecting RV failure rat models with respective treatment methods. Data normalized against TAPSE values prior to PAB surgery and represented as fold change. All data analyzed using One-way ANOVA with Tukey's Test for Post Hoc Analysis across all groups.

All treatment injections of neonate and child CPCs - alone, in encapsulated forms with LV cECM hydrogel, and LV cECM hydrogel only - lead to a significant increase in TAPSE values compared to negative control. In terms of age dependent effects, for neonate CPCs, there was a trend that using combined therapy method of CPCs and LV cECM hydrogels leads to a decreased TAPSE compared to injections of standalone therapeutics. However, for child CPCs, RV failure rat models injected with CPCs and LV cECM hydrogels have significantly improved TAPSE compared to using standalone injections and from the negative control. This indicates

promising effects of using combined therapy method especially for child CPCs. The previously published study of CPCs by the Davis laboratory demonstrated that neonate CPCs lead to greater improvements in cardiac functional outcomes compared to child CPCs, when using CPCs as a standalone method of injection. However, results from this study using the combined therapy method show that when LV cECM hydrogels are used as a platform of cell delivery for child CPCs, the enhanced benefit in cardiac functional outcome is significantly greater than that of neonate CPCs. Such results suggest that incorporating the combined therapy method of CPCs and LV cECM hydrogels would especially enhance the reparative potentials of child CPCs.

3.3. Histological analysis of myocyte hypertrophy in RV

While investigating the cardiac functional outcomes in live RV failure models is important for assessing the effect of CPC encapsulations, histological analysis must also be performed to evaluate the tissue-level effects of cardiac remodeling. RV failures of congenital heart defects are known to result from gradual progression of increased cardiomyocyte hypertrophy, indicating overworked RV.[9] So in this study, Wheat Germ Agglutinin (WGA) was used to quantify myocyte hypertrophy in RV. WGA binds to and stains the cell membranes of cardiomyocytes, allowing for fluorescence imaging to be used for quantifying the cross-sectional areas of cardiomyocytes. Below shows the results of measured cardiomyocyte areas after staining respective cryosectioned hearts with WGA.

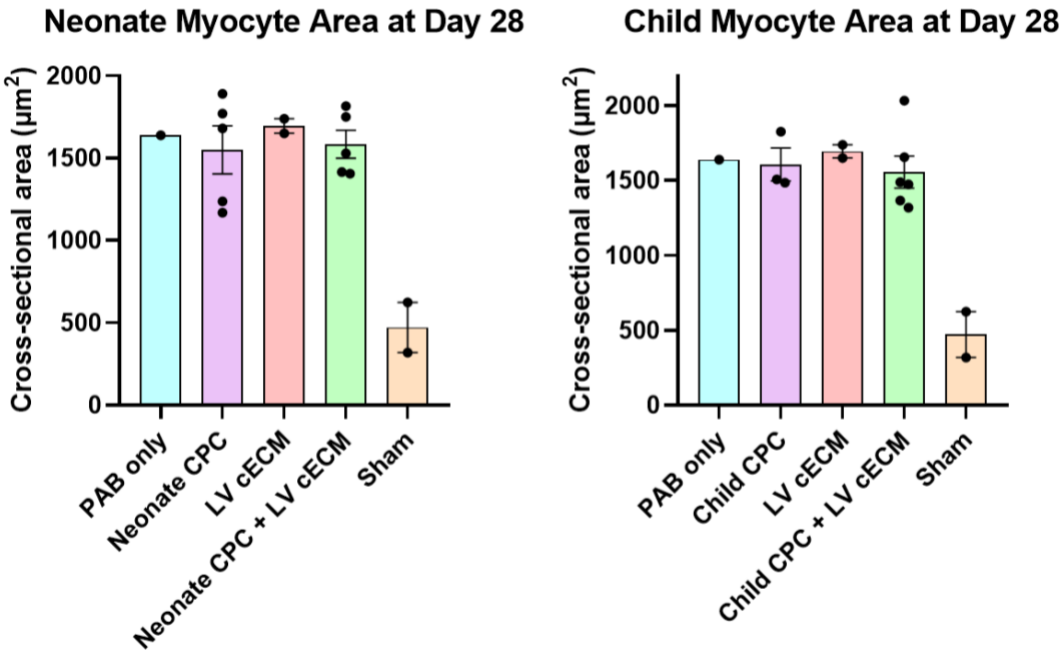

Figure 6. Histological Analysis. Cross-sectional areas of cardiomyocytes after WGA staining. All data measured in units of µm² and analyzed using One-way ANOVA with Tukey's Test for Post Hoc Analysis across all groups.

