

**Patterned Arteriole-Scale Vessels Enhance Engraftment,
Perfusion, and Maturation of Engineered Human
Myocardium for Heart Regeneration**

by

Rajeev Kant

Brown University

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor
of Philosophy in Biomedical Engineering

PROVIDENCE, RI

May 2022

© Copyright 2022 by Rajeev Kant

This dissertation by Rajeev Kant is accepted in its present form by the Biomedical Engineering graduate program as satisfying the dissertation requirement for the degree of Doctor of Philosophy.

Date: _____

Kareen L. K. Coulombe, Ph.D., Advisor

Recommend to the Graduate Council

Date: _____

Anita Shukla, Ph.D., Committee Chair

Date: _____

Jonghwan Lee, Ph.D., Reader

Date: _____

Peng Zhang, M.D., Reader

Date: _____

Michael Laflamme, M.D., Ph.D., External Reader

Approved by the Graduate Council

Date: _____

Andrew Campbell, Ph.D., Dean of the Graduate School

Curriculum Vitae

Rajeev J. Kant

903 Providence Place APT 287 | Providence, RI 02903 | rajeevjkant@gmail.com | (571) 314-1034

EDUCATION

Ph.D., Biomedical Engineering Sept. 2016 – May 2022

Brown University, Providence, RI

Dissertation: Patterned arteriole-scale vessels enhance engraftment, perfusion, and maturation of engineered human myocardium for heart regeneration^{1, 3, 7}

Advisor: Dr. Kareen Coulombe, Ph.D.

B.S., Materials Science and Engineering Sept. 2012 - June 2016

University of Washington, Seattle, WA; GPA: 3.47 (3.68 in major)

Thesis: Graphene Quantum Dots for Fluorescent Multimodal Bioimaging⁷

Advisor: Dr. Miqin Zhang, Ph.D.

Graduated with College Honors

PEER-REVIEWED PUBLICATIONS

1. **R. J. Kant**, K. D. Dwyer, J. H. Lee, C. Polucha, M. Kobayashi, S. Pyon, A. Soepriatna, J. Lee, and K. L. K. Coulombe, "Patterned arteriole-scale vessels enhance engraftment, perfusion, and maturation of engineered human myocardium for heart regeneration", in preparation.
2. C. M. Kofron, T. Y. Kim, F. Munarin, A. H. Soepriatna, **R. J. Kant**, U. Mende, B. R. Choi, and K. L. K. Coulombe, "A predictive *in vitro* screening platform for pro-arrhythmic toxicity testing using human 3D cardiac microtissues", *Sci Rep*, 11(1), May 2021.
3. **R. J. Kant**, C. F. Bare, and K. L. K. Coulombe, "Tissues with patterned vessels or protein release induce vascular chemotaxis in an *in vitro* platform", *Tissue Eng. Part A*, 27 (19-20), 1290-1304, Oct 2021.
4. F. Munarin, **R. J. Kant**, C.E. Rupert, A. Khoo, and K. L. K. Coulombe, "Engineered human myocardium with local release of angiogenic proteins improves vascularization and cardiac function in injured rat hearts", *Biomaterials*, 251(120033), Apr. 2020.
5. N. J. Kaiser, J. A. Bellows, **R. J. Kant**, and K. L. K. Coulombe, "Digital design and automated fabrication of bespoke collagen microfiber scaffolds," *Tissue Eng Part C: Methods*, 25(11), 687-700, Nov. 2019.
6. N. J. Kaiser, **R. J. Kant**, A. J. Minor, and K. L. K. Coulombe, "Optimizing blended collagen-fibrin hydrogels for cardiac tissue engineering with human iPSC-derived cardiomyocytes", *ACS Biomater Sci Eng*, 5(2), 887-899, Dec. 2018.
7. **R. J. Kant** and K. L. K. Coulombe, "Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues", *Acta Biomater*, 69, 42-62, Mar. 2018.
8. H. Wang, R. Revia, K. Wang, **R. J. Kant**, Q. Mu, Z. Gai, K. Hong, and M. Zhang, "Paramagnetic properties of metal-free boron-doped graphene quantum dots and their application for safe magnetic resonance imaging", *Adv Mat*, vol. 29, Mar. 2017.
9. Q. Mu, F. M. Kievit, **R. J. Kant**, G. Lin, M. Jeon, and M. Zhang, "Anti-HER2/neu peptide-conjugated iron oxide nanoparticles for targeted delivery of paclitaxel to breast cancer cells", *Nanoscale*, vol. 7, pg. 18010-18014, Nov. 2015.

RESEARCH EXPERIENCE

Graduate Research Assistant¹⁻⁷ Sept. 2016 – Present

Brown University, School of Engineering, Center for Biomedical Engineering

Ph.D. Advisor: Dr. Kareen Coulombe, Ph.D.

- Creating engineered cardiac tissues made of cardiomyocytes differentiated from human induced pluripotent stem cells.^{1, 2, 3, 4, 5}
- Designing a perfusion bioreactor to culture engineered cardiac tissues with sacrificially patterned conduits for hierarchical vascular formation.¹
- Developing surgical skills for *in vivo* studies pertaining to implantation of engineered cardiac tissues in a rodent model of ischemia/reperfusion myocardial infarction.^{1, 4}
- Devising methodologies for 3D reconstruction and origin tracing of host and implanted engineered cardiac tissue vasculature using optical computed tomography and micro-CT.¹
- Implementing scale-up procedures for translational applications of cellular therapeutics for heart regeneration.

Undergraduate Honors Thesis Research⁸ Sept. 2015 – June 2016

University of Washington, Department of Materials Science and Engineering

Thesis Advisor: Dr. Miqin Zhang, Ph.D.; Mentor: Dr. Hui Wang, Ph.D.

- Developed synthesis and processing procedures for novel photoluminescent quantum dots composed of boron-doped graphene.
- Performed characterization techniques including FTIR, Fluorescence X-ray Spectroscopy, UV/Vis Spectroscopy, cell viability, and confocal imaging.
- Helped design and implement an automated temperature acquisition monitor for near-IR laser exposure experiments with an Arduino.

Undergraduate Research Assistant⁹ Oct. 2014 – Sept. 2015

University of Washington, Department of Materials Science and Engineering

PI: Dr. Miqin Zhang, Ph.D.; Mentor: Dr. Qingxin Mu, Ph.D.

- Studied effectiveness of iron oxide nanoparticles for targeted delivery of cancer drugs to tumor sites via cell-based assays such as viability, uptake, and cell-cycle analysis.
- Measured iron content of nanoparticles using analytical chemistry techniques and characterization tools such as size exclusion chromatography, UV/Vis, and DLS.

Undergraduate Research Assistant June 2014 – Aug. 2014

University of Washington, Department of Pathology

PI: Dr. Lawrence Loeb, Ph.D.; Mentor: Dr. Ahn Eun, Ph.D.

- Investigated carcinogenesis using next generation sequencing methods to identify ultra-low frequency mutations in the mitochondrial genome of human breast epithelial cells.
- Optimized Python scripts to reduce sequencing times and developed Excel macros to speed up data analysis and processing workflow.

Undergraduate Research Assistant Feb. 2013 – Mar. 2014

University of Washington, Department of Pathology

PI: Dr. Michael Laflamme, Ph.D.; Mentor: Benjamin Van Biber

- Performed lab maintenance tasks including autoclaving and preparing reagents.

- Studied the effects of artificial selection in heterogeneous cultures of GFP-expressing human embryonic stem cells using flow cytometry.
- Developed advanced skills in tissue culture through lab projects and teaching aseptic technique to new lab members.

ACADEMIC PROJECTS

Physio-Control Corporation: Defibrillator Failure Analysis Sept. 2015 – Mar. 2016

MSE 491, 492 - Design in Materials Engineering

University of Washington, Department of Materials Science and Engineering

- Partnered with medical equipment manufacturer Physio-Control to determine root cause of intermittent displays issues on defibrillators.
- Developed and implemented cost-efficient screening solution into the company's workflow to prevent further defective units from passing the manufacturing line.
- Performed economic and Gage R&R reliability analysis to evaluate proposal feasibility.

UW Medicine: Limb Cooling for Acute Limb Ischemia Sept. 2015 – Dec. 2015

ME 414 - Engineering Innovation in Medicine

University of Washington, Department of Mechanical Engineering

- Collaborated with a team of students lead by a vascular surgeon to develop a medical device for limiting ischemic damage in limbs when blood circulation is cut off.
- Identified device needs and constraints from market and clinical research, interviews with physicians and patients, and design, selection, and analysis matrices.

Self-Assembling Organic Nanowires Jan. 2015 – June 2015

MSE 312, 313 - Integrated Junior Laboratory

University of Washington, Department of Materials Science and Engineering

- Optimized synthesis protocols of solution-processed polymer nanowires for use in organic transistors.
- Characterized crystal structure and optical properties of nanowires using techniques including XRD and UV/Vis Spectroscopy.

The Boeing Company: Torque-Tension Table Analysis Sept. 2014 – Dec. 2014

MSE 310 - Introduction to Materials Science and Engineering

University of Washington, Department of Materials Science and Engineering

- Collaborated with Boeing engineers to assess the accuracy of utilized torque-tension tables.
- Determined that using separate nut factor values for fasteners with dry lubricants significantly improved preload accuracy of bolted joints.

TEACHING & MENTORSHIP

Student Mentorship June 2017 - Present

Brown University, Center for Biomedical Engineering

- Mentorship of undergraduate and graduate students in various wet-lab experiments to complete rotation projects, undergraduate honors thesis projects, and undergraduate summer teaching and research award (UTRA) projects.

- Stephanie Roser, B.S., *Biomedical Engineering M.S. '22*
 - Project: “Efficient differentiation and arterial specification of human induced pluripotent stem cell-derived endothelial cells”
- Stephen Pyon, Sc.B., *Biomedical Engineering '24*
 - Project: “Histological evaluation of implanted ECTs containing patterned vessels”
- Momoka Kobayashi, Sc.B., *Biomedical Engineering '24*
 - UTRA: “Dynamic perfusion culture of engineered cardiac tissues”
- Andrea Lamas, Sc.B., *Biomedical Engineering '22*
 - UTRA: “Alignment of cardiomyocytes in engineered tissue through biophysical growth cues”
- Helen Belato, B.S., *Molecular Pharmacology and Physiology Ph.D. Candidate*
 - Rotation Project: “Biophysical creep conditioning of cardiomyocyte maturity in engineered human cardiac tissue”
- Yusuke Suita, B.S., *Molecular Pharmacology and Physiology Ph.D. Candidate*
 - Rotation Project: “Cardiac spheroid formation and evaluation in cardiotoxicity modeling”
- Colette Bare, Sc.B., *Biomedical Engineering '19*
 - UTRA and Honors Thesis: “An *in vitro* model of vascular ingrowth and inosculation”
- Amelia Khoo, Sc.B., *Biomedical Engineering '18*
 - UTRA and Honors Thesis: “Inducing vascular growth into patterned engineered cardiac tissue *in vivo*”

Course Instructor

Jan. – May 2019, Jan – May 2020

Lincoln School Introduction to Engineering
Brown University and The Lincoln School

- Taught a practical introductory engineering course to high school-level students from The Lincoln School, an all-girls private academy.
- Developed a 12-week long engineering-focused curriculum covering a variety of topics from the engineering design process, human-centered design, writing and presentation skills, material mechanics, biomaterials, and circuitry.
- Collaborated with the Brown Design Workshop to teach the students how to use various pieces of engineering equipment such as 3D printers, laser cutters, and woodworking equipment and apply them in projects to build an Arduino-controlled prosthetic device.
- Overhauled course curriculum in response to campus shutdown due to COVID-19 pandemic and developed online lab modules to provide an effective and interactive remote learning experience.
- Created open-source tutorial packets for project materials and curriculum to share with future instructors for training and implementation in other schools.

Course Instructor, Teaching Assistant

July 2017, July 2018, July 2019

CEEN 0924 – Engineering Biomedical Systems
Brown University, Summer@Brown Program

- Developing and teaching course curriculum including lectures, activities, labs, and lab tours to provide high school students with a university-level introduction to concepts and research in the biomedical engineering field.
- Led lab tours for high school students and held QA sessions for college applications.

Teaching Assistant

Fall 2017, Fall 2018

ENGN 1490 – Biomaterials

Brown University, School of Engineering

- Coordinated and lead team of four TAs in scheduling grading and office hours.
- Prepared and presented a review lecture for a comprehensive exam.
- Helped prepare weekly problem sets and organize course website materials.
- Held weekly office hours and graded student assignments and exams.

Guest Lecturer

Sept. 2017

ENGN 1520 – Cardiovascular Engineering

Brown University, School of Engineering

- Developed and presented a lecture on the structure and function of the cardiovascular system.
- Led discussion session aimed at understanding and synthesizing the results of a journal article regarding techniques in therapeutic angiogenesis.

Teaching Certificate Program

Sept. 2017 – Dec. 2017

Sheridan Teaching Seminar – Reflective Teaching

Sheridan Center for Teaching and Learning, Brown University

- Engaged in readings and workshops designed to improve teaching ability through learning and applying principles of learning design and evaluative learning, critical analysis and peer discussion of techniques, and practical teaching observation sessions.
- Led lectures, review sessions, and discussions of scientific articles in biomedical engineering undergraduate courses.

PRESENTATIONS & CONFERENCES**Brown/Yale Cardiovascular Student Research Symposium**

May 2021

Warren Alpert Medical School, Brown University, Providence, RI

Yale School of Medicine, Yale University, New Haven, RI

- Presentation (Virtual): “Vascular chemotaxis informs host-implant inosculation of engineered cardiac tissues for heart regeneration”

Cardiovascular Research Center Seminar

Mar. 2020, May 2021

Cardiovascular Institute and Warren Alpert Medical School, Brown University, Providence, RI

- Presentation (2021, virtual due to COVID-19): “*In vitro* evaluation of vascular chemotaxis to inform host-implant inosculation for heart regeneration”
- Presentation (2020 virtual due to COVID-19): “An *in vitro* model of host-implant inosculation using the aortic ring assay”

International Society for Applied Cardiovascular Biology Biennial Meeting

Mar. 2021

Tokyo, Japan (Shifted to virtual due to COVID-19)

- Platform Presentation (virtual due to COVID-19): “*In vitro* evaluation of vascular chemotaxis to inform host-implant inosculation for heart regeneration”

Biomedical Engineering Society Annual Meeting

Oct. 2019, Oct. 2017

Philadelphia, PA (2019); Phoenix, AZ (2017)

- Platform Presentation (2019): “*In vitro* modeling of host-implant vascular connections”
- Platform Presentation (2017): “Developmental Biomimicry for Maturation of Engineered Human Cardiac Tissue” (Presented in place of Dr. Kareen Coulombe, Ph.D. advisor)
- Poster (2017): “Sacrificial Fiber Patterning for Vascularization and Inosculation of hiPSC-Derived Cardiac Tissues”

North American Vascular Biology Organization - Vascular Biology 2018

Oct. 2018

Newport, RI

- Platform Presentation (2019): Poster: “Engineering Vascular Perfusion Between Host and Implanted Tissues for Myocardial Regeneration”

Cardiovascular Regenerative Engineering Symposium

July 2017, Feb. 2018

Yale University, New Haven, CT (2018); Brown University, Providence, RI (2017)

- Poster (2018): “Engineering Vascular Perfusion Between Host and Implanted Tissues for Myocardial Regeneration”
- Presentation (2017): “Sacrificial Fiber Patterning for Vascularization and Inosculation of hiPSC-Derived Cardiac Tissues”

AWARDS & HONORS

University of Washington Dean’s List

2014 – 2016

Eugene A. White Scholarship

2015

SERVICE

Habitat for Humanity of Greater Providence

Feb. 2021 - Present

Providence, RI

- Assisting in building new homes and repairing existing homes for local underserved communities of Providence, RI
- Helping coordinate recruitment and outreach efforts with the HabitatPVD Young Professionals Chapter (inaugural member) for community building events.

Temporary General Support – Rhode Island Hospital

Mar. 2021 - Present

Providence, RI

- Providing administrative and non-procedural support to healthcare provider teams to facilitate patient care during hospital staffing shortages due to COVID19 pandemic.
- Worked directly with patients as a liaison with their care team to communicate and address individualized needs.

Program Climate Committee, Diversity and Inclusion Action Committee - Biomedical Engineering and Biotechnology Graduate Advisory Board

June 2019 – Present

Brown University, Center for Biomedical Engineering

- Co-founded graduate student advocacy group to communicate needs of the student body to faculty and staff and build inter-program community.
- Arrange bi-annual town halls to evaluate climate of the program and enact positive changes.

- Develop modules for individual lab groups that focus on teaching how to have effective and meaningful discussions pertaining to diversity, equity, and inclusion.
- Assist in coordinating Center-wide events including Ph.D. recruitment and seminars.

Coulombe Lab Outreach Programs

June 2017 – Present

Brown University, School of Engineering

- Helping design and present activities to teach the anatomy and physiology of the heart, as well as promote higher education in STEM fields to K-12 students.
- Holding outreach events to expose students in the community (including Vartan Gregorian Elementary School, Lincoln School, and SPIRA Engineering Camp) to biomedical engineering and its connections to other math, science, and engineering disciplines.
- Developing free modules to teach cardiac anatomy and physiology for elementary, middle, and high school instructors to incorporate into their lesson plans.

Peer/Alumni Mentor Program

Oct. 2015 – Aug. 2019

University of Washington, College of Engineering

- Providing academic guidance for pre-engineering students regarding departmental expectations, coursework, research opportunities, and student organizations.
- Engaging students in quarterly events to foster peer-to-peer connections within the engineering community, and to publicize opportunities available to undergraduate students such as scholarships and internships.

Social Chair - Graduate Biomedical Engineering Society

May 2017 – May 2018

Brown University, Center for Biomedical Engineering

- Planned and hosted events for the biomedical engineering and biotechnology programs to foster community within and between departments.
- Supported faculty in the planning and execution of department-wide events and retreats.

Undergraduate Laboratory Course Revisions

July 2015 – Sept. 2015

University of Washington, Department of Materials Science and Engineering

- Collaborated with faculty to institute changes to junior laboratory experiments and course materials.
- Assisted in refitting and replacing department equipment including polishing wheels and a Jominy End Quench unit to meet laboratory and ASTM standards.
- Designed and 3D-printed supplemental equipment using SOLIDWORKS and MakerBot for a visual activity to demonstrate crystal lattices.
- Filmed and edited instructional videos to familiarize students with the experiments and improve time management during lab rotations.

Engineering Discovery Days

Mar. 2015 – Apr. 2015

University of Washington, College of Engineering

- Created and processed various ceramics using slip-casting techniques.
- Managed the ceramics booth and taught K-12 students the principles of slip-casting.

Community Hall Council

Oct. 2012 – June 2013

University of Washington, Residence Community Student Association

- Helped institute lower-cost room options in residence halls to address student concerns about increased housing fees due to administrative housing redevelopment plans.
- Planned and hosted events and social gatherings both within and between residence halls to foster community and spread awareness of campus resources.

WORK EXPERIENCE

Canvas & Catalyst Consultant

Dec. 2013 – Aug. 2014

University of Washington Information Technologies

- Provided administrative support for the University's learning management systems through emails, phone calls, and in-person consultations with students, staff, and faculty.
- Designed and taught workshops to improve consultant-client relations.
- Trained and helped acclimate new employees to office culture.

Housing Ambassador

Feb. 2013 – Apr. 2013

University of Washington Housing & Food Services

- Represented the University of Washington's Housing and Food Services in matters concerning the residence halls by leading tours and hosting open house events for prospective students.
- Marketed the dormitories to prospective students and cultivated community relations through events.

Dedication & Acknowledgements

I would like to express my heartfelt gratitude to so many people that have been integral to my time at Brown. Thank you to all members of the Coulombe Lab, who have contributed to such an incredibly fun and collaborative work environment for the past 6 years. Special thanks to Cassady, Nick, and Fabiola for teaching me everything I know about everything, and giving me the confidence to be the next senior grad student of the lab. To Kiera, your incredible expertise, compassion, and quirky wit made even the hardest of days enjoyable, and our research was all the better for it. Thank you for being there as both my friend and colleague as we pushed this work over the finish line.

To Kareen, thank you for your incredible mentorship, for continually pushing me to be a better scientist, for being my ally and teaching me how to advocate for myself, and being nothing but supportive of my aspirations. All that's left is to teach me how to win against Evalyn in duckpin bowling. Or cornhole. Or Code Names. Or anything. Please. Just once.

To my dissertation committee members both past and present (Dr. Anita Shukla, Dr. Jonghwan Lee, Dr. Peng Zhang, Dr. Michael Laflamme, Dr. Jeffery Scott, and Dr. Ruhul Abid), thank you for your generous guidance, expertise, and candid feedback. Your help in honing my science was instrumental in accomplishing all that I have.

To my family, thank you for your unconditional love and encouragement to continue pursuing my passion for science and medicine, even if you had no idea what I was talking about when I tried to explain what I did (I may have some light reading to recommend).

Finally to my friends, whether we first met in elementary school or just in the past month, thank you for your love and support all the way through to the end of 22nd grade. Thank you for making Brown feel like home, and for giving me an escape to Washington, Hawaii, Thailand, or countless other states and countries when I needed a break. I could not have done this without you all, and I'm lucky and grateful to have you all in my life.

Here's to the start of 23rd grade.

Contents

Curriculum Vitæ	iv
Dedication & Acknowledgements	xii
1 Introduction	1
1.1 The global burden of cardiovascular disease and heart failure	1
1.1.1 Pathophysiology of myocardial infarction	2
1.1.2 Current clinical interventions	4
1.2 The next generation of therapy for heart regeneration	6
1.2.1 Cell sources for heart regeneration	6
1.2.2 Differentiation of hPSCs into cardiomyocytes	7
1.2.3 3D cardiac tissue engineering	8
1.2.4 <i>In vitro</i> maturation strategies of hPSC-CMs	10
1.2.5 Preclinical application of hPSC-CMs	12
1.3 Clinical trials using hPSC-CMs	16
1.4 Project objective and scope	17
1.4.1 Specific Aim 1: To develop a platform for <i>in vitro</i> evaluation of pre-inosculative vascular migration	18
1.4.2 Specific Aim 2: To assess the structural and functional effects of patterning arteriole-scale channels in engineered human myocardium	19
1.4.3 Specific Aim 3: To evaluate the ability of vascularized EHM to engraft, improve perfusion, and remuscularize the heart using a ro- dent model of ischemia/reperfusion myocardial infarction	19

1.5	References	20
2	Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues	47
2.1	Abstract	47
2.2	Introduction	49
2.3	Vessel development and physiology	51
2.3.1	Vasculogenesis	52
2.3.2	Angiogenesis	52
2.3.3	Arteriogenesis	54
2.3.4	Lymphvasculogenesis and lymphangiogenesis	55
2.4	Cells in vascular tissue engineering research	56
2.4.1	Endothelial cell types	56
2.4.2	Co-culture with endothelial cells	59
2.5	Factors and signaling pathways in angiogenesis	60
2.5.1	Vascular endothelial growth factor (VEGF)	61
2.5.2	Fibroblast growth factor (FGF)	62
2.5.3	Platelet derived growth factor (PDGF)	63
2.5.4	Other growth factors and cytokines contributing to angiogenesis . .	64
2.5.5	The necessity of combinatorial growth factor use	67
2.6	Techniques for controlling angiogenesis with biomaterials	68
2.6.1	Matrix incorporation	69
2.6.2	Encapsulation	73
2.6.3	Topographical engineering	76
2.6.4	3D bioprinting	79
2.7	Beyond <i>in vitro</i> analysis of combinatorial angiogenesis strategies	83
2.7.1	<i>Ex vivo</i> models of angiogenesis	84

2.7.2	<i>In vivo</i> models of angiogenesis	85
2.7.3	Analysis of <i>in vivo</i> progress of vascularization	89
2.8	Conclusions	94
2.9	References	96
3	Tissues with patterned vessels or protein release induce vascular chemotaxis in an <i>in vitro</i> platform	127
3.1	Abstract	127
3.2	Introduction	128
3.3	Materials & methods	131
3.3.1	Sacrificial alginate fiber fabrication and construct gel casting	131
3.3.2	Endothelial cell culture	133
3.3.3	Engineered vessel formation	133
3.3.4	Aortic ring harvest and placement	133
3.3.5	Imaging	134
3.3.6	Outgrowth measurements	134
3.3.7	Model protein release	135
3.3.8	Immunohistochemistry	135
3.3.9	Image analyses	136
3.3.10	Statistical analysis	137
3.4	Results	137
3.4.1	Culture mold design enables co-culture of aortic rings with constructs containing engineered vessels	137
3.4.2	Endothelialized channels form functional engineered vasculature and exhibit sprouting	138
3.4.3	Global vascular outgrowth is natively influenced by physical cues but overridden by paracrine or chemical signaling	140
3.4.4	Vascular outgrowth distance is increased toward constructs by paracrine signaling from endothelial cells and growth factors	143

3.4.5	Paracrine endothelial cell signaling and growth factor release improve IF region outgrowth density and cell clustering	145
3.4.6	Network morphology shows cohesive structures with locally released growth factors or endothelial cells in a growth factor-rich environment	148
3.5	Discussion	150
3.6	Conclusion	159
3.7	References	159
4	Patterned arteriole-scale vessels enhance engraftment, perfusion, and maturation of engineered human myocardium for heart regeneration	171
4.1	Abstract	171
4.2	Introduction	172
4.3	Materials & methods	174
4.3.1	hiPSC maintenance	174
4.3.2	hiPSC-CM differentiation, expansion, and lactate selection	175
4.3.3	Cardiac fibroblast maintenance	176
4.3.4	Endothelial cell maintenance	177
4.3.5	Cellular viability, proliferation, and morphological assays	177
4.3.6	Mechanical characterization of EHMs	177
4.3.7	Gelatin-alginate fiber wetspinning	178
4.3.8	Collagen microfiber wetspinning	179
4.3.9	EHM fabrication for <i>in vivo</i> studies	179
4.3.10	GA fiber un-crosslinking and perfusion culture	180
4.3.11	Implantation of EHMs on infarcted hearts and explant	181
4.3.12	Optical coherence tomography	182
4.3.13	Vascular reconstructive analysis and immunohistochemistry	183
4.3.14	Bulk RNA sequencing	184
4.3.15	Data and statistical analysis	185

4.4	Results	186
4.4.1	Media composition influences hiPSC-CM and EC structure and function	187
4.4.2	Coating solution enables local delivery of ECs to lumen walls . . .	188
4.4.3	Dynamic perfusion culture of EHMs	192
4.4.4	P vEHMs demonstrate semi-conserved pattern morphology and improved engraftment	193
4.4.5	P vEHMs inosculate with host vessels and develop larger and more organized vasculature	195
4.4.6	Perfusion of vEHMs <i>in vitro</i> upregulates genes related to cardiomyocyte development and contractility	199
4.5	Discussion	200
4.6	Conclusions	206
4.7	References	206
5	A novel approach to cardiac revascularization	218
5.1	Synergy of Specific Aims	218
5.2	Broader impacts on heart regeneration	219
5.3	Future directions	223
5.4	Conclusions	227
5.5	References	227
A	Supplementary Information Related to Chapter 3	232
B	Supplementary Information Related to Chapter 4	239

List of Tables

2.1 Spatiotemporal control of physical and chemical cues. 126

3.1 List of antibodies used for immunohistochemistry. 136

4.1 Antibodies for immunohistochemical staining. 184

List of Figures

2.1	Graphical abstract.	48
2.2	Wound healing and angiogenesis.	68
2.3	Spatiotemporal control of angiogenesis.	94
3.1	Mold and experiment design.	132
3.2	Endothelialization of empty channels.	139
3.3	Population outgrowth from aorta-derived cells.	141
3.4	Regional outgrowth analysis.	144
3.5	Histological evaluation of outgrowth regions.	146
3.6	Vascular histomorphological evaluation in outgrowth regions.	149
4.1	Overview of hiPSC-CM differentiation, vEHM formation, and <i>in vivo</i> im- plantation.	175
4.2	Mixed media culture differentially impacts EC and CM structure and func- tion.	186
4.3	Formation of patterned vasculature using EC-coated gelatin-alginate fibers.	189
4.4	Fabrication and perfusion culture of vEHMs.	191
4.5	Implants mature <i>in vivo</i> and engraftment is best improved by <i>in vitro</i> per- fusion.	194
4.6	Patterned vasculature increases implant vessel diameter but only <i>in vitro</i> perfusion improves implant vessel organization.	196
4.7	Exploratory RNASeq analysis of implanted tissues suggests perfusion im- proves cardiac development.	198

A.1	Wet spinning of sacrificial alginate fibers.	232
A.2	Engineered vessel cross-section and regression.	233
A.3	Population outgrowth metrics.	234
A.4	Effect of 10% FBS (+ EC _{10FBS}) on + EC outgrowth metrics.	235
A.5	Representative images of vascular outgrowth.	236
A.6	Regional nuclear characterization.	237
A.7	IB4 histological stain marking outgrowing ECs and all other cells.	237
A.8	Subpopulation mask analysis of vimentin stains.	238
B.1	Viability and morphology of ECs and CMs.	239
B.2	ECT compaction and contractile kinetics.	240
B.3	Wet spun collagen microfiber bundles.	240
B.4	Echocardiographic metrics of heart function.	241
B.5	Identification of human cells in implants.	242
B.6	Implant vascularization and human vascular morphology.	243
B.7	Optical coherence tomography imaging workflow.	244
B.8	Supplemental RNASeq analysis of implants.	245
B.9	Gene ontology term analysis.	246

Chapter 1

Introduction

1.1 The global burden of cardiovascular disease and heart failure

Cardiovascular diseases (CVD) continue to be a leading cause of death in the world, with an estimated 17.9 million, or 32%, of deaths in 2019 globally [1]. Heart disease is a major contributor to CVD mortality, representing up to 16% of all deaths alone. This is in part due to increase in life expectancy, as age is a crucial predictive factor for cardiovascular health. Despite near universal increases in average life expectancy in the last couple decades across many countries, there has not been a commensurate increase in healthy life expectancy, leading to decreased quality of life in later years [1, 2]. Indeed, deterioration of cardiac tissue by mechanisms such as hypertrophy, cellular senescence, and molecular pathway dysregulation leads to a number of pathologies related to the heart and vasculature including the development of atherosclerotic plaques, chamber dilation, myocardial thickening, and fibrosis [3, 4].

With a continually aging population and lack of widely available or appropriate thera-

peutic interventions, the pervasiveness of heart disease had led to end stage heart failure (HF) for many patients [4]. In the US alone, the prevalence of HF in populations over 80 was 9.5% in men and 11% in women between 2015-2018 [5]. The prevalence of HF is expected to increase by 46% in the US population from 2012-2030, affecting over 8 million Americans over 18, and representing more than \$70 billion in related health care costs [6]. Thus, there is a great need for new therapies to relieve the burden of CVD before progression into end-stage HF.

1.1.1 Pathophysiology of myocardial infarction

One of the primary causes of heart failure is myocardial infarction (MI), or heart attack [7]. Over time, fatty plaques begin to develop and accumulate in arteries and obscure blood flow. This process, known as atherosclerosis, has several known contributing risk factors including age, diet, diabetes, smoking, and obesity [8]. Adherence of atherosclerotic plaques to coronary endothelium and resulting vascular inflammation causes narrowing of the blood vessel and lower flux of oxygen and nutrients to the myocardium. Myocardial infarction often occurs at these points where acute rupture of the plaque induces an inflammatory thrombotic response which can fully occlude the vessel, resulting in ischemia and necrosis of myocardial tissue [9]. Due to the lack of meaningful regenerative potential of adult myocardium in humans, an MI can cause the irreversible loss of 1 billion cardiomyocytes without clinical intervention within 15-20 minutes [7, 9–11]. MI occurs most often in the left ventricle (LV) due to its role in systemic circulation which necessitates much larger size, working pressures, and oxygen requirements relative to the right ventricle [12]. In practice, right ventricular MI typically co-presents with LV MI.

Consequently, the infarcted myocardium undergoes compensatory remodeling in an effort to stabilize the LV and retain contractile function. Within minutes to hours, the inflammatory response is triggered by ROS generation, chemokine release from necrotic cells,

and other inflammatory pathway-activating molecules termed alarmins [9, 10, 13]. Reperfusion of the myocardium itself by percutaneous intervention paradoxically provides much needed oxygen and nutrients to surviving ischemic cells, but also further injures the downstream tissue through mechanisms including damaging ROS species, calcium overload, massive immune infiltrates, and microvascular obstruction [14–17].

Clearance of necrotic myocardium and matrix debris occurs concomitantly with the formation of granulation tissue over the next few days, resulting in the loss of the initially hierarchical, fibrous architecture of the wall that enables twisting contractions for efficient pumping [18, 19]. Resident cardiac fibroblasts enter an activated myofibroblast-like state and reinforce the weakened ventricular wall through the remodeling of existing ECM as well as deposition and crosslinking of scar tissue over months, primarily composed of collagen [10, 13]. While this mechanism serves to counteract ventricular dilation and prevent possible wall rupture, the scar tissue itself is much stiffer than the native myocardium (55+ kPa versus 10-20 kPa in healthy tissue) and non-contractile [18, 19]. Vascular endothelial cells (ECs) re-enter a pro-angiogenic proliferative phase and begin to remodel vasculature to create new angiogenic sprouts, with later evidence of arteriogenic collateralization to varying degrees depending on species [20–22]. As early-responding neutrophils evacuate and the inflammatory response transitions into the pro-healing phase, supportive macrophages, lymphocytes, and other inflammatory cells remain in smaller numbers for paracrine regulation of neighboring cells [9]. Within 2-3 months, a mature scar has formed in place of the lost myocardium.

Diagnosis of heart failure can occur as early as discharge after MI, though late-onset HF due to continued decline in function in the longer term is more difficult to quantify [23]. The additional stress of contraction in the scar-laden LV to support systemic circulation can result in further dilation of the chamber, compensatory remodeling, arterial stiffening, and arrhythmogenesis [9]. Acute clinical and secondary interventions have shown to improve

prognosis, yet risks still remain [23–25]. Recurrent MI becomes more likely, and a study from 2012 suggested that cumulative risk of a recurrent cardiovascular event even 7 years after an initial MI was still 2-3 times higher than that of a healthy individual [26]. One such proposed mechanism suggests that inflammatory pathways initiated after MI were also atherogenic, leading to increased deposition of atherosclerotic plaque and thus greater susceptibility to further ischemic events [27]. Another retrospective study found that after acute MI, between 70-85% of patients depending on age range developed HF prior to death [28]. Thus HF diagnosis due to acute MI remains a difficult challenge to address.

1.1.2 Current clinical interventions

Acute clinical intervention for MI requires rapid reperfusion of the occluded artery. Current clinical guidelines suggest that intervention within 90 minutes of arrival at a hospital can best minimize permanent ischemic damage, and is often quantified as door-to-balloon (DTB) time [29]. Percutaneous coronary intervention (PCI) is the standard intervention due to its ability to rapidly reperfuse ischemic myocardium and reduce DTB time. Once an MI diagnosis has been confirmed by ECG, PCI is performed by catheterization via the femoral or radial artery into the coronary vascular bed, visually aided by intracoronary imaging to identify the site of obstruction [25]. In balloon angioplasty, an inflatable catheter is positioned at the site of obstruction and repeatedly inflated to compact the plaque against the vessel wall to relumenize the vessel. Atherectomy procedures can also be performed to remove calcified plaques, in which a small drill is used to excavate and remove the plaque buildup [30]. In both cases, a stent is often placed at the occlusion site to maintain patency of the vessel. PCI may also be used to bridge patients to more invasive procedures at a later point.

Following primary intervention, patients are placed on medications (most commonly a triple therapy of ACE inhibitors, beta-blockers, and aldosterone antagonists) to prevent

excess stress on the heart that would otherwise exacerbate maladaptive LV remodeling, dilation, and overall declines in cardiac function [9, 24, 31, 32]. In cases of contraindications to stent placement or stenosis of a placed stent, coronary artery bypass graft (CABG) surgery can be performed to bypass narrowed vessels [33]. However, medication timelines are indefinite, and often these interventions are often not sufficient to fully prevent decline in cardiac function. Eventually, some patients become refractory to medical interventions for HF and require a heart transplant. However, the lack of available organs means that many patients must be placed on mechanical circulatory support. Several technologies exist to facilitate systemic perfusion and can temporarily relieve stress on the heart, such as intra-aortic balloon pumps, Impella, and extra-corporeal membrane oxygenation, though these technologies are used in the short-term and/or otherwise tether patients to a health-care facility [34]. In the long-term, patients can receive a left-ventricular assist device (LVAD) as a longer-term bridge to transplant, though more often than not this has become a destination therapy owing to a severe lack of organs for transplantation [34]. The total artificial heart is another mechanical device that replaces both ventricles with mechanical pumps and is intended as bridge-to-transplant, though ongoing work (such as the clinical trial NCT02232659) is determining its effectiveness as a destination therapy [19, 35]. However, promising results and FDA-approved devices position these devices as primary competitors for cellular therapeutics.

Owing to medical advances in diagnosing HF as well as technical breakthroughs in intervention, improvements have been seen in short term and long-term outcomes of patients diagnosed with HF with decreases in 30-day and 5 year mortality [23, 36]. However, current acute clinical and secondary intervention techniques are still not sufficient to reactively address the post-MI development of HF.

1.2 The next generation of therapy for heart regeneration

1.2.1 Cell sources for heart regeneration

Cellularized therapeutics for heart regeneration is a relatively new field. Due to the inability of mature cardiomyocytes to proliferate in any significant capacity, harvesting or isolating large numbers of cells for therapeutic effect is monumental task. Skeletal myoblasts [37], paracrine signalling from somatic stem cells [38], direct transdifferentiation of fibroblasts [39, 40], cardiac progenitor cells [41], and non-human cardiomyocytes [42] have all been studied with variable success in order to provide therapeutic effect to the heart post-MI [7, 13]. The isolation of human embryonic stem cells (hESCs) became a major impetus for the development of cardiac tissue engineering as they offered the first directly renewable source of human cardiomyocytes [11]. However, despite common usage research to this day [43, 44], their use still has own set of challenges, notably ethical concerns with their sourcing from the inner cell mass of blastocysts, and continued immunosuppression due to the allogenic nature of hESC-derived implants. However, the introduction of human induced pluripotent stem cells (hiPSCs) in 2006 marked a grand milestone in the field, as it provided an ethically-sound source for patient-specific autologous implants. [45]. This technology has since also been used to generate patient-specific hiPSC-lines to better understand disease such as dilated cardiomyopathy [46], and is a powerful application of the hiPSC reprogramming technology to model disease *in vitro* and test potential treatments. hESCs continue to be used in research (though to a lesser extent as more groups adopt hiPSCs), and will collectively be referred to as human pluripotent stem cells (hPSCs).

1.2.2 Differentiation of hPSCs into cardiomyocytes

Recapitulation of the signals and processes present in embryonic development have guided the differentiation of human cardiomyocytes, but originally determined from the study of model organisms including *Xenopus*, zebrafish, and chick embryos [7]. In the early developing *Xenopus* embryo, induction of the mesodermal germ layer is primarily dictated by overlapping gradients spatiotemporal signalling. Maternal deposits of vegetal-localized VegT mRNA and equatorially-located Wnt protein enable dorsal stabilization of β -catenin and specification and anterior mesoderm through downstream Nodal signaling [47–50]. This Wnt signaling is later reversed by dorsoanterior expression of Wnt antagonists. In combination with BMP signaling from the formed mesoderm, cardiogenic mesodermal populations originating from the primitive streak begin to migrate down fibronectin highways and form the first and second heart fields that will define the chambers of the heart [51].

The 2000s proved to be an early renaissance for the field of cardiac tissue engineering beyond just the contributions of hiPSC development. Early differentiation of cardiomyocytes came in the form of gastrulation-mimicking embryoid-body methods, in which pluripotent stem cells (PSCs) were shown to aggregate and spontaneously differentiate into a number of different lineages, including ~ 1 -10% cardiomyocytes [52, 53]. Attempts at 2D monolayer PSC differentiation methods initially relied on additions of lot-dependent and non-defined serum, and xeno-conditioned media, but innovations enabled feeder-free culture and increasingly defined medias and differentiation efficiency [54–57]. Applying developmental biology-inspired understanding of the Wnt, TGF β , and Activin/Nodal signaling pathways led to the reliable recapitulation of mesoderm formation and ventricular cardiac specification *in vitro* with up to 80-90% purity (often evaluated by flow cytometry staining for cTnT) by the early 2010s [50, 58–61]. The most common procedures in hiPSC-CM differentiation today involve induction with BMP-4, bFGF, and Activin-A proteins [62]

and/or small molecule-based biphasic Wnt modulation [63, 64] to generate ventricular cardiomyocytes, and recent innovations in differentiation methodology have enabled the use of stirred tank bioreactors or roller bottles for massive hPSC-CM production on the scale necessary for clinical translation [65, 66]. Recent paradigm shifts have further begun to more closely examine subtype specification such as atrial differentiation by treatment with retinoic acid [67], purification by metabolic selection [68], and post-differentiation proliferation for *in vitro* expansion [69] or *in vivo* therapy [70, 71]. Further applications that have become the forefront of research in cellularized therapies for HF are in 3D cardiac tissue engineering and maturation of the otherwise fetal-like hPSC-CMs [66, 72–74].

1.2.3 3D cardiac tissue engineering

Developing replacement tissues for implantation is one of the main goals of cardiac tissue engineering. As a closer *in vivo* analogue, 3D culture in a tissue format provides more opportunities for cell-cell interactions, cellular co-culture, and importantly formation of a coherent syncytium in cardiac tissue [18]. Together, these components can enable better recapitulation of the 3D composition, structure, and function of native myocardium [75–78].

A popular subfield of *in vitro* cardiovascular research leverages the ability of cardiomyocytes and other cell types to spontaneously form cell aggregates for applications in *in vitro* disease modeling and drug screening [67, 79]. These individual aggregates, often termed microtissues or spheroids, are typically much smaller than would be useful for therapeutic intervention, though some research has examined using these tissues as building blocks for larger-scale tissues [80, 81]. However, the vast majority of cardiac tissue-focused *in vivo* research continues to rely on a biomaterial-based scaffold to provide a robust structure for cells to remodel and form a large cohesive tissue. These scaffolds are commonly made of natural polymers (e.g. collagen, decellularized ECM, and fibrin), or synthetic polymers

(e.g. PEG, PLGA, and PGS) [18]. The decision to use one type of material over another is often determined by the application, fabrication technique (e.g. replica molding [64, 82, 83] vs 3D bioprinting [84–86]), and research question being studied [87]. Typically, natural polymers are better tolerated by the body, have adhesion sites for cells or other bioactive interactions, but can be remodeled and degraded relatively quickly. In contrast, synthetic polymers often have a much wider design space for tuning stiffness and degradation properties, though generally must also be chemically modified to provide cell-adhesion sites and can be tolerated by the body without a negative immune reaction [18, 88].