Although not significant, the results show a slight decrease in myocyte areas for hearts injected with CPC only or encapsulated CPC in LV cECM hydrogels, compared to the negative control group that did not receive any therapeutic injections. Decreased areas indicate decreased myocyte hypertrophy. Trends were similar for both child and neonate CPC groups.

4. Discussion

Some possible areas of improvements for in-vitro tube formation assay include increasing consistency in evaluating the amount of paracrine release for each treatment group. Because of the 3-D encapsulated format of CPCs in either hydrogels or collagen gels, it is possible that growth factors in the gels did not fully get released into the conditioned media. One way to account for this would be to Perform BCA assay, a protein quantification assay, prior to tube formation assay to normalize the protein content in each of the groups. Time point could be one other limiting factor. The CPCs were encapsulated in hydrogels or collagen for only 7 days, and the conditioned media collected was the media added to these encapsulations only 1 day prior to media collection. Also, for the actual tube formation assay, the conditioned media was added to cardiac endothelial cells for 2 days only. Potentially over a longer time, more paracrine factors from the treatment groups could be fully released into the conditioned media we test, and this would more accurately represent how these treatments would behave when used for human patients.

For both in-vitro studies, we should increase the sample size of RV failure models to further explore the effects of therapeutics in cardiac functional outcome, especially because this is a pilot study. The surgery used to mimic RV dysfunction in HLHS patients was the PAB surgery; PAB surgery leads to RV ventricular failure, but the animal models still have the LV to function whereas real HLHS patients do not. This is an area of limitation, and therefore, adding more sample sizes to our study would especially be necessary to correctly mimic the heart of a real HLHS patient.

Overall, although the results are promising, we cannot conclude yet the therapeutic effects of CPCs encapsulated in LV cECM hydrogels for both in-vitro and in-vivo studies. In terms of paracrine release and improving cardiac reparative functions in RV failure rat models, using CPCs for both neonatal and child cells tend to show improvements for our study. This confirms with the previously published studies of CPCs in improving cellular function and cardiac remodeling and function. However, we would need further study to increase significance in understanding the enhanced benefits of using CPCs and LV cECM hydrogels as a combined method compared to standalone methods.

References

1. Grossfeld P, Ye M, Harvey R. Hypoplastic left heart syndrome: new insights. Circ Res. 2007;100(9):1246-8.
2. Bittle G. J., Morales D, Deatrick K.B., Parchment N, Progyaparamita S, Mishra R, Kaushal S. Stem Cell Therapy for Hypoplastic Left Heart Syndrome. *Circulation Research*. 2018;123:288–300
3. Bove EL. Current status of staged reconstruction for hypoplastic left heart syndrome. Pediatr Cardiol. 1998;19:308-15.
4. Robbins JM, Bird TM, Tilford JM, Cleves MA, Hobbs CA, Grosse SD and Correa A.
5. Hernandez, M. J., Gaetani, R., Pieters, V. M., Ng, N. W., Chang, A. E., Martin, T. R., . . . Christman, K. L. Decellularized Extracellular Matrix Hydrogels as a Delivery Platform for MicroRNA and Extracellular Vesicle Therapeutics. Advanced Therapeutics. 2018;1 (3):1800032.
6. Arnold, R. R., Loukanov, T., & Gorenflo, M. (2014). Hypoplastic left heart syndrome - unresolved issues. *Frontiers in pediatrics*, *2*, 125. https://doi.org/10.3389/fped.2014.00125
7. Si, M. S., & Ohye, R. G. (2017). Stem cell therapy for the systemic right ventricle. *Expert review of cardiovascular therapy*, *15*(11), 813–823. https://doi.org/10.1080/14779072.2017.1379898
8. Mahle, W. T., Spray, T. L., Wernovsky, G., Gaynor, J. W., & Clark III, B. J. (2000). Survival after reconstructive surgery for hypoplastic left heart syndrome: a 15-year experience from a single institution. *Circulation*, *102*(suppl_3), Iii-136.
9. Agarwal, U., Smith, A. W., French, K. M., Boopathy, A. V., George, A., Trac, D., Brown, M. E., Shen, M., Jiang, R., Fernandez, J. D., Kogon, B. E., Kanter, K. R., Alsoufi, B.,