Another important consideration in 3D tissue fabrication is the ability to easily co-culture hPSC-CMs with supportive cell types. The differentiation process inherently lends itself to a artificial type of co-culture, as "contaminating" nonmyocytes may not fully be removed by purification processes [68]. While the identity of these nonmyocytes is still not well understood, they are generally considered to be "fibroblast-like" based off of morphology, immunohistochemical staining (e.g. vimentin, α -SMA, and matrix remodeling capability, though they are notoriously difficult to characterize and can vary across differentiation batches [89, 90]. While their exact impacts on hPSC-CMs are unclear, they may prove to be beneficial as fibroblasts make up a significant portion of the native heart and contribute significantly to supportive functions for cardiomyocytes including paracrine signaling, ECM remodeling, and electrophysiological function [61, 90]. Research has consistently found that cardiac tissue formation and function is more robust with the inclusion of fibroblasts or other supportive stromal cell types [75, 91, 92] Notably, Tiburcy et al. reported that engineered cardiac tissues composed of ~ 60 -70% hPSC-CMs and ~ 30 -40% fibroblasts were able to generate the greatest amount of force in mechanical testing, suggesting a significant contribution of fibroblasts to cardiac tissue contractile function [93].

Endothelial cells are the most abundant nonmyocytes found in the heart, with a microvascular density of 2000-4000/mm² in the myocardium compared to ~ 800 /mm² in other

tissues[94–97]. While their contributions to vascularization will be discussed in greater detail in Chapters 2 and 4, ECs also influence cardiomyocytes via juxtacrine and paracrine signaling as well to modulate contractility and remodeling [61, 97]. In *in vitro* culture, studies have shown EC-CM co-culture can enable hPSC-CM reorganization, maturation, and apoptosis prevention through the secretion of cardioprotective factors [98–101]. While previous studies examining CM-EC co-implantation have demonstrated intra-implant vascularization and perfusion [82, 102–104], few have been able to show improvements in CM engraftment [105–108].

1.2.4 *In vitro* maturation strategies of hPSC-CMs

The immature nature of differentiated hPSC-CMs remains a great challenge for clinical application. Developmentally, fetal cardiomyocytes have underdeveloped sarcomeres and rely primarily on glycolysis as energy sources. Over the first two decades after birth, the human heart matures through a combination of proliferation, hypertrophy, sarcomere organization, ion channel expression, and mitochondrial biogenesis to support a metabolic switch towards oxidative phosphorylation of fatty acids in order to support the high ATP demand of constant contraction [73, 90, 109, 110]. The structure and function of hPSC-CM in comparison is akin to fetal cardiomyocytes, and while studies examining long-term culture (30-200+ days) of hPSC-CMs have shown improvements in structural, metabolic, and functional markers, it is not presently a feasible timeline for clinical translation [78, 111, 112]. Thus, the field has turned to a number of methods to improve hPSC-CM maturation to provide suitable therapeutic effect.

Media supplementation is a common method of metabolic maturation owing to its relative ease to apply during culture. The standard medium used for hPSC-CM differentiation is RPMI B27, which while suitable was originally used for neural cell culture is not optimal for differentiated cardiomyocytes to mature [60]. The low levels of calcium (0.4

mM in media versus 2.2 mM extracellularly [113]) affects calcium gradients necessary for optimal signaling and can prevent maturation of calcium handling and buffering capacity of cardiomyocytes [73]. RPMI B27 further contains high glucose and lacks fatty acids, which together can encourage a prolonged, glycolysis-dependent metabolic profile [114, 115]. Thus groups have examined media manipulations such as calcium addition [116], growth factors/soluble cues [62, 117, 118], fatty acids [78, 119], and even entirely new media formulations to improve markers of cardiomyocyte maturation [90, 115, 120].

Structural maturation can also be influenced by substrate stiffness and topographical cues. These factors play important roles not only in the culture and structural maturation of cardiomyocytes, but in the stability and differentiation of PSCs as well [121, 122]. Research has shown that *in vitro* culture of CMs on stiff substrates can have a negative impact on ultrastructural organization, contraction kinetics, action potentials, and calcium handling, even despite matrix coatings like Matrigel and laminin [123–125]. Thus, engineering materials to achieve variable stiffness and surface topographies have become popular for *in vitro* perturbation, optimization, and study of hiPSC-CM sarcomere structure and function [66, 104, 120, 126–128].

Cardiomyocyte alignment can also be achieved through cell-induced passive tension due to the remodeling capabilities of engineered cardiac tissues, particularly when doped with a stromal population like fibroblasts. By arresting the ends of tissues with posts, frames, or other materials, remodeling and compaction generates tension lines that cardiomyocytes align with, enabling greater force generation [87, 93, 103, 129]. Post-based methods of cardiac tissue formation are particularly effective at aligning larger tissues when patterned throughout the bulk of the tissue. Despite the void spaces created which reduce the effective number of cells to be delivered per unit area, the unconstrained edges of the tissue can remodel and effectively align cells along free boundaries [130–133]. However, care must be taken to account for this remodeling process, as overcompaction can drive greater stress

generation, resulting in necking and failure of the tissue [134].

Electrical maturation of hPSC-CMs seeks to improve ion channel expression, gap junction formation, and calcium handling abilities to ensure proper activation and propagation of action potentials, coupling to contraction, and repolarization kinetics [90]. Immature hPSC-CMs typically exhibit spontaneous contraction akin to nodal cells due to funny current expression, but entrainment with electrical field stimulation is a common way to reduce or eliminate automaticity, while additionally improving electrophysiological properties [135, 136]. hPSC-CMs are also notorious for lacking T-tubules which function to transmit the action potential signal and enable sarcolemmal calcium-induced calcium release and excitation-contraction coupling. Formation of these structures is typically not seen *in vitro*, with only a few groups reporting their presence [137–139]. Co-culture with fibroblasts has also shown improvements in electrical function as they provide the ability to effectively remodel tissues to form a cohesive syncytium [75, 91]. In particular, Zhang et al. demonstrated distinct differences in modulating the electrical properties of hPSC-CMs when cocultured with hPSC-derived cardiac fibroblasts in comparison to human dermal fibroblasts, suggesting lineage-specific interactions may be crucial for proper electromechanical function [140].

1.2.5 Preclinical application of hPSC-CMs

Preclinical evaluation of cardiac therapies remains the gold standard for determining clinical effectiveness of cell-based therapeutics. In this pursuit, both small (e.g. mice, rats, guinea pigs) and large (e.g. pigs, non-human primates) animal models have been used owing to their similar biology to humans [141, 142]. Methods of inducing MI can vary, leading to different injury and remodeling responses. Permanent ligation of the left anterior descending artery is one of the most common techniques as it creates a massive acute transmural injury [143]. However, this injury often results in higher mortality, as opposed

to ischemia-reperfusion (IR) methods with enable restoration of blood flow after an allotted time of ischemia. IR MI is typically regarded as a clinically relevant model as it parallels the revascularization treatments that are considered the standard of clinical care for MI [143]. This model typically achieves similar transmural infarctions equivalent to that of permanent ligation methods (though this is dependent on the duration of ligation), and provides opportunities to study ischemic preconditioning and reperfusion injury. Ablative cryoinfarction models are another popular model of acute MI, which utilize liquid nitrogen-frozen probes to create cryoinjuries that mimic MI, though with less remodeling and scarring. While less clinically relevant, this method creates more consistent injuries across animals [143, 144]. Lastly, chronic models typically use ameroid/angioplastic constriction, delayed implantation (3-4 weeks), or less commonly hyperlipidemic/atherosclerosis-inducing diets to model chronic HF [143, 144]. These may be considered the most clinically-similar to patient incidence of HF as it models damage due to relatively slow constriction of blood flow over a longer period of time, however limited studies in hPSC-CM implantation in chronic MI models have suggested subpar engraftment and lack of functional improvement [145, 146]. This suggests there is a critical period for implantation of a cellular therapeutic to be incorporated into the remodeling process of the injured myocardium for therapeutic implant, such as at the time of peak inflammation 3-4 days after onset of MI [19].

The idea of using stem cells to regenerate the heart led to the study of adult stem cell-based therapies (e.g. bone marrow-derived stem cells, mesenchymal stem cells, c-kit⁺ cardiac stem cells) prior to the rise of hPSC-specific interventions, and there continues to be debate in the field regarding their efficacy in cardiac repair relative to cardiomyocyte-specific cellular interventions [147]. However, current hypotheses suggest that these interventions offer no long-term engraftment and primarily act in a paracrine fashion, and thus are insufficient for complete repair of the heart post-MI where direct remuscularization therapy may be necessary [7, 145, 147, 148]. Thus, most work in cell-based cardiac remuscularization therapies are focused on the use of hPSC-CMs for therapeutic remuscu-

larization. Though a number of delivery routes have been attempted including pericardial injection and systemic infusion, cell retention and engraftment are commonly accepted to be highest though intramuscular injections directly in the myocardium or an epicardially affixed patch [149].

Intramyocardial injections confer a number of benefits in experimental design. A primary benefit for clinical translation is the ability for minimally invasive delivery, which reduces the potential of intra- and post-operative complications and faster recovery time. Syringe injections also enable direct integration into the wall of the myocardium, which may assist in reinforcing areas of the LV that would otherwise be occupied by noncontractile scar tissue. Direct injections of hPSC-CMs have also shown the ability to couple to the electrical activity of the host, though with some instances of graft-derived focal automaticity which resulted in arrhythmic activity [65, 150, 151]. However, injections innately cause mechanical damage both to the delivered cells as well as injection site as well as cell wash-out [149]. The cells must also be collected by thawing or enzymatic harvest, which are stressful events that can cause cell death, prevents the ability to pattern or otherwise organize the cells, and requires the cells to reassemble their contractile framework upon engraftment.

In contrast, epicardially-implanted tissues have their own set of advantages and challenges. Perhaps the greatest benefit is the ability to engineer the tissue in 3D based on the needs of the study, such as incorporation of growth factor-releasing substrates [64, 141], vascular channels [82, 106], and anisotropic cues [19, 130, 132, 152]. *In vitro* culture time in a tissue format can enable structural and functional maturation as described in the previous section. Cellular incorporation in a biomaterial can also theoretically improve retention and thus engraftment [147]. However, cardiac tissue implantation typically require invasive surgery which invites possible intra- and post-operative complications, and the tissues must also be robust enough for a surgical team to handle. However, there is promising work

in developing flexible [153] or injectable [154] cardiac tissue scaffolds as well as surgical device innovations [155] for minimally invasive tissue implantation. Epicardial attachment is typically achieved with sutures, which damages the myocardium and can also cause tearing in the implant itself. The greatest challenge with ECTs, however, is their electrical isolation from the host heart by scar tissue. Studies examining electrical coupling have shown that while injected cells are able to engraft and couple to the myocardium (despite some arrhythmic issues), cardiac tissue coupling is sparse at best [65, 145, 150]. Current research is seeking ways to overcome this issue through monitoring with external electronics and telemetry, conductive biomaterials, *ex vivo* fluorescence imaging and antifibrotic methodologies [129, 154, 156–159].

Irrespective of implantation method used, recent research has demonstrated that hPSC-CM implantation strategies have collectively demonstrated stable engraftment (measured up 8 weeks), myocardial sparing, functional improvement (based on fractional shortening and/or ejection fraction), and even *in vivo* maturation of implanted cells and tissues across both small and large animal studies [43, 65, 83, 129, 131, 151, 160]. However, there are some common issues that necessitate continued scientific and technological innovation to overcome. While teratoma formation is no longer considered an issue for hPSC-CM implantation, retention and engraftment remain low at about 10-25% of input [157]. Recent estimates suggest a necessary therapeutic hPSC-CM dose of 5M/g heart as a patch or 20M/g via injection [145], which accounts for billions of cardiomyocytes when scaled up to clinical size. Thus, improving engraftment and survival remains a key limiting step in remuscularization therapies. Heat shock protocols and co-implantation with a pro-survival cocktail has shown efficacy in improving engraftment [59, 145, 149]. Stable engraftment is also challenging due to the immune response to implants which necessitate chronic immune suppression protocols. Newer work is seeking to attenuate immune rejection through methods including engineering hypoimmune cells [161] and developing major histocompatibility complex-matched allografts [162]. Arrhythmia incidence is another key concern,

as was described and shown in several large animal models [43, 65, 151, 163]. Lastly, vascularization is a surprisingly often overlooked component of hPSC-CM therapies (particularly those based on intramyocardial injections), as the survival and engraftment of implanted cells is ultimately dependent on oxygen and nutrient flux [105]. As opposed to injections, engineered tissue-based approaches enable greater consideration of this crucial aspect through both *in vitro* and *in vivo* techniques, and is discussed in greater detail in Chapter 2.

1.3 Clinical trials using hPSC-CMs

A number of first-in-human clinical trials have begun using hPSC-derived cardiac progenitors or cardiac cells. The ESCORT trial (NCT02057900) was among the first to deliver CD15+/ISL1+ hESC-derived cardiac progenitors epicardially in a fibrin gel with concomitant CABG to patients with severe left ventricle dysfunction [147, 164]. The study reported no intra- or post-operative events including teratoma formation, arrhythmia, or immune response. Though not intended as a direct remuscularization therapy, it demonstrated the feasibility of biologic interventions for HF and has helped pave the way for hiPSC-CM-based interventions.

Among these are a trial (NCT04982081) for transendocardial injection of 400M allogenic hiPSC-CMs in 20 patients with a focus on safety out to 12 months. The HEAL-HCF trial (NCT03763136) aims to delivery 200M allogenic hiPSC-CM intramyocardially with concomitant CABG in 20 patients with congestive heart failure, and has primary outcomes focused on evaluation of teratoma formation and arrhythmia incidence out to 6 months. The IDCVTCHF trial (NCT03759405) is seemingly the first clinical trial using autologous hiPSC-CM transplants and is seeking to recruit 3 patients, though the trial has been registered since 2018 with few status updates. Another trial from Japan (NCT04696328) is

seeking to implant allogenic hiPSC-CM cell sheets in ischemic cardiomyopathy patients, with primary outcomes evaluating LV ejection fraction up to 26 weeks and incidence of adverse events out to 12 months. The BioVAT HF trial (NCT04396899) is seeking to epicardially transplant allogenic hiPSC-CMs and stromal cells in a bovine type-1 collagen patch, with primary outcomes focused on measuring heart wall thickness and thickening fraction up to 12 months. Lastly, the HECTOR trial (NCT05068674) is seeking to examine the dose response of injecting 50, 150, and eventually 200M allogeneic hESC-CMs intramyocardially to alleviate chronic LV dysfunction.

Cellular therapies using hPSC-CMs continue to show promise with exciting clinical trials that will provide a deeper understanding of how hPSC-CM technology can be leveraged and engineered to improve heart function after MI and prevent further decline.

1.4 Project objective and scope

Despite incredible achievements in cardiac tissue engineering over the past two decades, clinical application remains in its infancy due to insufficient preclinical understanding of engineered cardiac tissue engraftment, mature function, and overall therapeutic effect. We proposed that the latter two criteria are significantly bottlenecked by the former, and thus hypothesized that patterning sufficiently large vasculature conduits in cardiac tissue *in vitro* could be used as a means of rapid perfusion once implanted on the otherwise harsh ischemic infarct region of the heart, thus enabling greater retention, *in vivo* integration, maturation, and ultimately restore contractility to the heart. To evaluate our hypothesis through this dissertation work, we developed a multi-faceted approach separated into three distinct Specific Aims to investigate the fabrication of patterned, arteriole-scale channels and understand their role in vascular inosculation, incorporation into cardiac tissues to form functional engineered human myocardium (EHM), and therapeutic effect in a preclinical model of

ischemia-reperfusion myocardial infarction using state-of-the-art vascular reconstruction and imaging techniques.

1.4.1 Specific Aim 1: To develop a platform for *in vitro* evaluation of pre-inoscultative vascular migration

In Aim 1, we hypothesized that patterned vasculature in engineered tissues could initiate vascular chemotaxis from host tissue and thus facilitate inosculation, rapid perfusion, and cell survival. To test this we developed a channel-patterning technique to create arteriole-scale vessels embedded in collagen 1 gels which acted as a simplified engineered tissue. We then devised a platform for modeling pre-inoscultative vascular migration by adapting the aortic ring assay in a co-culture format with engineered vessels in a collagen gel to mimic *in vivo* host-implant interactions in a controlled and defined *in vitro* system. Broadly, our results demonstrated application of the model to study vascular migration between surrogate host and implantable tissues and the ability to perturb various factors related to angiogenesis including media composition and growth factor release. Our platform and developed tools provide a strong foundation for evaluating complex host-implant pre-inoscultative interactions to better understand, develop, and implement vascular therapeutics *in vivo*.

R. J. Kant, C. F. Bare, and K. L. K Coulombe, "Tissues with patterned vessels or protein release induce vascular chemotaxis in an *in vitro* platform", *Tissue Eng. Part A*, 27 (19-20), 1290-1304, Oct 2021.

1.4.2 Specific Aim 2: To assess the structural and functional effects of patterning arteriole-scale channels in engineered human myocardium

In Aim 2, we sought to improve upon and apply our established vessel-patterning technique in the context of cardiovascular regenerative engineering. We hypothesized that incorporating a large vascular framework into an engineered tissue would necessarily affect the structure and function of the tissue, and understanding this heterogeneous system would be important in developing a stable, implantable tissue. In fabricating and culturing EHMs *in vitro*, we demonstrated the positive impacts of co-cultured media on hiPSC-CM force production and sarcomere hypertrophy relative to control and minimized consequences on EC function. We also developed a dynamic culture system for EHMs containing patterned, arteriole-scale vessels (termed vEHMs) with constant perfusion. Structural evaluation of vEHMs through 2D area measurements and histology showed greater compaction as a result of perfusion and reduced sarcomere development in the bulk of tissues due to an expected loss of passive alignment cues beyond 100-200 μm of tissue edges.

1.4.3 Specific Aim 3: To evaluate the ability of vascularized EHM to engraft, improve perfusion, and remuscularize the heart using a rodent model of ischemia/reperfusion myocardial infarction

In Aim 3 we applied our developed vEHMs in a rodent model of ischemia reperfusion MI to better understand the *in vivo* response of host vasculature, hypothesizing that arteriole-scale perfused vasculature would provide rapid perfusion of nutrients to implanted hiPSC-CMs, allowing for greater survival, engraftment, and consequently improved CM maturation. We demonstrated significantly improved engraftment of perfused vEHMs (P

vEHMs) relative to control EHMs EHMs and non-perfused vEHMs (NP vEHMs), and greatly improved maturation of implanted tissues relative to *in vitro* controls. Vascular analysis through revealed increased vessel diameter and organization due to *in vitro* perfusion of patterned vessels and robust intra-implant vasculature in microCT reconstructions. RNA sequencing of explanted tissues revealed upregulation of pathways implicated in heart development, particularly related to contractility and electrophysiology of hiPSC-CMs.

1.5 References

1. WHO. *Cardiovascular diseases* 2021. [https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-\(cvds\)](https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)).
2. Cao, X. *et al.* A comparative, correlate analysis and projection of global and regional life expectancy, healthy life expectancy, and their GAP: 1995-2025. *Journal of Global Health* **10**. ISSN: 2047-2978. <http://jogh.org/documents/issue202002/jogh-10-020407.pdf> (Dec. 2020).
3. North, B. J. & Sinclair, D. A. The Intersection Between Aging and Cardiovascular Disease. *Circulation Research* **110**, 1097–1108. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.111.246876> (Apr. 2012).
4. Strait, J. B. & Lakatta, E. G. Aging-Associated Cardiovascular Changes and Their Relationship to Heart Failure. *Heart Failure Clinics* **8**, 143–164. ISSN: 15517136. <https://linkinghub.elsevier.com/retrieve/pii/S1551713611001012> (Jan. 2012).
5. Virani, S. S. *et al.* Heart Disease and Stroke Statistics-2021 Update A Report from the American Heart Association. *Circulation* **143**, E254–E743. ISSN: 15244539.

- <https://www.ahajournals.org/doi/10.1161/CIR.0000000000000950>
(Feb. 2021).
6. Heidenreich, P. A. *et al.* Forecasting the Impact of Heart Failure in the United States. *Circulation: Heart Failure* **6**, 606–619. ISSN: 1941-3289. <http://my.americanheart.org/statements%20https://www.ahajournals.org/doi/10.1161/HHF.0b013e318291329a> (May 2013).
 7. Laflamme, M. A. & Murry, C. E. Heart regeneration. *Nature* **473**, 326–335. ISSN: 0028-0836. <http://www.nature.com/doi/10.1038/nature10147> (2011).
 8. Palasubramaniam, J., Wang, X. & Peter, K. Myocardial Infarction—From Atherosclerosis to Thrombosis. *Arteriosclerosis, Thrombosis, and Vascular Biology* **39**, E176–E185. ISSN: 1079-5642. <https://www.ahajournals.org/doi/10.1161/ATVBAHA.119.312578> (Aug. 2019).
 9. Frangogiannis, N. G. in *Comprehensive Physiology* 4, 1841–1875 (Wiley, Sept. 2015). <https://onlinelibrary.wiley.com/doi/10.1002/cphy.c150006>.
 10. Ertl, G. & Frantz, S. Healing after myocardial infarction. *Cardiovascular Research* **66**, 22–32. ISSN: 00086363 (2005).
 11. Gepstein, L. Derivation and Potential Applications of Human Embryonic Stem Cells. *Circulation Research* **91**, 866–876. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/01.RES.0000041435.95082.84> (Nov. 2002).
 12. Ondrus, T. *et al.* Right ventricular myocardial infarction: From pathophysiology to prognosis. *Experimental and clinical cardiology* **18**, 27–30. ISSN: 1205-6626. <http://www.ncbi.nlm.nih.gov/pubmed/24294033%20http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3716484> (2013).

13. Laflamme, M. A. & Murry, C. E. Regenerating the heart. *Nature Biotechnology* **23**, 845–856. ISSN: 1087-0156. <http://www.nature.com/articles/nbt1117> (July 2005).
14. Hashmi, S. & Al-Salam, S. Acute myocardial infarction and myocardial ischemia-reperfusion injury: a comparison. *Int J Clin Exp Pathol* **8**, 8786–8796. ISSN: 1936-2625. www.ijcep.com (2015).
15. Merz, S. F. *et al.* Contemporaneous 3D characterization of acute and chronic myocardial I/R injury and response. *Nature Communications* **10**, 1–14. ISSN: 20411723. <http://dx.doi.org/10.1038/s41467-019-10338-2> (2019).
16. Yellon, D. M. & Hausenloy, D. J. Myocardial Reperfusion Injury. *New England Journal of Medicine* **357**, 1121–1135. ISSN: 0028-4793. [https://academic.oup.com/eurheartjsupp/article-lookup/doi/10.1016/S1520-765X\(02\)90013-1](https://academic.oup.com/eurheartjsupp/article-lookup/doi/10.1016/S1520-765X(02)90013-1) <http://www.nejm.org/doi/abs/10.1056/NEJMra071667> (Sept. 2007).
17. Niccoli, G., Scalone, G., Lerman, A. & Crea, F. Coronary microvascular obstruction in acute myocardial infarction. *European Heart Journal* **37**, 1024–1033. ISSN: 0195-668X. <https://academic.oup.com/eurheartj/article-lookup/doi/10.1093/eurheartj/ehv484> (Apr. 2016).
18. Kaiser, N. J. & Coulombe, K. L. K. Physiologically inspired cardiac scaffolds for tailored in vivo function and heart regeneration. *Biomedical materials (Bristol, England)* **10**, 034003. ISSN: 1748-605X. <http://www.ncbi.nlm.nih.gov/pubmed/25970645> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4696555> (2015).
19. Dwyer, K. D. & Coulombe, K. L. Cardiac mechanostucture: Using mechanics and anisotropy as inspiration for developing epicardial therapies in treating myocardial infarction. *Bioactive Materials* **6**, 2198–2220. ISSN: 2452199X. <https://>

- doi.org/10.1016/j.bioactmat.2020.12.015%20https://linkinghub.elsevier.com/retrieve/pii/S2452199X20303406 (July 2021).
20. Helisch, A. & Schaper, W. Arteriogenesis: The development and growth of collateral arteries. *Microcirculation* **10**, 83–97. ISSN: 10739688 (2003).
 21. Weyers, J. J. *et al.* Effects of cell grafting on coronary remodeling after myocardial infarction. *Journal of the American Heart Association* **2**, 1–13. ISSN: 20479980 (2013).
 22. Tatli, E., Altun, A., Büyüklü, M. & Barotçu, A. Coronary collateral vessel development after acute myocardial infarction. *Experimental and clinical cardiology* **12**, 97–9. ISSN: 1205-6626. <http://www.ncbi.nlm.nih.gov/pubmed/18650990>%20<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC2359597> (2007).
 23. Cahill, T. J. & Kharbanda, R. K. Heart failure after myocardial infarction in the era of primary percutaneous coronary intervention: Mechanisms, incidence and identification of patients at risk. *World Journal of Cardiology* **9**, 407. ISSN: 1949-8462. <http://www.wjgnet.com/1949-8462/full/v9/i5/407.htm> (2017).
 24. Bahit, M. C., Kochar, A. & Granger, C. B. Post-Myocardial Infarction Heart Failure. *JACC: Heart Failure* **6**, 179–186. ISSN: 22131779. <https://doi.org/10.1016/j.jchf.2017.09.015>%20<https://linkinghub.elsevier.com/retrieve/pii/S2213177917306789> (Mar. 2018).
 25. Hoole, S. P. & Bambrough, P. Recent advances in percutaneous coronary intervention. *Heart* **106**, 1380–1386. ISSN: 1355-6037. <https://heart.bmj.com/lookup/doi/10.1136/heartjnl-2019-315707> (Sept. 2020).
 26. Smolina, K., Wright, F. L., Rayner, M. & Goldacre, M. J. Long-Term Survival and Recurrence After Acute Myocardial Infarction in England, 2004 to 2010. *Circula-*

- tion: Cardiovascular Quality and Outcomes* **5**, 532–540. ISSN: 1941-7713. <https://www.ahajournals.org/doi/10.1161/CIRCOUTCOMES.111.964700> (July 2012).
27. Dutta, P. *et al.* Myocardial infarction accelerates atherosclerosis. *Nature* **487**, 325–329. ISSN: 0028-0836. <http://www.nature.com/articles/nature11260> (July 2012).
 28. Torabi, A., Cleland, J. G., Rigby, A. S. & Sherwi, N. Development and course of heart failure after a myocardial infarction in younger and older people. *Journal of geriatric cardiology : JGC* **11**, 1–12. ISSN: 1671-5411. <http://www.ncbi.nlm.nih.gov/pubmed/24748875> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3981977> (Mar. 2014).
 29. Namdar, P., YekeFallah, L., Jalalian, F., Barikani, A. & Razaghpoor, A. Improving Door-to-Balloon Time for Patients With Acute ST-Elevation Myocardial Infarction: A Controlled Clinical Trial. *Current Problems in Cardiology* **46**, 100674. ISSN: 01462806. <https://doi.org/10.1016/j.cpcardiol.2020.100674> <https://linkinghub.elsevier.com/retrieve/pii/S0146280620301511> (Mar. 2021).
 30. Rao, G., Sheth, S. & Grines, C. Percutaneous coronary intervention: 2017 in review. *Journal of Interventional Cardiology* **31**, 117–128. ISSN: 08964327. <https://onlinelibrary.wiley.com/doi/10.1111/joic.12508> (Apr. 2018).
 31. Yoshiyama, M. Angiotensin converting enzyme inhibitor prevents left ventricular remodelling after myocardial infarction in angiotensin II type 1 receptor knockout mice. *Heart* **91**, 1080–1085. ISSN: 1355-6037. <https://heart.bmj.com/lookup/doi/10.1136/hrt.2004.035618> (Aug. 2005).
 32. Cole, G. D. *et al.* “Triple Therapy” of Heart Failure With Angiotensin-Converting Enzyme Inhibitor, Beta-Blocker, and Aldosterone Antagonist May Triple Survival

- Time. *JACC: Heart Failure* **2**, 545–548. ISSN: 22131779. <https://linkinghub.elsevier.com/retrieve/pii/S2213177914002704> (Oct. 2014).
33. Gu, Y. L. *et al.* Role of coronary artery bypass grafting during the acute and sub-acute phase of ST-elevation myocardial infarction. *Netherlands Heart Journal* **18**, 348–354. ISSN: 1568-5888. <http://link.springer.com/10.1007/BF03091790> (July 2010).
34. Shah, K. S., Kittleson, M. M. & Kobashigawa, J. A. Updates on Heart Transplantation. *Current Heart Failure Reports* **16**, 150–156. ISSN: 1546-9530. <http://link.springer.com/10.1007/s11897-019-00432-3> (Oct. 2019).
35. Khan Minhas, A. M. Total Artificial Heart as the Destination Therapy: A Review. *American Journal of Cardiovascular and Thoracic Surgery* **2**, 1–6. ISSN: 2573864X. <https://symbiosisonlinepublishing.com/thoracic-and-cardiovascular-surgery/cardiovascular-thoracic-surgery15.php> (June 2017).
36. Bui Anh, L., Horwisch Tamara, B. & Fonarow Gregg, C. Epidemiology and risk profile of heart failure. *Nature Publishing Group* **8**, 1–25. ISSN: 2164-8344 (2012).
37. Taylor, D. A. *et al.* Regenerating functional myocardium: Improved performance after skeletal myoblast transplantation. *Nature Medicine* **4**, 929–933. ISSN: 1078-8956. <http://www.nature.com/articles/nm0898-929> (Aug. 1998).
38. Mirotsov, M. *et al.* Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proceedings of the National Academy of Sciences* **104**, 1643–1648. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.0610024104> (Jan. 2007).
39. Klose, K., Gossen, M. & Stamm, C. Turning fibroblasts into cardiomyocytes: technological review of cardiac transdifferentiation strategies. *The FASEB Journal* **33**,

- 49–70. ISSN: 0892-6638. <https://onlinelibrary.wiley.com/doi/abs/10.1096/fj.201800712R> (Jan. 2019).
40. Wada, R. *et al.* Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. *Proceedings of the National Academy of Sciences* **110**, 12667–12672. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.1304053110> (July 2013).
41. Le, T. & Chong, J. Cardiac progenitor cells for heart repair. *Cell Death Discovery* **2**, 16052. ISSN: 2058-7716. <http://dx.doi.org/10.1038/cddiscovery.2016.52>
<http://www.nature.com/articles/cddiscovery201652> (Dec. 2016).
42. Dvir, T. *et al.* Prevascularization of cardiac patch on the omentum improves its therapeutic outcome. *Proceedings of the National Academy of Sciences* **106**, 14990–14995. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.0812242106> (2009).
43. Liu, Y.-W. *et al.* Human embryonic stem cell–derived cardiomyocytes restore function in infarcted hearts of non-human primates. *Nature Biotechnology* **36**, 597–605. ISSN: 1087-0156. <http://www.nature.com/articles/nbt.4162> (Aug. 2018).
44. Bargehr, J. *et al.* Epicardial cells derived from human embryonic stem cells augment cardiomyocyte-driven heart regeneration. *Nature Biotechnology* **37**, 895–906. ISSN: 1087-0156. <http://dx.doi.org/10.1038/s41587-019-0197-9> (2019).
45. Takahashi, K. & Yamanaka, S. Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* **126**, 663–676. ISSN: 00928674. <https://linkinghub.elsevier.com/retrieve/pii/S0092867406009767> (Aug. 2006).

46. Lee, C. *et al.* Generation of three iPSC lines from dilated cardiomyopathy patients carrying a pathogenic LMNA variant. *Stem Cell Research* **59**, 102638. ISSN: 18735061. <https://doi.org/10.1016/j.scr.2021.102638> <https://linkinghub.elsevier.com/retrieve/pii/S1873506121004852> (Mar. 2022).
47. Hikasa, H. & Sokol, S. Y. Wnt Signaling in Vertebrate Axis Specification. *Cold Spring Harbor Perspectives in Biology* **5**, a007955–a007955. ISSN: 1943-0264. <http://cshperspectives.cshlp.org/lookup/doi/10.1101/cshperspect.a007955> (Jan. 2013).
48. Schohl, A. & Fagotto, F. A role for maternal β -catenin in early mesoderm induction in *Xenopus*. *EMBO Journal* **22**, 3303–3313. ISSN: 02614189 (2003).
49. Yamaguchi, T. P. Heads or tails: Wnts and anterior–posterior patterning. *Current Biology* **11**, R713–R724. ISSN: 09609822. <https://linkinghub.elsevier.com/retrieve/pii/S0960982201004171> (Sept. 2001).
50. Ueno, S. *et al.* Biphasic role for Wnt/beta-catenin signaling in cardiac specification in zebrafish and embryonic stem cells. *Proceedings of the National Academy of Sciences* **104**, 9685–9690. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.0702859104> (June 2007).
51. Lopez-Sanchez, C. & Garcia-Martinez, V. Molecular determinants of cardiac specification. *Cardiovascular Research* **91**, 185–195. ISSN: 0008-6363. <https://academic.oup.com/cardiovasces/article-lookup/doi/10.1093/cvr/cvr127> (July 2011).
52. Doevendans, P. A. *et al.* Differentiation of cardiomyocytes in floating embryoid bodies is comparable to fetal cardiomyocytes. *Journal of Molecular and Cellular Cardiology* **32**, 839–851. ISSN: 00222828 (2000).

53. Kehat, I. *et al.* Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. *Journal of Clinical Investigation* **108**, 407–414. ISSN: 0021-9738. <http://www.jci.org/articles/view/12131> (Aug. 2001).
54. Wiles, M. V. & Johansson, B. M. Embryonic Stem Cell Development in a Chemically Defined Medium. *Experimental Cell Research* **247**, 241–248. ISSN: 00144827. <https://linkinghub.elsevier.com/retrieve/pii/S0014482798943531> (Feb. 1999).
55. Amit, M. & Itskovitz-Eldor, J. in *Methods in Enzymology* 06, 37–49 (2006). <https://linkinghub.elsevier.com/retrieve/pii/S007668790620003X>.
56. Murry, C. E. & Keller, G. Differentiation of Embryonic Stem Cells to Clinically Relevant Populations: Lessons from Embryonic Development. *Cell* **132**, 661–680. ISSN: 00928674. <https://linkinghub.elsevier.com/retrieve/pii/S009286740800216X> (Feb. 2008).
57. Burridge, P. W. *et al.* Chemically defined generation of human cardiomyocytes. *Nature Methods* **11**, 855–860. ISSN: 1548-7091. <http://www.nature.com/doifinder/10.1038/nmeth.2999> (2014).
58. Kattman, S. J. *et al.* Stage-Specific Optimization of Activin/Nodal and BMP Signaling Promotes Cardiac Differentiation of Mouse and Human Pluripotent Stem Cell Lines. *Cell Stem Cell* **8**, 228–240. ISSN: 19345909. <http://dx.doi.org/10.1016/j.stem.2010.12.008><https://linkinghub.elsevier.com/retrieve/pii/S1934590910007034> (Feb. 2011).
59. Laflamme, M. A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnology* **25**, 1015–1024. ISSN: 10870156 (2007).

60. Lian, X. *et al.* Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. *Proceedings of the National Academy of Sciences* **109**, E1848–E1857. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.1200250109> (2012).
61. Coulombe, K. L., Bajpai, V. K., Andreadis, S. T. & Murry, C. E. Heart Regeneration with Engineered Myocardial Tissue. *Annual Review of Biomedical Engineering* **16**, 1–28. ISSN: 1523-9829. <http://www.annualreviews.org/doi/10.1146/annurev-bioeng-071812-152344> (2014).
62. Funakoshi, S. *et al.* Generation of mature compact ventricular cardiomyocytes from human pluripotent stem cells. *Nature Communications* **12** (2021).
63. Rupert, C. E., Irofuala, C. & Coulombe, K. L. K. Practical adoption of state-of-the-art hiPSC-cardiomyocyte differentiation techniques. *PLOS ONE* **15** (ed Guo, Y.) e0230001. ISSN: 1932-6203. <http://dx.doi.org/10.1371/journal.pone.0230001> %20<https://dx.plos.org/10.1371/journal.pone.0230001> (Mar. 2020).
64. Munarin, F., Kant, R. J., Rupert, C. E., Khoo, A. & Coulombe, K. L. Engineered human myocardium with local release of angiogenic proteins improves vascularization and cardiac function in injured rat hearts. *Biomaterials* **251**, 120033. ISSN: 01429612. <https://doi.org/10.1016/j.biomaterials.2020.120033> %20<https://linkinghub.elsevier.com/retrieve/pii/S0142961220302799> (Aug. 2020).
65. Romagnuolo, R. *et al.* Human Embryonic Stem Cell-Derived Cardiomyocytes Regenerate the Infarcted Pig Heart but Induce Ventricular Tachyarrhythmias. *Stem Cell Reports* **12**, 967–981. ISSN: 22136711 (2019).
66. Dhahri, W. *et al.* In Vitro Matured Human Pluripotent Stem Cell-derived Cardiomyocytes Form Grafts With Enhanced Structure and Function in Injured Hearts. *Cir-*

- ulation*. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.121.053563> (Jan. 2022).
67. Soepriatna, A. H. *et al.* Human Atrial Cardiac Microtissues for Chamber-Specific Arrhythmic Risk Assessment. *Cellular and Molecular Bioengineering* **14**, 441–457. ISSN: 1865-5025. <https://link.springer.com/10.1007/s12195-021-00703-x> (Oct. 2021).
68. Tohyama, S. *et al.* Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell* **12**, 127–137. ISSN: 19345909. <http://dx.doi.org/10.1016/j.stem.2012.09.013> (2013).
69. Buikema, J. W. *et al.* Wnt Activation and Reduced Cell-Cell Contact Synergistically Induce Massive Expansion of Functional Human iPSC-Derived Cardiomyocytes. *Cell Stem Cell* **27**, 50–63. ISSN: 18759777. <https://doi.org/10.1016/j.stem.2020.06.001> (2020).
70. Abouleisa, R. R. *et al.* Cell cycle induction in human cardiomyocytes is dependent on biosynthetic pathway activation. *Redox Biology* **46**, 102094. ISSN: 22132317. <https://doi.org/10.1016/j.redox.2021.102094> <https://linkinghub.elsevier.com/retrieve/pii/S2213231721002536> (Oct. 2021).
71. Zhu, F., Meng, Q., Yu, Y., Shao, L. & Shen, Z. Adult Cardiomyocyte Proliferation: a New Insight for Myocardial Infarction Therapy. *Journal of Cardiovascular Translational Research* **14**, 457–466. ISSN: 1937-5387. <https://link.springer.com/10.1007/s12265-020-10067-8> (June 2021).
72. Rowton, M., Guzzetta, A., Rydeen, A. B. & Moskowitz, I. P. Control of cardiomyocyte differentiation timing by intercellular signaling pathways. *Seminars in Cell & Developmental Biology* **118**, 94–106. ISSN: 10849521. <https://doi.org/>

- 10.1016/j.semcd.2021.06.002%20https://linkinghub.elsevier.com/retrieve/pii/S1084952121001531 (Oct. 2021).
73. Guo, Y. & Pu, W. T. Cardiomyocyte Maturation. *Circulation Research* **126**, 1086–1106. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.119.315862> (Apr. 2020).
 74. Tani, H., Tohyama, S., Kishino, Y., Kanazawa, H. & Fukuda, K. Production of functional cardiomyocytes and cardiac tissue from human induced pluripotent stem cells for regenerative therapy. *Journal of Molecular and Cellular Cardiology* **164**, 83–91. ISSN: 10958584. <https://doi.org/10.1016/j.yjmcc.2021.11.008> (2022).
 75. Beauchamp, P. *et al.* 3D Co-culture of hiPSC-Derived Cardiomyocytes With Cardiac Fibroblasts Improves Tissue-Like Features of Cardiac Spheroids. *Frontiers in Molecular Biosciences* **7**, 1–17. ISSN: 2296-889X. <https://www.frontiersin.org/article/10.3389/fmolb.2020.00014/full> (Feb. 2020).
 76. Branco, M. A. *et al.* Transcriptomic analysis of 3D Cardiac Differentiation of Human Induced Pluripotent Stem Cells Reveals Faster Cardiomyocyte Maturation Compared to 2D Culture. *Scientific Reports* **9**, 9229. ISSN: 2045-2322. <http://www.nature.com/articles/s41598-019-45047-9> (Dec. 2019).
 77. Kerr, C. M., Richards, D., Menick, D. R., Deleon-Pennell, K. Y. & Mei, Y. Multicellular Human Cardiac Organoids Transcriptomically Model Distinct Tissue-Level Features of Adult Myocardium. *International Journal of Molecular Sciences* **22**, 8482. ISSN: 1422-0067. <https://www.mdpi.com/1422-0067/22/16/8482> (Aug. 2021).
 78. Lopez, C. A. *et al.* Physiological and pharmacological stimulation for in vitro maturation of substrate metabolism in human induced pluripotent stem cell-derived cardiomyocytes. *Scientific Reports* **11**, 7802. ISSN: 2045-2322. <https://doi.org/>

- 10.1038/s41598-021-87186-y%20http://www.nature.com/articles/s41598-021-87186-y (Dec. 2021).
79. Kofron, C. M. *et al.* A predictive in vitro risk assessment platform for pro-arrhythmic toxicity using human 3D cardiac microtissues. *Scientific Reports* **11**, 10228. ISSN: 2045-2322. <https://doi.org/10.1038/s41598-021-89478-9> %20http://www.nature.com/articles/s41598-021-89478-9 (Dec. 2021).
 80. Blakely, A. M., Manning, K. L., Tripathi, A. & Morgan, J. R. Bio-Pick, Place, and Perfuse: A New Instrument for Three-Dimensional Tissue Engineering. *Tissue Engineering Part C: Methods* **21**, 737–746. ISSN: 1937-3384. <https://www.liebertpub.com/doi/10.1089/ten.tec.2014.0439> (July 2015).
 81. Arai, K. *et al.* Drug response analysis for scaffold-free cardiac constructs fabricated using bio-3D printer. *Scientific Reports* **10**, 8972. ISSN: 2045-2322. <http://dx.doi.org/10.1038/s41598-020-65681-y> %20http://www.nature.com/articles/s41598-020-65681-y (Dec. 2020).
 82. Brady, E. L. *et al.* Guided vascularization in the rat heart leads to transient vessel patterning. *APL Bioengineering* **4**, 016105. ISSN: 2473-2877. <http://aip.scitation.org/doi/10.1063/1.5122804> (Mar. 2020).
 83. Gao, L. *et al.* Large Cardiac Muscle Patches Engineered From Human Induced-Pluripotent Stem Cell-Derived Cardiac Cells Improve Recovery From Myocardial Infarction in Swine. *Circulation* **137**, 1712–1730. ISSN: 0009-7322. <http://circ.ahajournals.org/lookup/doi/10.1161/CIRCULATIONAHA.117.030785> %20https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.117.030785 (Apr. 2018).