Wagner, M. B., Platt, M. O., & Davis, M. E. (2016). Age-Dependent Effect of Pediatric Cardiac Progenitor Cells After Juvenile Heart Failure. Stem cells translational medicine, 5(7), 883–892. https://doi.org/10.5966/sctm.2015-0241

10. Agarwal U, Smith AW, French KM., et al. Stem Cells Transl Med. 2016;5:883-92. PMC4922847
11. Gordon BM, Rodriguez S, Lee M, Chang RK. Decreasing number of deaths of infants with hypoplastic left heart syndrome. J Pediatr. 2008;153:354–358
12. Ishigami S, Ohtsuki S, Tarui S, Ousaka D, Eitoku T, Kondo M, Okuyama M, Kobayashi J, Baba K, Arai S, Kawabata T, Yoshizumi K, Tateishi A, Kuroko Y, Iwasaki T, Sato S, Kasahara S, Sano S and Oh H. Intracoronary autologous cardiac progenitor cell transfer in patients with hypoplastic left heart syndrome: the TICAP prospective phase 1 controlled trial. Circ Res. 2015;116:653-64.
13. Ungerleider, J. L., Johnson, T. D., Rao, N., & Christman, K. L. (2015). Fabrication and characterization of injectable hydrogels derived from decellularized skeletal and cardiac muscle. METHODS, 15–17. https://doi.org/10.1016/j.ymeth.2015.03.024
14. Gaetani R, Yin C, Srikumar N, Braden R, Doevendans PA, Sluijter JP and Christman KL. Cardiac-Derived Extracellular Matrix Enhances Cardiogenic Properties of Human Cardiac Progenitor Cells. Cell Transplant. 2016;25:1653-1663.
15. Seif-Naraghi SB, Horn D, Schup-Magoffin PJ and Christman KL. Injectable extracellular matrix derived hydrogel provides a platform for enhanced retention and delivery of a heparin-binding growth factor. Acta biomaterialia. 2012;8:3695-703. PMC3429632

16. Trac, D., Maxwell, J. T., Brown, M. E., Xu, C., & Davis, M. E. (2019). Aggregation of Child Cardiac Progenitor Cells Into Spheres Activates Notch Signaling and Improves Treatment of Right Ventricular Heart Failure. Circulation Research. 2019;124 (4):526-538.
17. Kritzmire, S. M., & Cossu, A. E. (2020). Hypoplastic Left Heart Syndrome.
18. Imoto, Y. U. T. A. K. A., Kado, H. I. D. E. A. K. I., Asou, T., Shiokawa, Y., Takeda, K. O. I. C. H. I. R. O., & Yasui, H. I. S. A. T. A. K. A. (1995). Valve replacement in an infant with congenital mitral stenosis--report of a case which showed hemodynamics similar to that of hypoplastic left heart syndrome in neonatal period. [Zasshi][Journal]. Nihon Kyobu Geka Gakkai, 43(1), 55-59.
19. Centers for Disease Control and Prevention. (2022, January 24). Congenital heart defects - facts about hypoplastic left heart syndrome. Centers for Disease Control and Prevention. Retrieved April 4, 2022, from https://www.cdc.gov/ncbddd/heartdefects/hlhs.html.
20. Ohye, R. G., Schranz, D., & D'Udekem, Y. (2016). Current therapy for hypoplastic left heart syndrome and related single ventricle lesions. Circulation, 134(17), 1265-1279.