84. Gao, L. *et al.* Myocardial Tissue Engineering with Cells Derived from Human-Induced Pluripotent Stem Cells and a Native-Like, High-Resolution, 3-Dimensionally Printed Scaffold. *Circulation Research* **120**, 1318–1325. ISSN: 15244571 (2017).
85. Zhang, Y. S. *et al.* Bioprinting 3D microfibrinous scaffolds for engineering endothelialized myocardium and heart-on-a-chip. *Biomaterials* **110**, 45–59. ISSN: 01429612. <http://linkinghub.elsevier.com/retrieve/pii/S014296121630477X> (Dec. 2016).
86. Noor, N. *et al.* 3D Printing of Personalized Thick and Perfusable Cardiac Patches and Hearts. *Advanced Science* **6**, 1900344. ISSN: 2198-3844. <https://onlinelibrary.wiley.com/doi/abs/10.1002/advs.201900344> %20https://onlinelibrary.wiley.com/doi/10.1002/advs.201900344 (June 2019).
87. Zuppinger, C. 3D Cardiac Cell Culture: A Critical Review of Current Technologies and Applications. *Frontiers in Cardiovascular Medicine* **6**, 1–9. ISSN: 2297-055X. <https://www.frontiersin.org/article/10.3389/fcvm.2019.00087/full> (June 2019).
88. Malafaya, P. B., Silva, G. A. & Reis, R. L. Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. *Advanced Drug Delivery Reviews* **59**, 207–233. ISSN: 0169409X (2007).
89. Kaiser, N. J., Kant, R. J., Minor, A. J. & Coulombe, K. L. Optimizing Blended Collagen-Fibrin Hydrogels for Cardiac Tissue Engineering with Human iPSC-derived Cardiomyocytes. *ACS Biomaterials Science and Engineering* **5**, 887–899. ISSN: 23739878 (Feb. 2019).
90. Gomez-Garcia, M. J., Quesnel, E., Al-attar, R., Laskary, A. R. & Laflamme, M. A. Maturation of human pluripotent stem cell derived cardiomyocytes in vitro and in vivo. *Seminars in Cell & Developmental Biology*. ISSN: 10849521. <https://doi.org/10.1016/j.sembio.2019.05.001> (May 2019).

- // doi . org / 10 . 1016 / j . semcdb . 2021 . 05 . 022 % 20https : // linkinghub . elsevier . com / retrieve / pii / S1084952121001312 (May 2021).
91. Rupert, C. E., Kim, T. Y., Choi, B. R. & Coulombe, K. L. Human Cardiac Fibroblast Number and Activation State Modulate Electromechanical Function of hiPSC-Cardiomyocytes in Engineered Myocardium. *Stem Cells International* **2020**. ISSN: 16879678 (2020).
 92. Roberts, M. A. *et al.* Stromal cells in dense collagen promote cardiomyocyte and microvascular patterning in engineered human heart tissue. *Tissue engineering. Part A* **22**, ten.TEA.2015.0482. ISSN: 1937-335X. <http://online.liebertpub.com/doi/abs/10.1089/ten.TEA.2015.0482%5Cnhttp://online.liebertpub.com/doi/10.1089/ten.TEA.2015.0482%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/26955856> (2016).
 93. Tiburcy, M. *et al.* Defined Engineered Human Myocardium With Advanced Maturation for Applications in Heart Failure Modeling and Repair. *Circulation* **135**, 1832–1847. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.116.024145> (May 2017).
 94. Stoker, M. E., Gerdes, A. M. & May, J. F. Regional differences in capillary density and myocyte size in the normal human heart. *The Anatomical Record* **202**, 187–191. ISSN: 0003-276X. <http://doi.wiley.com/10.1002/ar.1092020203> (Feb. 1982).
 95. Kaneko, N., Matsuda, R., Toda, M. & Shimamoto, K. Three-dimensional reconstruction of the human capillary network and the intramyocardial micronecrosis. *AJP: Heart and Circulatory Physiology* **300**, H754–H761. ISSN: 0363-6135. <http://ajpheart.physiology.org/cgi/doi/10.1152/ajpheart.00486.2010> (2011).

96. Hsieh, P. C., Davis, M. E., Lisowski, L. K. & Lee, R. T. ENDOTHELIAL-CARDIOMYOCYTE INTERACTIONS IN CARDIAC DEVELOPMENT AND REPAIR. *Annual Review of Physiology* **68**, 51–66. ISSN: 0066-4278. <https://www.annualreviews.org/doi/10.1146/annurev.physiol.68.040104.124629> (Jan. 2006).
97. Segers, V. F. M., Brutsaert, D. L. & De Keulenaer, G. W. Cardiac Remodeling: Endothelial Cells Have More to Say Than Just NO. *Frontiers in Physiology* **9**. ISSN: 1664-042X. <http://journal.frontiersin.org/article/10.3389/fphys.2018.00382/full> (Apr. 2018).
98. Narmoneva, D. A., Vukmirovic, R., Davis, M. E., Kamm, R. D. & Lee, R. T. Endothelial Cells Promote Cardiac Myocyte Survival and Spatial Reorganization. *Circulation* **110**, 962–968. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/01.CIR.0000140667.37070.07> (Aug. 2004).
99. Leucker, T. M. *et al.* Endothelial–cardiomyocyte crosstalk enhances pharmacological cardioprotection. *Journal of Molecular and Cellular Cardiology* **51**, 803–811. ISSN: 00222828. <https://linkinghub.elsevier.com/retrieve/pii/S0022282811002744> (Nov. 2011).
100. Zhang, Y. *et al.* Endothelial Cells Regulate Cardiac Myocyte Reorganisation Through β 1-Integrin Signalling. *Cellular Physiology and Biochemistry* **35**, 1808–1820. ISSN: 1015-8987. <https://www.karger.com/Article/FullText/373992> (2015).
101. Dunn, K. K. *et al.* Coculture of Endothelial Cells with Human Pluripotent Stem Cell-Derived Cardiac Progenitors Reveals a Differentiation Stage-Specific Enhancement of Cardiomyocyte Maturation. *Biotechnology Journal* **14**, 1800725. ISSN: 1860-6768. <https://onlinelibrary.wiley.com/doi/10.1002/biot.201800725> (Aug. 2019).

102. Kreutziger, K. L. *et al.* Developing Vasculature and Stroma in Engineered Human Myocardium. *Tissue Engineering Part A* **17**, 1219–1228. ISSN: 1937-3341. <http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2010.0557> (2011).
103. Schaefer, J. A., Guzman, P. A., Riemenschneider, S. B., Kamp, T. J. & Tranquillo, R. T. A cardiac patch from aligned microvessel and cardiomyocyte patches. *Journal of Tissue Engineering and Regenerative Medicine* **12**, 546–556. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.2568> (Feb. 2018).
104. Fleischer, S., Shapira, A., Feiner, R. & Dvir, T. Modular assembly of thick multi-functional cardiac patches. *Proceedings of the National Academy of Sciences* **114**, 1898–1903. ISSN: 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.1615728114> (2017).
105. Sun, X. *et al.* Transplanted microvessels improve pluripotent stem cell-derived cardiomyocyte engraftment and cardiac function after infarction in rats. *Science Translational Medicine* **12**, 1–11. ISSN: 19466242 (2020).
106. Redd, M. A. *et al.* Patterned human microvascular grafts enable rapid vascularization and increase perfusion in infarcted rat hearts. *Nature Communications* **10**, 1–14. ISSN: 20411723. <http://dx.doi.org/10.1038/s41467-019-08388-7> (2019).
107. Su, T. *et al.* Cardiac Stromal Cell Patch Integrated with Engineered Microvessels Improves Recovery from Myocardial Infarction in Rats and Pigs. *ACS Biomaterials Science & Engineering* **6**, 6309–6320. ISSN: 2373-9878. <https://pubs.acs.org/doi/10.1021/acsbiomaterials.0c00942> (Nov. 2020).
108. Sekine, H. *et al.* Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation* **118**, 145–152. ISSN: 15244539 (2008).

109. Mollova, M. *et al.* Cardiomyocyte proliferation contributes to heart growth in young humans. *Proceedings of the National Academy of Sciences* **110**, 1446–1451. ISSN: 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.1214608110> (2013).
110. Cui, Y. *et al.* Single-Cell Transcriptome Analysis Maps the Developmental Track of the Human Heart. *Cell Reports* **26**, 1934–1950. ISSN: 22111247. <https://doi.org/10.1016/j.celrep.2019.01.079><https://linkinghub.elsevier.com/retrieve/pii/S2211124719301081> (Feb. 2019).
111. Kamakura, T. *et al.* Ultrastructural Maturation of Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes in a Long-Term Culture. *Circulation Journal* **77**, 1307–1314. ISSN: 1346-9843. https://www.jstage.jst.go.jp/article/circj/77/5/77_CJ-12-0987/_article (2013).
112. Ebert, A. *et al.* Proteasome-Dependent Regulation of Distinct Metabolic States During Long-Term Culture of Human iPSC-Derived Cardiomyocytes. *Circulation Research* **125**, 90–103. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.118.313973> (June 2019).
113. Atchison, D. K. & Beierwaltes, W. H. The influence of extracellular and intracellular calcium on the secretion of renin. *Pflügers Archiv - European Journal of Physiology* **465**, 59–69. ISSN: 0031-6768. <http://link.springer.com/10.1007/s00424-012-1107-x> (Jan. 2013).
114. Nakano, H. *et al.* Glucose inhibits cardiac muscle maturation through nucleotide biosynthesis. *eLife* **6**, 1–23. ISSN: 2050-084X. <https://elifesciences.org/articles/29330> (Dec. 2017).
115. Feyen, D. A. *et al.* Metabolic Maturation Media Improve Physiological Function of Human iPSC-Derived Cardiomyocytes. *Cell Reports* **32**, 107925. ISSN: 22111247.

- <https://linkinghub.elsevier.com/retrieve/pii/S2211124720309062>
(July 2020).
116. Minor, A. J. & Coulombe, K. L. K. Stimulating Calcium Handling in hiPSC-Derived Engineered Cardiac Tissues Enhances Force Production. *Stem Cells Translational Medicine*, 1–10. ISSN: 2157-6564. <https://academic.oup.com/stcltm/advance-article/doi/10.1093/stcltm/szab002/6528832> (Feb. 2022).
 117. Rupert, C. E. & Coulombe, K. L. IGF1 and NRG1 Enhance Proliferation, Metabolic Maturity, and the Force-Frequency Response in hESC-Derived Engineered Cardiac Tissues. *Stem Cells International* **2017**. ISSN: 16879678 (2017).
 118. Hu, D. *et al.* Metabolic Maturation of Human Pluripotent Stem Cell-Derived Cardiomyocytes by Inhibition of HIF1 α and LDHA. *Circulation Research* **123**, 1066–1079. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.118.313249> (Oct. 2018).
 119. Horikoshi, Y. *et al.* Fatty Acid-Treated Induced Pluripotent Stem Cell-Derived Human Cardiomyocytes Exhibit Adult Cardiomyocyte-Like Energy Metabolism Phenotypes. *Cells* **8**, 1095. ISSN: 2073-4409. <https://linkinghub.elsevier.com/retrieve/pii/S2666166721006183><https://www.mdpi.com/2073-4409/8/9/1095> (Sept. 2019).
 120. Knight, W. E., Cao, Y., Dillon, P. & Song, K. A simple protocol to produce mature human-induced pluripotent stem cell-derived cardiomyocytes. *STAR Protocols* **2**, 100912. ISSN: 26661667. <https://doi.org/10.1016/j.xpro.2021.100912><https://linkinghub.elsevier.com/retrieve/pii/S2666166721006183> (Dec. 2021).
 121. Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **126**, 677–689. ISSN: 00928674. <https://>

- linkinghub.elsevier.com/retrieve/pii/S0092867406009615 (Aug. 2006).
122. Acevedo-Acevedo, S. & Crone, W. C. Substrate stiffness effect and chromosome missegregation in hIPS cells. *Journal of Negative Results in BioMedicine* **14**, 22. ISSN: 1477-5751. <http://dx.doi.org/10.1186/s12952-015-0042-8> <https://jnrbm.biomedcentral.com/articles/10.1186/s12952-015-0042-8> (Dec. 2015).
123. Engler, A. J. *et al.* Embryonic cardiomyocytes beat best on a matrix with heart-like elasticity: scar-like rigidity inhibits beating. *Journal of Cell Science* **121**, 3794–3802. ISSN: 1477-9137. <https://journals.biologists.com/jcs/article/121/22/3794/35302/Embryonic-cardiomyocytes-beat-best-on-a-matrix> (Nov. 2008).
124. Körner, A., Mosqueira, M., Hecker, M. & Ullrich, N. D. Substrate Stiffness Influences Structural and Functional Remodeling in Induced Pluripotent Stem Cell-Derived Cardiomyocytes. *Frontiers in Physiology* **12**. ISSN: 1664-042X. <https://www.frontiersin.org/articles/10.3389/fphys.2021.710619/full> (Aug. 2021).
125. Schroer, A., Pardon, G., Castillo, E., Blair, C. & Pruitt, B. Engineering hiPSC cardiomyocyte in vitro model systems for functional and structural assessment. *Progress in Biophysics and Molecular Biology* **144**, 3–15. ISSN: 00796107. <https://linkinghub.elsevier.com/retrieve/pii/S0079610718301160> (July 2019).
126. Batalov, I., Jallerat, Q., Kim, S., Bliley, J. & Feinberg, A. W. Engineering aligned human cardiac muscle using developmentally inspired fibronectin micropatterns. *Scientific Reports* **11**, 11502. ISSN: 2045-2322. <https://doi.org/10.1038/>

- s41598-021-87550-y%20http://www.nature.com/articles/s41598-021-87550-y (Dec. 2021).
127. Wang, P.-Y., Yu, J., Lin, J.-H. & Tsai, W.-B. Modulation of alignment, elongation and contraction of cardiomyocytes through a combination of nanotopography and rigidity of substrates. *Acta Biomaterialia* **7**, 3285–3293. ISSN: 17427061. <http://dx.doi.org/10.1016/j.actbio.2011.05.021>%20<https://linkinghub.elsevier.com/retrieve/pii/S1742706111002224> (Sept. 2011).
 128. Takada, T. *et al.* Aligned human induced pluripotent stem cell-derived cardiac tissue improves contractile properties through promoting unidirectional and synchronous cardiomyocyte contraction. *Biomaterials* **281**, 121351. ISSN: 01429612. <https://doi.org/10.1016/j.biomaterials.2021.121351>%20<https://linkinghub.elsevier.com/retrieve/pii/S0142961221007079> (Feb. 2022).
 129. Shadrin, I. Y. *et al.* Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues. *Nature Communications* **8**, 1825. ISSN: 2041-1723. <http://dx.doi.org/10.1038/s41467-017-01946-x>%20<http://www.nature.com/articles/s41467-017-01946-x> (Dec. 2017).
 130. Munarin, F., Kaiser, N. J., Kim, T. Y., Choi, B.-R. & Coulombe, K. L. K. Laser-Etched Designs for Molding Hydrogel-Based Engineered Tissues. *Tissue engineering. Part C, Methods* **23**, 311–321. ISSN: 1937-3392. <http://www.ncbi.nlm.nih.gov/pubmed/28457187>%0A<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5444510> (2017).
 131. Querdel, E. *et al.* Human Engineered Heart Tissue Patches Remuscularize the Injured Heart in a Dose-Dependent Manner. *Circulation* **143**, 1991–2006. ISSN: 0009-

7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.120.047904> (May 2021).
132. Bian, W., Jackman, C. P. & Bursac, N. Controlling the structural and functional anisotropy of engineered cardiac tissues. *Biofabrication* **6**, 024109. ISSN: 1758-5082. <https://iopscience.iop.org/article/10.1088/1758-5082/6/2/024109> (Apr. 2014).
133. Costa, K. D., Lee, E. J. & Holmes, J. W. Creating Alignment and Anisotropy in Engineered Heart Tissue: Role of Boundary Conditions in a Model Three-Dimensional Culture System. *Tissue Engineering* **9**, 567–577. ISSN: 1076-3279. <https://www.liebertpub.com/doi/10.1089/107632703768247278> (Aug. 2003).
134. Wang, H. *et al.* Necking and failure of constrained 3D microtissues induced by cellular tension. *Proceedings of the National Academy of Sciences* **110**, 20923–20928. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.1313662110> (2013).
135. Sun, X. & Nunes, S. S. Maturation of human stem cell-derived cardiomyocytes in biowires using electrical stimulation. *Journal of Visualized Experiments* **2017**, 1–8. ISSN: 1940087X (2017).
136. Godier-Furnémont, A. F. *et al.* Physiologic force-frequency response in engineered heart muscle by electromechanical stimulation. *Biomaterials* **60**, 82–91. ISSN: 01429612. <https://linkinghub.elsevier.com/retrieve/pii/S0142961215003397> (Aug. 2015).
137. Abilez, O. J. *et al.* Passive Stretch Induces Structural and Functional Maturation of Engineered Heart Muscle as Predicted by Computational Modeling. *STEM CELLS* **36**, 265–277. ISSN: 10665099. <http://doi.wiley.com/10.1002/stem.2732> (Feb. 2018).

138. Ronaldson-Bouchard, K. *et al.* Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature* **556**, 239–243. ISSN: 0028-0836. <http://www.nature.com/articles/s41586-018-0016-3> (Apr. 2018).
139. Miki, K. *et al.* ERR γ enhances cardiac maturation with T-tubule formation in human iPSC-derived cardiomyocytes. *Nature Communications* **12**, 3596. ISSN: 2041-1723. <http://dx.doi.org/10.1038/s41467-021-23816-3> <http://www.nature.com/articles/s41467-021-23816-3> (Dec. 2021).
140. Zhang, J. *et al.* Functional cardiac fibroblasts derived from human pluripotent stem cells via second heart field progenitors. *Nature Communications* **10**, 2238. ISSN: 2041-1723. <http://dx.doi.org/10.1038/s41467-019-09831-5> <http://www.nature.com/articles/s41467-019-09831-5> (Dec. 2019).
141. Kant, R. J. & Coulombe, K. L. Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues. *Acta Biomaterialia* **69**, 42–62. ISSN: 17427061. <https://doi.org/10.1016/j.actbio.2018.01.017> <https://linkinghub.elsevier.com/retrieve/pii/S174270611830028X> (Mar. 2018).
142. Milani-Nejad, N. & Janssen, P. M. Small and large animal models in cardiac contraction research: Advantages and disadvantages. *Pharmacology & Therapeutics* **141**, 235–249. ISSN: 01637258. <http://linkinghub.elsevier.com/retrieve/pii/S016372581300212X> (Mar. 2014).
143. Martin, T. P. *et al.* Preclinical models of myocardial infarction: from mechanism to translation. *British Journal of Pharmacology*, 1–22. ISSN: 0007-1188. <https://onlinelibrary.wiley.com/doi/10.1111/bph.15595> (Aug. 2021).

144. Lindsey, M. L. *et al.* Guidelines for experimental models of myocardial ischemia and infarction. *American Journal of Physiology-Heart and Circulatory Physiology* **314**, H812–H838. ISSN: 0363-6135. <http://www.physiology.org/doi/10.1152/ajpheart.00335.2017> (2018).
145. Eschenhagen, T., Ridders, K. & Weinberger, F. How to repair a broken heart with pluripotent stem cell-derived cardiomyocytes. *Journal of Molecular and Cellular Cardiology* **163**, 106–117. ISSN: 00222828. <https://doi.org/10.1016/j.yjmcc.2021.10.005> <https://linkinghub.elsevier.com/retrieve/pii/S0022282821001991> (Feb. 2022).
146. Fernandes, S. *et al.* Human embryonic stem cell-derived cardiomyocytes engraft but do not alter cardiac remodeling after chronic infarction in rats. *Journal of Molecular and Cellular Cardiology* **49**, 941–949. ISSN: 00222828. <https://linkinghub.elsevier.com/retrieve/pii/S0022282810003391> (Dec. 2010).
147. Menasché, P. Cell Therapy With Human ESC-Derived Cardiac Cells: Clinical Perspectives. *Frontiers in Bioengineering and Biotechnology* **8**. ISSN: 2296-4185. <https://www.frontiersin.org/articles/10.3389/fbioe.2020.601560/full> (Oct. 2020).
148. Katarzyna, R. Adult Stem Cell Therapy for Cardiac Repair in Patients After Acute Myocardial Infarction Leading to Ischemic Heart Failure: An Overview of Evidence from the Recent Clinical Trials. *Current Cardiology Reviews* **13**, 223–231. ISSN: 1573403X. <http://www.eurekaselect.com/152041/article> (July 2017).
149. Li, J. *et al.* All Roads Lead to Rome (the Heart): Cell Retention and Outcomes From Various Delivery Routes of Cell Therapy Products to the Heart. *Journal of the American Heart Association* **10**, 1–17. ISSN: 2047-9980. <https://www.ahajournals.org/doi/10.1161/JAHA.120.020402> (Apr. 2021).

150. Gerbin, K. A., Yang, X., Murry, C. E. & Coulombe, K. L. K. Enhanced electrical integration of engineered human myocardium via intramyocardial versus epicardial delivery in infarcted rat hearts. *PLoS ONE* **10**, 1–20. ISSN: 19326203 (2015).
151. Shiba, Y. *et al.* Electrical integration of human embryonic stem cell-derived cardiomyocytes in a guinea pig chronic infarct model. *Journal of Cardiovascular Pharmacology and Therapeutics* **19**, 368–381. ISSN: 19404034 (2014).
152. Riemenschneider, S. B. *et al.* Inosculation and Perfusion of Pre-Vascularized Tissue Patches Containing Aligned Human Microvessels after Myocardial Infarction. *Biomaterials* **97**, 51–61. ISSN: 1527-5418. <https://pubmed.ncbi.nlm.nih.gov/27162074/> (2016).
153. Montgomery, M. *et al.* Flexible shape-memory scaffold for minimally invasive delivery of functional tissues. *Nature Materials* **16**, 1038–1046. ISSN: 1476-1122. <http://www.nature.com/articles/nmat4956> (Oct. 2017).
154. Wang, L. *et al.* Injectable and conductive cardiac patches repair infarcted myocardium in rats and minipigs. *Nature Biomedical Engineering* **5**, 1157–1173. ISSN: 2157-846X. <https://www.nature.com/articles/s41551-021-00796-9> (Oct. 2021).
155. Roche, C. D., Zhou, Y., Zhao, L. & Gentile, C. A World-First Surgical Instrument for Minimally Invasive Robotically-Enabled Transplantation of Heart Patches for Myocardial Regeneration: A Brief Research Report. *Frontiers in Surgery* **8**, 1–10. ISSN: 2296-875X. <https://www.frontiersin.org/articles/10.3389/fsurg.2021.653328/full> (Oct. 2021).
156. Zimmermann, W.-H. *et al.* Engineered heart tissue grafts improve systolic and diastolic function in infarcted rat hearts. *Nature Medicine* **12**, 452–458. ISSN: 1078-8956. <http://www.nature.com/articles/nm1394> (Apr. 2006).

157. Zimmermann, W.-H. Remuscularization of the failing heart. *The Journal of Physiology* **595**, 3685–3690. ISSN: 00223751. <https://onlinelibrary.wiley.com/doi/10.1113/JP273098> (June 2017).
158. Pecha, S. *et al.* Human iPS cell-derived engineered heart tissue does not affect ventricular arrhythmias in a guinea pig cryo-injury model. *Scientific Reports* **9**, 9831. ISSN: 2045-2322. <http://www.nature.com/articles/s41598-019-46409-z> (Dec. 2019).
159. Li, Y. *et al.* Conductive biomaterials for cardiac repair: A review. *Acta Biomaterialia*. ISSN: 17427061. <https://linkinghub.elsevier.com/retrieve/pii/S1742706121002555> (Apr. 2021).
160. Kadota, S., Pabon, L., Reinecke, H. & Murry, C. E. In Vivo Maturation of Human Induced Pluripotent Stem Cell-Derived Cardiomyocytes in Neonatal and Adult Rat Hearts. *Stem Cell Reports* **8**, 278–289. ISSN: 22136711. <http://dx.doi.org/10.1016/j.stemcr.2016.10.009> <https://linkinghub.elsevier.com/retrieve/pii/S2213671116302478> (Feb. 2017).
161. Deuse, T. *et al.* Hypoimmune induced pluripotent stem cell-derived cell therapeutics treat cardiovascular and pulmonary diseases in immunocompetent allogeneic mice. *Proceedings of the National Academy of Sciences* **118**, e2022091118. ISSN: 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.2022091118> (July 2021).
162. Shiba, Y. *et al.* Allogeneic transplantation of iPS cell-derived cardiomyocytes regenerates primate hearts. *Nature* **538**, 388–391. ISSN: 0028-0836. <http://dx.doi.org/10.1038/nature19815> <http://www.nature.com/articles/nature19815> (Oct. 2016).

163. Chong, J. J. *et al.* Human Embryonic Stem Cell-Derived Cardiomyocytes Regenerate Non-Human Primate Hearts. *Nature* **510**, 273–277. ISSN: 0028-0836 (Print) (2014).
164. Menasché, P. *et al.* Transplantation of Human Embryonic Stem Cell-Derived Cardiovascular Progenitors for Severe Ischemic Left Ventricular Dysfunction. *Journal of the American College of Cardiology* **71**, 429–438. ISSN: 07351097. <https://linkinghub.elsevier.com/retrieve/pii/S0735109717417281> (Jan. 2018).

Chapter 2

Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues

Reproduced with permission from R. J. Kant and K. L. K Coulombe, “Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues”, *Acta Biomater*, 69, 42-62, Mar 2018.

2.1 Abstract

The field of tissue engineering has turned towards biomimicry to solve the problem of tissue oxygenation and nutrient/waste exchange through the development of vasculature. Induction of angiogenesis and subsequent development of a vascular bed in engineered tissues is actively being pursued through combinations of physical and chemical cues, notably through the presentation of topographies and growth factors. Presenting angiogenic signals in a spatiotemporal fashion is beginning to generate improved vascular networks, which

will allow for the creation of large and dense engineered tissues. This review provides a brief background on the cells, mechanisms, and molecules driving vascular development (including angiogenesis), followed by how biomaterials and growth factors can be used to direct vessel formation and maturation. Techniques to accomplish spatiotemporal control of vascularization include incorporation or encapsulation of growth factors, topographical engineering, and 3D bioprinting. The vascularization of engineered tissues and their application in angiogenic therapy *in vivo* is reviewed herein with an emphasis on the most densely vascularized tissue of the human body - the heart.

Statement of significance: Vascularization is vital to wound healing and tissue regeneration, and development of hierarchical networks enables efficient nutrient transfer. In tissue engineering, vascularization is necessary to support physiologically dense engineered tissues, and thus the field seeks to induce vascular formation using biomaterials and chemical signals to provide appropriate, pro-angiogenic signals for cells. This review critically examines the materials and techniques used to generate scaffolds with spatiotemporal cues to direct vascularization in engineered and host tissues *in vitro* and *in vivo*. Assessment of the field's progress is intended to inspire vascular applications across all forms of tissue engineering with a specific focus on highlighting the nuances of cardiac tissue engineering for the greater regenerative medicine community.

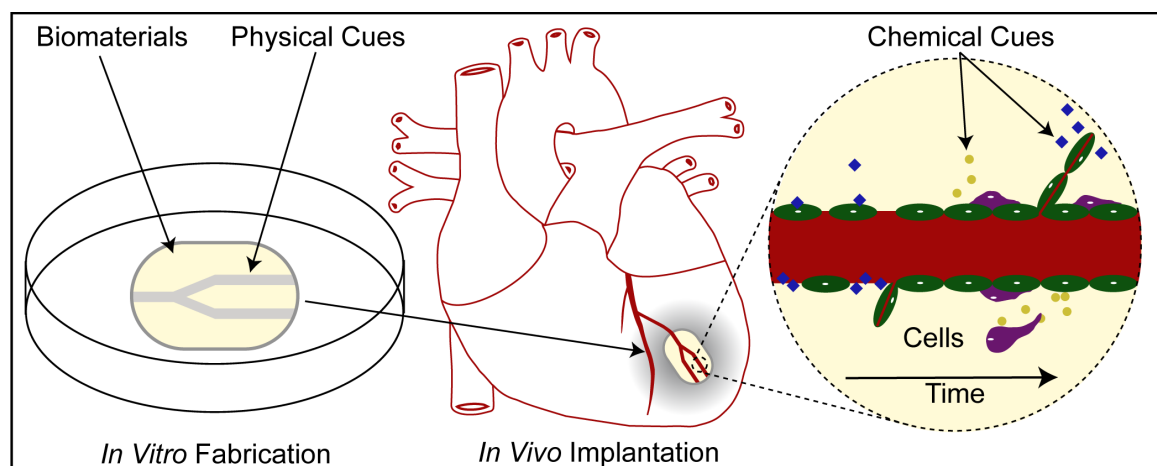


Figure 2.1: Graphical abstract.

2.2 Introduction

The regenerative potential of the human body after injury or disease is variable across different organs and tissues. In any healing scenario, there is a need for local microvasculature so that cells and proteins can be transported to the site of the injury to initiate and support the wound-healing response [1]. Furthermore, a hierarchical vascular bed is necessary to efficiently deliver blood to tissues, which necessitates a balance of capillaries and larger arteriolar vessels. The wound healing process is particularly paramount in organs like the brain and heart that have little functional regenerative potential, but where functional recapitulation of the damaged tissue is necessary for the continued health of the host. However, even with therapeutic intervention it is not yet possible to achieve this. For example, the high metabolic demand of cardiac tissue necessitates a high density, perfusable vascular network for sufficient nutrient and oxygen flux throughout the tissue. Consequently, the human heart has a dense vessel network of 2000-3000 capillaries/mm² and ~ 108 arterioles/mm², which supports the terminally differentiated, non-proliferative cardiomyocytes that endow contractile function [2–5]. Major coronary events such as a myocardial infarction (MI) can cause localized cell death if the tissue is not quickly supplied with adequate oxygen and nutrients, followed by fibrous scar tissue formation over the infarct area [6]. Post-MI complications such as reduced ejection fraction, ventricular hypertrophy, and arrhythmias can arise and lead to progressive heart failure wherein the heart is unable to pump enough blood to the rest of the body [7]. Cardiac tissue engineering seeks to regenerate the contractility of the heart that is lost following MI, but efforts up to this point have been unable to achieve full functional restoration, which may be partially attributed to the fact that currently implantable engineered constructs evaluated *in vivo* develop microvascular densities ~ 7 -fold lower than that of the native heart [8]. The only suitable therapy for end-stage heart failure patients is a heart transplant, but a lack of available donors and numerous complications make this a limited option for clinicians and

patients [3] and tissue engineering solutions hold promise to alleviate this problem through the development of therapies to restore tissue function.

In tissue engineering, scaffolds made of degradable biomaterials are commonly used to facilitate the creation, growth, implantation, and integration of cells with the host [7, 9]. However, there are a significant number of considerations involved in creating these tissues. One such challenge is the generation of thick, complex engineered tissues with sufficient microvasculature to support oxygen and nutrient transport between cells. The diffusional limit of oxygen restricts cells to being in close proximity to a capillary in order to acquire an adequate flux of oxygen, and the highly metabolic cardiomyocytes of the heart must be within 100-200 μm of a capillary for this reason [10–12]. The presence of a developed vascular network would have significant downstream effects on the success of the whole engineered tissue, namely enabling the development of more dense construct that better approximates the native tissue, allowing for superior inosculation with host vasculature upon implantation, and even having implications in remodeling and maturation, such as electrical syncytium formation in cardiac tissue [9, 10]. Thus, one of the major current goals of tissue engineering and regenerative medicine is the vascularization of 3D tissue constructs to mimic the networks of host tissue.

Complex physical and chemical mechanisms act on endothelial cells (ECs) to initiate *in vivo* angiogenesis followed by further processes to remodel and mature vasculature, which tissue engineers seek to emulate *in vitro* and *in vivo* through several methods [13]. Of interest in this review are techniques involving the manipulation of biomaterials to act as topographical signals to direct angiogenesis, and exposure to select growth factors (GFs) to similarly influence network formation [3, 10, 14]. Excellent reviews on biomaterial formulation and production for tissue engineering are found elsewhere [3, 9, 15–17]. However, a major obstacle and yet unmet need in the field is developing angiogenic systems of sufficient complexity such that dense and perfused microvascular networks form. Re-

cent studies have attempted to introduce more sophisticated uses of physical and chemical cues to selectively induce angiogenesis in engineered tissues, including *in vitro* prevascularization and encouraging penetration of host vasculature into implants via *in situ* tissue engineering methods. This review will summarize recent methods of spatiotemporally controlling angiogenesis in engineered tissues using biomaterials and combinatorial biological cues, including growth factors. Techniques will be drawn from all forms of vascular tissue engineering with the purpose of understanding, inspiring, and applying design considerations for spatiotemporal control across all of tissue engineering and within the subfield of cardiac tissue engineering.

2.3 Vessel development and physiology

The formation of vascular networks in the body begins early during embryonic development, and dynamic changes to the network occur throughout life in response to both stimulatory and inhibitory signals. Neovascularization arises through both vasculogenesis and angiogenesis, in which EC precursors differentiate into arterial, venous, or lymphatic endothelium. While angiogenesis is more commonly utilized in tissue engineering applications, it is crucial to fully understand the physiological processes that dictate vessel formation and remodeling to discern both the inspirations for studies as well as their current limitations. Vascularization is also integral in the overall wound-healing process, wherein neovessel formation is necessary to facilitate the transport of cells and proteins to the site of injury in order to repair the damaged tissue. Readers are directed to Barrientos et al. for a comprehensive review of the mechanisms of wound healing [1].

2.3.1 Vasculogenesis

Vasculogenesis is the *de novo* formation of blood vessels, most commonly via angioblast differentiation in prenatal development [10, 18]. Hemangioblasts originating from the lateral plate mesoderm are directed to aggregate and form “blood islands”, or aggregates of cells, comprised of hematopoietic stem cells surrounded by angioblasts. Endothelial progenitor cells (EPCs) arising from the angioblasts differentiate into ECs and the aggregates fuse into a primitive vascular plexus, generating lumens throughout the body that are perfused following the onset of the heartbeat [19]. The driving mechanisms of embryonic vasculogenesis are difficult to determine, and there exist several hypotheses and computational models suggesting that vascular development and arrangement occurs via cell motility guided by the extracellular matrix (ECM), paracrine and autocrine-mediated chemotaxis, and cellular contact signaling [20, 21]. Postnatal vasculogenesis is less well understood, but is thought to be dictated by EPCs residing in various locations around the body, such as within stem cell niches of the vascular basement membrane, in the circulation, or in the bone marrow that are mobilized in response to stimuli like growth factors and hypoxic stress [22]. These immature cell types are clearly crucial for *de novo* vessel formation, and studies have pursued methods such as the use of angioblast-like cells and co-culture to initiate vasculogenic processes in *in vitro* and *in vivo* settings [23, 24]. The use of developmental precursor cells such as pluripotent stem cell-derived angioblasts is promising as an *in vitro* model of vasculogenesis and for translational applications [25].

2.3.2 Angiogenesis

Angiogenesis is the major determinant for postnatal vascularization in which new capillaries are generated from existing vasculature through sprouting and intussusception processes [13, 26]. Sprouting occurs in response to events like inflammation or ischemia that

cause the release of stimulatory autocrine and paracrine cytokines, such as the very potent vascular endothelial growth factor (VEGF). Subsequent degradation of the local basement membrane and migration of vessel ECs, led by a specialized tip cell, results in leaky vasculature. Buildup and polymerization of plasma-derived proteins such as fibrinogen and fibronectin from the blood causes the formation of a provisional ECM that guides EC extension into the surrounding environment [27, 28]. Proliferation of stalk cells behind the tip cell and recruitment of mural cell populations allow for elongation, stability, and lumen formation of the sprout [13, 26, 29]. The process of intussusception involves the remodeling of existing vasculature via the protrusion and fusion of opposite vessel walls within a lumen, effectively splitting an existing vessel into two and creating a branched structure. In comparison to sprouting, intussusceptive angiogenesis is significantly faster in expanding capillary networks as it doesn't rely on cellular division, but rather reorganization of existing ECs [29]. Stress triggers like hypoxia and injury induce angiogenesis to form immature microvascular networks through both sprouting and intussusception processes, followed by pericyte invasion and subsequent cytokine-mediated cellular recruitment to stabilize and remodel the vessel walls and basement membrane [13, 27, 29]. The network of premature vessels is then pruned and remodeled to establish an efficiently perfused, hierarchical vascular bed that meets the oxygenation needs of the tissue. Vessel ablation in this manner is mediated primarily by hemodynamic cues from local vasculature, in which ECs migrate and integrate with nearby perfused vessels or otherwise undergo vascular regression via apoptosis due to lack of pro-survival stimuli such as shear flow and growth factor gradients [30]. As discussed below, many tissue engineering applications aim to induce an angiogenic response from host endothelial cells that takes advantage of the body's natural capacity to vascularize tissue through angiogenesis when regenerative strategies are employed in specific wound beds, such as an infarcted heart.

2.3.3 Arteriogenesis

In contrast to the capillary-forming process of angiogenesis, arteriogenesis is the compensatory remodeling of existing collateral blood vessels into functional arterial vessels in response to physical stimuli like flow-related stresses due to large pressure differentials between upstream arteries and downstream branches [31]. This process effectively increases vessel diameter and wall thickness through EC activation of downstream cellular pathways such as proliferation, and recruitment of supportive cell types for further autocrine and paracrine signaling. In instances where vascular networks are implanted, compensatory arteriogenesis does not occur until inosculation with the host network is achieved, as the absence of a flow stimulus in vasculature instead promotes vessel regression [30]. Caution is advised in how the tissue engineering community uses the term “arteriogenesis,” as published literature has defined it as the appearance of new artery-sized vessels (e.g., $< 50 \mu\text{m}$), although this is not the accepted definition historically in the field of vascular biology [32]. Arteriogenesis does not induce new vessel formation, but can act independent of angiogenesis through related pathways [33, 34]. It is an important post-anastomotic maturation process that operates in conjunction with vascular pruning to direct efficient vascular adaptations based on a tissue’s oxygenation needs [34]. For example, there is evidence that suggests that existing coronary arteries can undergo arteriogenesis in response to a cellular implant after MI [35]. Indeed, this remodeling mechanism is an excellent target of opportunity to ensure sufficient blood flow to implanted engineered tissues or to remodel bypass routes for increased blood flow such as in the case of peripheral artery disease or around a stenotic site.

2.3.4 Lymphvasculogenesis and lymphangiogenesis

The vasculature of the lymphatic system is a uniquely blind-ended vascular tract that is mainly derived from the venous lineage of endothelial cells during embryonic development (sometimes referred to as lymphvasculogenesis), and can be identified by lymphatic-specific markers such as PROX-1 and VEGFR3 [36, 37]. The lymphatic endothelial cells (LECs) that form lymph vessels are structured with loose intercellular junctions and a discontinuous basement membrane to facilitate the functions of the lymphatic system, which ranges from supporting the immune response to introducing interstitial fluid back into the circulatory system [38]. In the context of wound healing, lymphangiogenesis occurs in lymphatic capillaries after injury, wherein recruited inflammatory cells like macrophages release growth factors that stimulate lymphatic vascular growth, further facilitating transport of immune cells and cytokines to the site of damage as well as promoting interstitial drainage [37]. Lymphangiogenesis thus acts in concert with angiogenesis, yet there are far fewer studies pursuing or even considering lymphatic tissue engineering applications despite the equal importance of the former [39]. For example, Klotz et al. demonstrated that MI induction in mice causes upregulation of pro-lymphangiogenic genes in the surrounding heart tissue, which aids and may even expedite the resolution of the inflammatory response [36, 40]. Güc et al. demonstrated that an engineered VEGF-C variant that binds fibrin induced local lymphangiogenesis in a subcutaneous rodent model, and observed increased granulation tissue thickness and ECM deposition indicative of improved wound healing [41]. While not a focus of this review, it is important to understand how lymphangiogenesis interfaces with angiogenesis in wound repair, and how this process can be effectively integrated into current vascular tissue engineering efforts.

2.4 Cells in vascular tissue engineering research

The development of vascular endothelium and its specialized arterial and venous lineages are a central focus in vascular tissue engineering efforts and of interest to this review. The differences between individual cell types, what phenomena are trying to be recapitulated, and how relevant those results are to *in vivo* processes must be considered when choosing an EC lineage for vascularization studies. Furthermore, the interactions between ECs and other cell types must be carefully dissected and understood as well, either for co-culture studies or *in vivo* interactions with host cells.

2.4.1 Endothelial cell types

In vascular biology and tissue engineering research, ECs are commonly identified by staining for highly expressed markers such as platelet endothelial cell adhesion molecule-1 (PECAM-1, more commonly known as CD31), RECA-1 (rat endothelial cell antigen-1, the rodent equivalent of CD31), VE-Cadherin (CD144), or von Willebrand factor (vWF) [42]. Human umbilical vein endothelial cells (HUVECs) remain one of the more popular endothelial cell lines to use due to their low cost, simple isolation, and high angiogenic potential [43]. However, these are not the most physiologically relevant cell type to use, as angiogenic efforts often focus on microvascular bed development which is mediated by different, organ-specific EC lineages. Alternatively, studies have used primary aortic, vein, lymphatic, and organ-specific microvascular ECs from both humans and rats, donor human samples, and most recently ECs derived from human embryonic or induced pluripotent stem cells (hESC-ECs and hiPSC-ECs) [39, 44–46]. Rodent cells, while acceptable for *in vitro* and *in vivo* studies, are not translationally feasible given their xenogenic origin and thus incompatibility with the human immune system. Furthermore, species-specific differences, like the differential response of human and rodent aortic smooth muscle cells

(SMCs) to the anti-inflammatory molecule dexamethasone, limits the application of research findings in translational, clinical studies [47].

hiPSC-ECs provide a target of opportunity for translational application in that autologous therapies may be viable in the future [2]. Differentiation protocols to generate hiPSC-ECs previously relied on stromal cell co-culture, embryoid body, and small molecule monolayer strategies [45]. However, these methods are devoid of physical stimuli like flow which help specify particular EC fates, and often have low yields which necessitates time-consuming and expensive purification processes like fluorescence-activated cell sorting. It is suggested that genetic background has a bearing on the differentiation potential of stem cells across many lineages, including hiPSC-ECs [48]. Thus, lineages must be properly characterized before use whether differentiated or commercially sourced, and in the former case the differentiation protocol must be optimized for that specific lineage. More recently, attempts to generate hiPSC-ECs have used 3D matrices and/or induced flow to encourage hiPSC-EC differentiation for higher yields. The following articles are suggested for further reading on the differentiation, characterization, and application of hiPSC-ECs [45, 49].

EPCs are unspecialized, but determined vascular cells which have become a recent subject of interest in recapitulating embryonic vasculogenic development processes *in vitro* and *in vivo*. There is an important debate around what hemangioblast lineages constitute “true” EPCs, as these cells can be sourced from various organs, have variable differentiation efficiency into endothelial cells, and thus contribute differentially to vessel development [38, 50, 51]. EPCs can also arise from the differentiation of other cell types, such as the transdifferentiation of macrophages into lymphatic EPCs [52]. Peters et al. describes the requirements of EPCs to be expression of EC-associated markers (CD31, CD105, CD144, CD146), as well as a lack of certain hematopoietic markers (CD14, CD45, CD115), and readers are further referred to this review for an in depth review of the underlying biology of EPCs [38]. The main criticisms behind use of these cells is their relatively unknown

characterization and thus unpredictable effectiveness in culture, and the inability to easily source them commercially; many studies rely on human donor cells which are expensive and time-consuming to harvest.

In vascular tissue engineering and angiogenesis research more broadly, there are overarching concerns with the use of ECs and their related lineages *in vitro* and *in vivo*. Most endothelium in the body is naturally quiescent, and only takes on a proliferative phenotype in response to injury [43]. In this active state, ECs undergo a change in surface marker expression and can lose organ- and function-specific characteristics. Furthermore, the specific characteristics and responses of ECs are dependent on the tissue they originate from [53, 54]. This is a major challenge when developing studies involving ECs, as the need for proliferative and tubulogenic cells to form a vascular bed conflicts with the desire for those cells to recapitulate the structure and function of a native tissue. Furthermore, characteristics such as fenestrated, continuous, and discontinuous endothelium are unique and crucial to the proper function of organs, but experimental design to develop a capillary phenotype other than continuous endothelium is not considered in vascular tissue engineering studies, with “leakages” often being attributed to unstable, immature vessels [55]. However, these characteristics are specified in a paracrine fashion by local tissue, suggesting that specific phenotypes could be induced by surrounding host tissue after implantation [54]. Lastly, endothelial cell types tend to suffer from inactivation of proliferative and tubulogenic capacity at higher passages in culture [43].

Vascular endothelium is supported by cell types that are recruited at various steps of vascular development, homeostasis, and remodeling. SMCs are present around all vessels except capillaries and contract circumferentially to regulate transport of blood around the body, and can thus be used to identify arterioles by staining for α -smooth muscle actin (α -SMA) [27]. Pericytes maintain the basement membrane of capillaries and provide a number of supportive roles for ECs depending on the organ, but notably mediate paracrine

signaling, hemostasis, and vessel stabilization [56]. Multipotent lineages such as bone marrow- or adipose-derived mesenchymal stem cells (MSCs) can also be recruited and differentiate into vascular mural cell types [57]. Leukocytes like macrophages and monocytes provide cues for vascular remodeling such as matrix metalloproteinases (MMPs) and pro-angiogenic growth factors.

2.4.2 Co-culture with endothelial cells

Co-culture of ECs with these supportive cell types is a natural option given the multitude of cell types necessary to develop and maintain vasculature. This enables further specialization of an engineered tissue for specific applications, but requires consideration of the cell-cell signaling interactions of different lineages and their dependence on ratios of different cell types, as well as culture conditions such as substrates, matrix proteins, and media exposure *in vitro* [58]. For example, SMCs have been cultured with ECs to encourage the formation of arteriolar vessels [59]. Supportive stromal cell types are necessary for long-term (< 1 year) stabilization of EC networks by inducing a paracrine-mediated quiescent phenotype in the vascular cells [60, 61]. Paracrine signaling between ECs and supporting cell types also confers effects on gene expression, such as the upregulation of pro-angiogenic cytokines which affect vascular morphology [62, 63]. Optimal ratios of cell types for co-culture is less clear, as there does not appear to be a consensus due to the vast number of parameters to control, even within applications of a specific tissue type. Metabolic and proliferative potential, angiogenic potential of the ECs, lineage differences, paracrine interactions, culture media, and cell-biomaterial interactions are among the many potentially confounding variables that complicate the determination of definitive co-culture ratios [58]. However, it is known that high amounts of endothelial cells relative to other cell types typically induces vessel regression due to the redundancy of vascular structures [30]. In applications regarding the heart, cardiomyocytes have been cultured with ECs

or SMCs for cardiac tissue engineering applications and shown beneficial effects on cardiac tissue metrics [64, 65]. Tri-cultures of cardiomyocytes, ECs, and supportive stromal cells have been performed, but most studies are relegated to two cell populations due to the additional biological complexity that arises when > 2 cell populations exist in a system, which in certain cases produced less favorable results than co-culture [66–68]. The additional biological complexity of co-culture systems enables a greater and more physiologically relevant understanding of cellular interactions both with other cells and their microenvironment, which is critical in implementing vascularization efforts in specific tissue engineering applications.

2.5 Factors and signaling pathways in angiogenesis

Angiogenesis is dependent on cell-cell and cell-matrix interactions, meaning that careful orchestration of the appropriate signaling factors and pathways is required to induce neovascularization in engineered tissues. The most well-known molecules used to encourage vascularization are angiogenic growth factors, morphogenetic proteins that induce and support angiogenic processes such as proliferation, differentiation, and migration of cells through a variety of unique signal transduction pathways [69]. Indeed, the use of “traditional” angiogenic factors and their recombinant variants is ubiquitous in the literature in a variety of applications, and research has focused heavily on improving the stability, expression, delivery, and retention of single factors to target tissues [69]. The natural complexity of angiogenesis stems from the multiple isoforms of growth factors and cytokines that interact with target receptors in time-dependent and spatially-controlled ways to elicit a specific response [70]. The nuances of these signaling pathways is a great challenge for translational application of angiogenic proteins as therapeutics. Temporal exposure of growth factors must be carefully considered, such as a controlled release system can be designed to maintain a local concentration that elicits a prolonged physiological response.

However, inhibition, sequestration, or downregulated production of growth factors and their downstream pathways is just as important to prevent uncontrolled growth and potentially harmful consequences [70]. Thus, therapeutic angiogenesis must be precisely regulated by limiting it to the intended therapeutic areas for an appropriate time frame. There are even studies showing that controlled inhibition can assist in angiogenesis, such as through anti-growth factor drugs like anti-VEGF or by specific inhibition of proteins like glycogen synthase kinase 3β in post-MI hearts to activate the Wnt signaling pathway [55, 71–73]. Despite being utilized less in vascular tissue engineering, these inhibitory compounds are as important as their angiogenic-inducing analogs [42]. A few common growth factor families that are important in angiogenic cascades and utilized in vascular regeneration will be briefly discussed to better understand their role in developing mature vascular networks.

2.5.1 Vascular endothelial growth factor (VEGF)

The VEGF family of angiogenic growth factors is considered the gold standard, playing a significant role in both vasculogenesis and angiogenesis [19, 74]. These homodimeric polypeptides consist of six main isoforms of VEGF (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and VEGF-F), as well as placental growth factor (PlGF), and the associated receptors VEGFR-1, VEGFR-2, VEGFR-3, Neuropilin-1 (NRP-1), and NRP-2 [55, 74, 75]. VEGF-A was the first factor of the family discovered and is presently the most well-known and widely used factor, as it plays a major role in mediating vascular EC survival, proliferation, and migration [13]. VEGF-A and its spliced variants can bind both VEGFR-1 and VEGFR-2, although binding of the latter receptor is far better understood and mediates pathways with the strongest angiogenic responses [76]. Binding to VEGFR-2 causes dimerization of the receptor and enables phosphorylation at multiple tyrosine domains, which dictates further downstream signaling cascades to orchestrate angiogenesis. VEGF-B assists in the maintenance and maturation of formed vessels, and interestingly a recent study

implicated VEGF-B as a non-angiogenic cardioprotective factor through mitigation of oxidative stress-induced apoptosis [73, 77, 78]. VEGF-C and VEGF-D can bind VEGFR3 (primarily), VEGFR2, NRP-1, and NRP2, causing downstream upregulation of lymphangiogenesis to support the wound-healing process [75, 76]. PlGF is a key player in vascular development during embryogenesis, and additionally displays synergistic roles with other VEGF isoforms in wound healing, ischemia, and other stressed conditions [1, 13, 55, 79]. VEGF-E and VEGF-F are special angiogenic proteins; the former is encoded by the Orf virus and only binds VEGFR-2, and the latter encompasses a subfamily of VEGF-like molecules present in snake venom which bind mainly VEGFR-1 and promote high vascular permeability [79]. VEGF-A remains the most commonly used angiogenic growth factor within the VEGF family in vascular engineering applications due to its ubiquitous presence in initiating angiogenic processes and its potent effects, with the other isoforms often relegated to more niche applications. However, despite this pervasive presence, studies have demonstrated that VEGF alone is limited in its ability to induce the formation of functional, dense, and persistent vessel networks [70, 80]. Disorganized and often leaky vessels result from the sole delivery of VEGF, which can be indicative of unstable, immature vessels that require additional modulation from other factors or cells [81].

2.5.2 Fibroblast growth factor (FGF)

The FGF family consists of 22 growth factors and 4 associated receptors with their alternative splicing variants, which mediate a wide variety of functions from angiogenesis to neural development [82, 83]. Despite the name, some of the molecules in this family are not considered traditional growth factors, but they all share the common ability to bind to FGF receptor (FGFR) isoforms [83]. Of significant relevance to angiogenesis are FGF-1 (acidic FGF, or aFGF) and FGF-2 (basic FGF, or bFGF), although other isoforms have also been implicated in angiogenic pathways [83, 84]. Binding to the appropriate receptor (such as

FGF-1 to FGFR-1 or FGFR-2) in the presence of heparin-sulfate proteoglycans in ECM and on the surface of cells causes tyrosine phosphorylation and subsequent signal transduction, such as through binding of the cytoplasmic effectors FGF receptor substrate 2 α or phospholipase C γ [55, 83]. FGF promotes tubulogenesis and migration of ECs and EPCs, and can assist in signaling for both ECM proteolysis and synthesis of collagen, fibronectin, and proteoglycans, as well as enhancing VEGF production, suggesting synergistic actions for angiogenesis [84–86]. Further, both VEGF and FGF provide cues for initiating angiogenesis by inducing EC proliferation and migration, but act through different pathways [55]. For example, a study by Lee et al. compared the effects of VEGF and bFGF in subcutaneously implanted alginate hydrogels and found that both growth factors were individually able to induce granulation tissue and vessel formation [87]. However, FGF signaling was unable to increase blood vessel density to levels as high as in VEGF-loaded hydrogels. Studies have also posited that FGF isoforms assist in cardiac remodeling and endow cardioprotective properties through angiogenesis and lesser-understood paracrine pathways [83].

2.5.3 Platelet derived growth factor (PDGF)

Closely related to VEGF is the PDGF family of growth factors which encompasses PDGF-A, PDGF-B, PDGF-C, PDGF-D, the heterodimer PDGF-AB, and the receptors PDGFR- α and PDGFR- β [88, 89]. Homodimer PDGF isoforms remain inactive until dimerization and receptor binding occurs, allowing for receptor dimerization, auto-phosphorylation and subsequent downstream signaling dependent on the variety of dimer complexes formed, phosphorylated domains, and associated adapter molecules involved [89]. While normally secreted by circulating platelets in the bloodstream, other cell types have been shown to release specific isoforms of the factor. PDGF-AA, BB, and CC are the most well-known supporting factors for angiogenesis. ECs, fibroblasts, macrophages, and other vascular support cells can release this growth factor after neovessels are established, generating

a chemotactic gradient that further attracts pericytes, leukocytes, fibroblasts, and vascular SMCs to stabilize, remodel, and mature vessels [55, 90–92]. PDGF isoforms exhibit VEGF-independent stimulation of angiogenic pathways, such as the necessity of PDGF-B in recruiting SMCs/pericytes to immature vessels for stabilization and remodeling [92, 93]. Absence of this factor has been found to result in a maintenance of the proliferative EC phenotype which is not conducive to maturation, or even vessel regression in the event of arrested VEGF signaling [91, 93].

2.5.4 Other growth factors and cytokines contributing to angiogenesis

Many other cytokines participate in angiogenesis in addition to the dominant effects of those described above. Some are not considered traditional growth factors at all, but cytokines and other proteins that produce downstream effects on angiogenesis in tissues which can be dependent on specific conditions, including location, cell type, and timing. For example, the angiopoietin (Ang) family of growth factors are critically involved in embryonic vascular development via the Tie pathway, and are also necessary for sprouting angiogenesis, vessel wall remodeling, EC survival, and mural cell recruitment [84, 90, 94, 95]. Transforming growth factor- β (TGF- β) is released by ECs as part of the inflammatory response to recruit macrophages, and further can help mediate the differentiation of vascular SMCs and contribute to EC proliferation, migration, and tubulogenesis to form and maintain granulation tissue [84, 91]. Sonic hedgehog (SHH) is a morphogen that is present in both embryonic development as well as postnatal angiogenesis, and has been shown to mediate expression of angiogenic growth factors like VEGF-A and Ang isoforms, promote angiogenic cytokine production from stromal populations, and enhance recruitment of EPCs to remodel microvascular networks [96–101]. SHH is upregulated in the ischemic heart, and has been shown to act in a cardioprotective manner via paracrine-mediated growth factor upregulation, arrested cardiomyocyte apoptosis, and neovascular-

ization [102, 103]. MMPs initiate ECM remodeling events that are required for EC sprouting and neovessel intussusception [13, 84]. MMP-14 (also called MT1-MMP) in particular is a membrane-bound MMP that has been shown to play a crucial role in facilitating angiogenesis by degrading the ECM for cell migration and activating membrane-bound growth factors [104]. MMP-9 and MMP-12 have been noted to play a role in angiogenesis and vessel stabilization as well, such as in creation of angiostatin to inhibit vessel formation, although many studies examining these proteins are found in the context of inhibiting tumor angiogenesis and metastasis via MMP inhibition [84, 105].

Specific events can induce a variable angiogenic response as well, as is the case of factors that provide some level of compensatory cardioprotective effect after a heart attack. When myocardial ischemia occurs, hepatocyte growth factor (HGF) is induced in the myocardium, and nerve growth factor (NGF) and insulin-like growth factor-1 (IGF-1) are secreted to mitigate cardiac damage after infarction and assist in activating angiogenic pathways [13, 106–108]. Neovascularization-related growth factors play an important role in ischemic heart disease (reviewed by Henning, 2016) including stromal cell-derived factor-1 (SDF-1), which has been shown to attenuate cardiomyocyte apoptosis and stimulate angiogenesis in both *in vitro* angiogenic assays and *in vivo* rat models of MI [74, 109, 110]. Other types of exogenously delivered molecules can be used to induce vascularization in contexts like cardiac repair. For instance, the drug ONO1301, an agonist for Prostaglandin I₂, was shown to induce expression of VEGF, SDF, and HGF in the myocardium and acted in a cardioprotective role by increasing local vascularization and overall cardiac performance in a hamster model of dilated cardiomyopathy [108]. Growth factor fragments and peptide sequences derived from larger molecules involved in angiogenesis pathways have demonstrated improved function versus full-length molecules due to modified binding affinities and protein stability [111–114]. For example, a peptide from the Notch ligand Jagged1 improved the vascular bed in an *in vivo* model of cardiac failure and recruited cardioprotective cytokines through downstream pathway activation [115]. Adipose-derived microvascular

fragments isolated from rodent fat pads have been found to induce significant angiogenesis and lymphangiogenesis when implanted subcutaneously, and may be a translationally relevant methodology for implant prevascularization or direct transplantation [116, 117].

The vast number of molecules that participate in angiogenesis via different pathways prompts the notion that preferential molecule choice will be required to stimulate the intended angiogenic phenotype. For example, Benest et al. demonstrated *in vivo* that VEGF signaling induced several short, highly branched vessels, while Ang-1 signaling produced longer but less branched vessels as well as higher pericyte recruitment [118]. At the same time, there may be some leeway in angiogenic growth factor selection if another molecule can sufficiently supplant the original factor's angiogenic functions, and surrounding host tissue can be left to provide the necessary maturation and remodeling signals. Grigorescu et al. demonstrated in their study of angiogenic properties of EPCs that the depletion of VEGF-A alone did not significantly interrupt angiogenic processes in culture, but simultaneous knockout of both VEGF-A and SDF-1 resulted in severely stunted angiogenesis [119]. This calls into question the assumption that VEGF is the primary initiator of angiogenesis and encourages discourse around where interventions should target along angiogenic signaling cascades to produce the intended therapeutic effect. Exposure to multiple factors to robustly stimulate this cascade in key pathways may be enough to develop a new, permanent vasculature in tailored regenerative medicine applications. There are still many unknown intermediate mechanisms, such as the interplay of CD31 in the angiogenesis pathway, and often *in vitro* isolation studies are unable to capture the complexity of growth factor response *in vivo* [42]. What has been made clear is that these signaling molecules elicit multi-pathway effects and induce paracrine activity [14, 108]. Thus, angiogenic systems must be designed knowing as much as possible about systemic effects across the regulatory network, and be inclusive of the waterfall effects of the biochemical stimuli used.

2.5.5 The necessity of combinatorial growth factor use

The study of single-use growth factor release has revealed the great complexity of angiogenic signaling responses. The vast research into the actions of growth factors has made it clear that the use of a single growth factor is not sufficient to create a stable vascular structure, as many growth factors work either in tandem or sequentially [120]. For example, it has been shown that VEGF-C promotes PDGF-BB expression in ischemic tissues [13]. A study by Stone et al. found that endogenous VEGF-A alone cannot properly induce both angiogenesis and arteriogenesis, and that additional modulation with exogenous Ang-1 to act as a maturation agent could produce more physiologically relevant vascular networks [80]. Brudno et al. further demonstrated that simultaneous exposure of VEGF-A and Ang2 additively induced angiogenesis *in vitro* and *in vivo* [94]. This response was inhibited by synchronous release of PDGF-BB and Ang1, but delayed release of the latter two factors did not interfere with angiogenesis but instead promoted vessel maturation and remodeling. As a final example, bone morphogenetic proteins (BMPs) have been shown to promote angiogenesis by stimulating osteoblasts to produce VEGF-A [121]. Thus, understanding these relationships within the organ of interest is crucial to designing angiogenic tissue engineering systems. **Fig. 2.2** demonstrates the temporal complexity and overlap of multiple prominent pathways in angiogenesis and its relation to the wound healing cascade. Implementing this notion of combinatorial growth factor use in experimental design has enabled a more thorough examination of multi-pathway effects and paracrine signaling in angiogenesis, which can then be leveraged to generate more robust vascular beds in engineered tissues by creatively presenting cues that initiate these interactions. This beneficial shift in direction has been accompanied by the popularity of engineering biomaterials for tissue engineering applications, providing the field with the tools necessary to develop and examine more complex systems for spatiotemporal administration of multiple angiogenic growth factors.

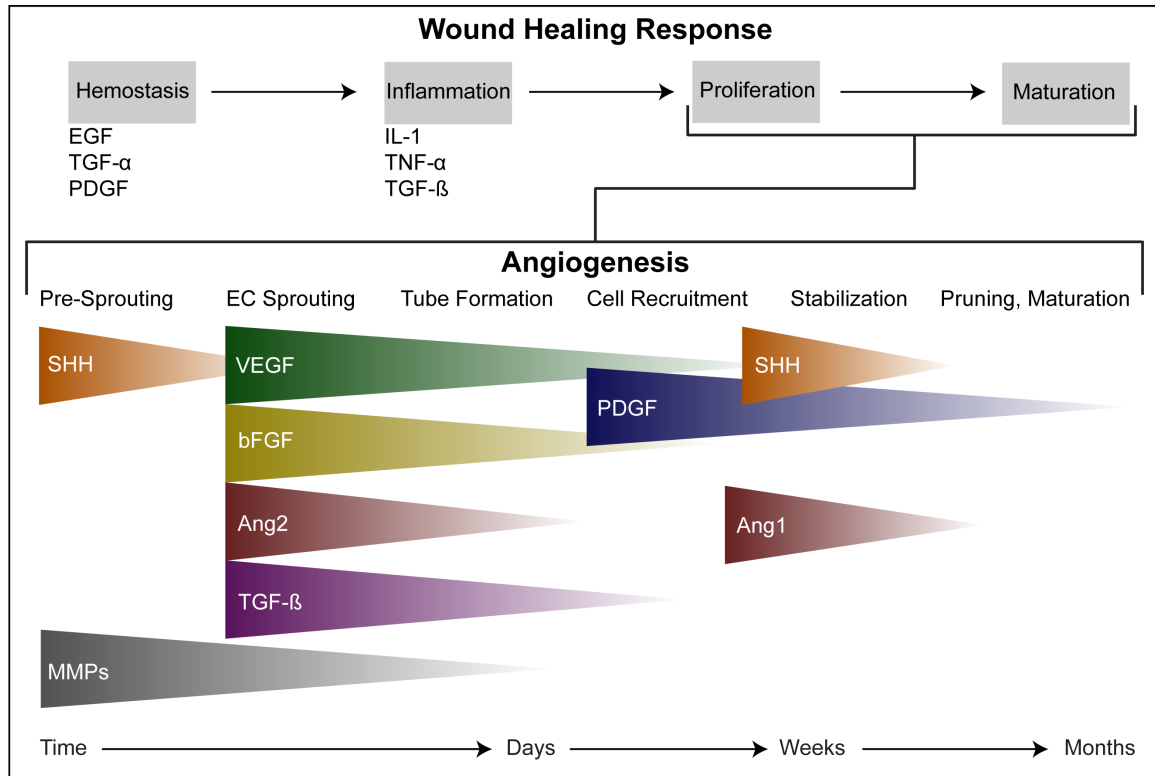


Figure 2.2: Temporally controlled, overlapping, parallel, and repetitive signaling events (abridged) in angiogenesis and their role in the wound healing response. Examples of activated factors are listed below each stage of wound healing. Ang: Angiopoietin; bFGF: Basic fibroblast growth factor; EGF: Epidermal growth factor; bFGF: Basic fibroblastic growth factor; IL-1: Interleukin-1; MMPs: Matrix metalloproteinases; PDGF: Platelet-derived growth factor; SHH: Sonic hedgehog; TGF- β : Transforming growth factor beta; TNF: Tumor necrosis factor; VEGF: Vascular endothelial growth factor.

2.6 Techniques for controlling angiogenesis with biomaterials

The use of biomaterials has expanded in the past decade with the development of innovative techniques to support the growth of tissues both *in vitro* and *in vivo* and provide cells with well-defined spatiotemporal cues to initiate angiogenesis [10]. The use of synthetic and natural materials imposes constraints dependent on the specific application of the biomaterial. These materials must be chosen carefully, as there will inevitably be interactions when placed into a physiological environment, whether that be through cell-material inter-

actions *in vitro* or an implant *in vivo*. Cells that require adhesions should be able to adhere and interact with the matrix and neighboring cells, whereas cells not requiring adhesions should be able to survive and function within the microenvironment of the biomaterial. In short, the material in question must be biocompatible and tailored to the specific engineered tissue [3, 9]. Material selection is thus a significant component of tissue engineering, and certainly there is no shortage of studies examining optimal material choices and even developing composites and entirely new materials with which to engineer replacement tissues [122–125]. Of interest to the subject of this review, however, is the manipulation of these materials and their interactions with angiogenic growth factors to control angiogenesis and neovascularization in engineered tissues.

2.6.1 Matrix incorporation

Incorporation of angiogenic growth factors within a scaffold matrix is a common method of presenting bioactive agents to cells. Direct interactions between proteins and materials provides growth factors with some level of protection from natural processes in the body that could inhibit activity, such as cellular inactivation or enzymatic digestion [44, 126]. Angiogenic growth factor release kinetics are mediated by factors including concentration, size, diffusion rate, porosity, scaffold degradation rate, and extent of polymer crosslinking. Material formulations and modifications can enable release by specific stimuli like pH and temperature change or biochemical activation, which lends increased spatiotemporal control to the system [14, 27, 125]. Retention of factors in the scaffold encourages cellular responses originating from cells seeded into the scaffold as well as ingrowth from host tissue in *in vivo* settings. Growth factor incorporation is commonly achieved through covalent binding interactions of the cytokine with the matrix or binding domain modification, but can also be achieved through techniques like adsorption (adhesion of substances to a surface) and ionic complexation (formation of polyion structures through combinations of

various ions and molecules) [27, 125].

Tuning EC responses by covalently bound growth factor incorporation demonstrates how both understanding the cell biology and materials chemistry is critical for advancing angiogenic therapies. For example, Chiu et al. showed that covalent immobilization of VEGF-165 (a recombinant variant of VEGF-A) and/or Ang-1 on collagen scaffolds using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) chemistry resulted in increased proliferation and tubulogenesis of seeded ECs *in vitro* after 7 days in comparison to controls containing solubilized growth factors [127]. Stepwise angiogenic growth factor immobilization after EDC activation in a phosphate buffered saline (PBS) reaction buffer also resulted in higher protein incorporation than simultaneous processing in PBS, DI water, or 2-(N-morpholino)ethanesulfonic acid (MES) buffers. In a related study by the same group, Odedra et al. created radial gradients of immobilized VEGF-165 in EDC-activated cylindrical collagen scaffolds using point source, flow, and source-sink methods [128]. Enhanced D4T EC (an embryoid body-derived mouse lineage) migration from the periphery of the 12 mm diameter, 2.5 mm thick scaffolds created a cell distribution mimicking the angiogenic growth factor gradient, although a corresponding enhanced proliferation was not observed. This study demonstrates that mimicking the chemotactic gradients generated by angiogenic growth factors is a useful tool for organizing and directing ECs *in vitro*.

Introducing heparin or heparin-binding domains into a biomaterial allows for increased specificity to sequester heparin-binding growth factors such as VEGF, PDGF, and bFGF in the material matrix, and even utilize it to sequester endogenous factors in a reservoir when implanted *in vivo*. Wu et al. bound heparin to decellularized liver scaffolds using an endpoint attachment technique (terminal reductive amination of the sugar chain and attachment with an amine linker), followed by incubation of the scaffolds overnight with VEGF-165 to allow for adsorption [129, 130]. Significant proliferation of HUVECs *in vitro* (~65%

in comparison to 20-25% for controls, $p < 0.05$) was present in the growth-factor bound scaffolds as measured by Ki67 expression. One area of consideration is uniform binding of heparin to a scaffold surface, as heterogeneous distribution of heparin means that there will also be a heterogeneous distribution of growth factor throughout the matrix. Techniques like the toluidine blue metachromatic assay can be used to stain and thus visually confirm the presence of a heparin coating [131]. Quade et al. utilized a bolus injection strategy reminiscent of Odedra et al. in which VEGF was incorporated in a single central biopolymer depot within a collagen scaffold, and the kinetics of release and resulting migration on human dermal microvascular ECs were examined *in vitro* over 28 days [46]. Heparin, alginate, hyaluronic acid, alginate-heparin, and alginate-hyaluronic acid biopolymers were all tested, with the authors finding that the alginate and alginate-heparin depots could best retard burst release of VEGF within the first four days and allow for more sustained release in subsequent days. Migration assays revealed that the increase in cellular proliferation was correlated with the release of VEGF in each biopolymer group, with higher burst release materials resulting in increased proliferation at early time points. In contrast, the heparin and alginate-heparin groups demonstrated higher proliferation rates at later time points. Migration assays revealed that the alginate-heparin group enabled the highest migration towards the central depot, with a > 2 -fold increase in the number of cells found 2000-2500 μm deep in comparison to controls.

As an example of dual growth factor incorporation, Lakshmanan et al. demonstrated the incorporation of VEGF and bFGF in a scaffold blend made of poly(L-lactide-co-caprolactone) (PLCL) and poly(2-ethyl-2-oxazoline) (PEOz) by dissolving the factors in PBS and adding it to the prepolymer solution prior to electrospinning [132]. Release of the growth factors from the matrix *in vitro* resulted in HUVEC proliferation and migration, and gene expression analysis confirmed the presence of angiogenic gene upregulation in the form of VEGFR, PDGFR, and FGFR isoform expression. However, the authors reported an encapsulation efficiency of $< 30\%$ across different combinations of their growth factors and

materials, and a significant burst release likely due to the lack of strong binding interactions between the growth factors and biomaterials.

Despite being a widespread method of modulating growth factor retention and release, matrix incorporation has inherent drawbacks. Angiogenic growth factors in a matrix can fail to generate a truly guided vessel network because random distribution and immobilization within the matrix can generate imperfect chemotactic gradients upon release, resulting in less well-defined spatial control of angiogenesis [10]. While biological vascular development is not necessarily structured and organized, tissue engineers may seek to have greater spatial control of angiogenic processes in their systems. There are studies that address this issue, such as a study by Yuen et al. wherein the use of stimulatory VEGF and inhibitory anti-VEGF signals restricted angiogenesis of human dermal microvascular ECs to 1 mm thick zones within a scaffold using a layer-by-layer incorporation strategy, although specific vessel geometries could not be controlled [71]. This dual incorporation method attenuated the initial net VEGF burst release due to the antagonistic effect of the two molecules, allowing for a temporally stable release profile over 30 days *in vitro* in the specified angiogenic zones. A final issue is that properties like loading and release rates of angiogenic growth factors are directly tied to the parent scaffold, meaning altering the properties and kinetics of growth factors in a system necessitates adjusting the scaffold properties [126]. These problems indicate the need for more sophisticated modulation of angiogenesis within a scaffold matrix through spatiotemporal interactions of growth factors and biomaterials with constituent cells in the scaffold, which has motivated the development of encapsulation methods to increase the control over material, protein, and/or cell localization.

2.6.2 Encapsulation

The encapsulation of angiogenic growth factors is another method of controlling growth factor release similar to incorporation, but the key difference is that the protein is incorporated in a separate, often micro- or nano-sized carrier composed of a material different from that of the bulk scaffold. This effectively decouples the scaffold properties from the properties and kinetics of the growth factor release, and provides an additional layer of protection to maintain angiogenic growth factor bioactivity [126]. The carriers themselves are typically spherical in shape and can be created by way of solvent extraction/evaporation, coacervation (phase separation), or spray-drying [133]. Solvent extraction/evaporation consists of dispersing a bioactive molecule in the particle precursor solvent, emulsifying the solvent in an immiscible phase, then extraction of the solvent to obtain microparticles. Coacervation is the separation of a coating material's liquid phase from a polymer-rich solution containing the molecule of interest, followed by solidification of the coating material around the core polymers. Spray drying is formation of microparticles by spraying an amphipathic prepolymer solution loaded with a bioactive compound through a heated atomizer to form micelles. Encapsulation of bioactive compounds is achieved through various methods such as sequestration inside a material, property-based interactions based on chemical properties such as solubility and hydrophobicity, and covalent chemical binding, and can be accomplished either during or after carrier formation [27, 125, 134]. The overall process is often referred to as micro- or nanoencapsulation depending on the size scale of the synthesized carriers. The loaded particles can then be embedded in a scaffold or delivered separately to the target site, then released via diffusion. Sequestration of proteins in microspheres provides an opportunity for spatiotemporal control of angiogenic growth factor bioactivity beyond that capable with incorporation, which can aid in generating chemotactic gradients to facilitate angiogenesis and cell recruitment [135, 136].

Many of the same strategies in growth factor incorporation can be applied to micro-

sphere encapsulation. For example, Roberts et al. demonstrated heparin-mediated loading and release of VEGF and bFGF from poly(vinyl alcohol) (PVA)-heparin hydrogels with decreased burst release when compared to control PVA gels [135]. This dual but independent angiogenic growth factor release resulted in a 4.2-fold increase in the proliferation of HUVECs over 3 days in comparison to cells cultured only in endothelial cell media. In a study by Akar et al., 10 mm bilayer disk scaffolds composed of a 3.7 mm thick layer of PEG-fibrin under a 0.3 mm layer of PEG-poly(lactic acid) (PEG-PLLA) with PDGF-BB-loaded poly(lactic-co-glycolic acid) (PLGA) microspheres seeded into the thin layer were implanted subcutaneously in rats next to skeletal muscle [137]. The release and diffusion of the growth factor generated a chemotactic gradient that caused dose-dependent invasion of cells and increased blood vessel density in the PEG-fibrin scaffold over six weeks.

Encapsulation techniques enable greater flexibility in designing complex growth factor release systems. For example, Lai et al. demonstrated the independent release of four growth factors from a layered electrospun scaffold made of collagen and hyaluronic acid with embedded gelatin nanoparticles [120]. As the construct degraded, bFGF and epidermal growth factor (EGF) were released from the scaffold as well as VEGF and PDGF from the nanoparticles over the course of a month, significantly increasing HUVEC proliferation, tubulogenesis, and branching *in vitro* in comparison to controls. *In vivo* models showed that the growth factors resulted in approximately 148 capillaries/mm² (control = 90 capillaries/mm²) and significantly higher CD31-positive and α -SMA-positive vessels in the wound area by week 4 as determined by quantitative histological staining, which is indicative of stabilized vasculature. In an example of multiphase growth factor release, Izadifar et al. developed a model for nanoparticle release kinetics, and validated it experimentally with bilayer nanoparticles made of PLLA and PLGA which released VEGF and bFGF followed by PDGF from the nanoparticle core after 5 days [138]. The authors further found that nanoparticles with a smooth polymer surface reduced surface area and abated the burst release. In another study, Dashnyam et al. promoted *in vitro* tubulogenesis

in HUVECs using mesoporous silica nanoparticles that released silicon ions and VEGF [139]. The bioactive silicon ions had been shown to induce angiogenesis in ECs following studies investigating the paracrine effects of bioactive glasses on angiogenesis signaling *in vitro* and *in vivo* [140]. Interestingly, gene and protein expression analysis indicated that the silicon molecules upregulated expression of hypoxia inducing factor 1 (HIF1 α) ($p < 0.05$), causing downstream angiogenic signaling that resulted in increased bFGF protein expression as determined by western blot analysis [139].

Recent innovations in the field have begun to ameliorate what were once significant disadvantages of growth factor encapsulation. Encapsulation method, carrier size, material selection, and protein to carrier ratios are among the considerations that affect encapsulation efficiency and thus directly impact downstream properties in the system like release kinetics [133]. Burst release is common due to the high ratio of surface area to volume of the encapsulation vehicles but can be prevented by developing multi-layer particles or modifying the particle characteristics [125, 138]. Quantification of release profiles has been problematic as assumptions of *in vitro* release do not necessarily translate to *in vivo* outcomes, as demonstrated in the study by Izadifar et al. who were unable to determine the benefits of co-release of three growth factors at once opposed to sequential release [14, 138]. This can be approached through the use of model proteins and development of analytical programs to predict loading efficiency and release kinetics [136, 138], yet tracking actual protein release *in vivo* is the gold standard and can be achieved using unique protein tags such as biotinylation [141]. Understanding the properties of the biomaterials and the proteins or other biologicals themselves used in microencapsulation methods is key to designing an effective delivery system.

2.6.3 Topographical engineering

Vascular beds can alternatively be generated with specific and detailed geometries through topographical engineering, which is the manipulation of materials or chemicals to act as regulatory cues for the directed assembly of multicellular structures, often at the hundreds of microns scale and often containing micro- and nanoscale topographical features. In the context of vascularization, this generally entails physical or chemical methods of patterning vessels by restricting EC attachment through methods like specific adherence and alignment to predefined strips of material or fibers, or organization on or within patent channels [142].

Lithography is the largest subgroup within topographical engineering which can generate specific geometries using microfabrication techniques. Soft lithography is commonly used to induce these physical cues through the creation of an elastomeric mold with defined topographies such as oriented channels. These techniques often involve the creation of intermediate molds, typically made of silicon, acrylic, or glass, and which allow for high throughput replication of the final mold, often made of polydimethylsiloxane (PDMS), and lower overall production costs. For example, Zorlutuna et al. utilized X-ray interference lithography to generate nanopatterned tubular collagen scaffolds which were then seeded with human vascular SMCs and human internal thoracic artery ECs [143]. The topography of the scaffold assisted in orienting the SMCs circumferentially around the lumens and ECs in the interior lumen, all while maintaining the proliferative capacity of each cell type. In another study by Zheng et al., soft lithography was used to create a microfluidic device made of collagen gel with continuous channels that generated patent 3D microvessels when perfused with HUVECs in growth medium for 7 days followed by pro-angiogenic media (containing exogenous VEGF, bFGF, and phorbol12-myristate-13-acetate) for another 7 days [144]. In the presence of human brain vascular pericytes, the constructs demonstrated differential sprouting angiogenesis into the collagen bulk, with half of the constructs

sprouting an average of 75.4 μm into the bulk, and other samples not sprouting, and even retracting from the walls. The authors posited several hypotheses for these conflicting results, including variable density of cells and different conditions when gelling the collagen. Lithographic microfluidics are also useful in characterization of cell lines, as demonstrated by Kurokawa et al. who developed a PDMS microfluidic device composed of sequential tissue chambers flanked on either side with fibronectin-coated fluid channels that could be used to characterize the physiological functionality of their iPSC-ECs [145]. In this study, the authors first differentiated mCherry-transfected iPSCs into ECs, then sorted and seeded the CD31+ cells with fibrin into the main chambers and channel flow was set to generate a shear stress of 4 dyn/cm^2 . Over the course of 14 days, the ECs aligned in the direction of flow and formed vessels 60 μm in diameter on average which inosculated with the adjacent fluid channels, as determined by perfusion of FITC-dextran.

Other forms of topographical modification can also be performed, such as stamping films of self-assembling monolayers as in microcontact printing, or circulating chemicals and cells as in microfluidics [146, 147]. Chemical stamping induces patterning, either by preferential adsorption of a peptide to a substrate or surface patterning to mediate cellular behavior [142]. Photolithography and electron beam lithography enable the use of UV-curable materials by applying a mask and photoresist layer to materials in order to fabricate specific topographies on a substrate with resolutions as low as a 1 μm [142, 147]. In a study aimed at inducing angiogenesis through other means than exogenous growth factors, Lei et al. stamped SVVYGLR peptide strips (found adjacent to the RGD sequence in thrombin-cleaved osteopontin, and reported to activate ECs *in vitro* and induce angiogenesis *in vivo*) onto a polyethylene terephthalate film using photolithography, then seeded HUVECs to the substrate [148]. Cells began to align within 4 hours of seeding and underwent morphogenesis into lumen shapes, with greater alignment and shape change occurring in lower-width peptide strips of 10 and 50 μm as opposed to 100 μm .

Topographical engineering has been utilized to generate vessel-like conduits in cardiac tissues engineered *in vitro*. Thomson et al. developed a porous fibrin scaffold from a micro-template of parallel-aligned 60 μm polycarbonate fibers bundled in a poly(methyl methacrylate) (PMMA) shell and spaces infiltrated with 27 μm diameter PMMA beads [68]. Once the fibrin polymerized around the shell template, the polycarbonate fibers and beads were sacrificed to generate a porous microstructure with micro-channels spanning the length of the scaffold. With subsequent centrifuge-assisted loading of a tri-culture of primary neonatal rat ventricular myocytes (NRVMs), neonatal rat cardiac fibroblasts, and ECs (HUVECS or rat aortic ECs), the constructs supported *in vitro* cellular organization into the scaffold micro-channels, with the ECs forming aligned lumen structures, the NRVMs aligning and spontaneously beating, and ECM deposition by the fibroblasts. In another study, Vollert et al. created vessel channels by embedding alginate fibers in a fibrin cardiac scaffold with a murine cell mixture of cardiomyocytes, fibroblasts, SMCs, and ECs, sacrificing the alginate, then perfusing the generated lumens over 15-25 days [149]. The average luminal diameter was estimated to be 100 μm , which expanded to approximately 500 μm during perfusion, and histological analysis indicated that ECs from the migrated to the lumen walls. Recently Zhang et al. developed a synthetic microvascular chip coined 'Angiochip' for loading cells into bulk spaces and ECs into patterned lumens that could be surgically anastomosed to host vasculature *in vivo* for immediate blood perfusion of the implant [150]. Soft lithography was used to shape layers of poly(octamethylene maleate (anhydride) citrate) (POMaC) prepolymer, which were then UV-cured and adhered to a glass substrate. The resulting patterned chip had internal and perfusable branching networks with channels as small as 50-100 μm and with 25-50 μm thick walls, designed with pores throughout the POMaC layers to facilitate molecule diffusion and exchange. *In vitro* experiments demonstrated that lumens could be endothelialized with HUVECs and the parenchymal spaces filled with other cell types, such as cardiomyocytes or hepatocytes, demonstrating that Angiochip could be a versatile platform for engineering patterned, vas-

cularized tissue *in vitro*.

Topographical engineering is amongst the broadest of categories for manipulating biomaterials to induce angiogenesis. There are a multitude of techniques available to generate intricate geometries for vascularization, but each technique also has drawbacks. Soft lithographic techniques have inherent height and size-scale limitations, and some materials and processing methods can be toxic to cells or unfavorably affect substrate surfaces [142, 147]. Protein and peptide-based techniques suffer from protein stability and short half-lives, and sacrificial materials can leave toxic degradation products if not properly removed [10]. Microfluidic devices are often designed with non-implantable materials, making them incompatible for *in vivo* studies. There is great potential for this subfield to spawn a new generation of vascularized constructs due to the freedom afforded to it, but current implementations are often fabricated with *in vitro* modeling in mind, rather than *in vivo* implantation. As a result, there is often conflict between the disadvantages of topographical engineering techniques and the criteria for implantation [11].

2.6.4 3D bioprinting

The advent of 3D printing inspired the regenerative medicine community to adapt the technology to address biomedical problems. Coined “3D bioprinting,” this technique allows for the bottom-up production of 3D constructs with complex geometries and micron-scale resolution through the deposition of cells, materials, and biological factors, as well as fabrication techniques for patterning vasculature [10, 151, 152]. Geometric features can be modeled using computer-aided design (CAD) methodologies, enabling quick and precise alteration of variables to affect properties such as scaffold size and engineered macroporosity via patterned channels or a lattice. As a new fabrication tool, 3D bioprinting processes are continuing to be developed, and this review will focus on the main principles that define different 3D bioprinting strategies and their current applications in vascular

tissue engineering.

Inkjet bioprinting is a non-contact, layer-by-layer method wherein a piezoelectric or thermal actuator is used to disperse bioink droplets onto a substrate [151]. This is an inexpensive method of bioprinting because regular inkjet printers can be easily modified to be compatible with bioprinting. However, nozzle extrusion generates shear stresses in addition to pressure or thermal stress from the respective actuator types, which can cause damage to extruded cells and uneven dispersion [153, 154]. Lee et al. demonstrated the use of inkjet bioprinting to create 3D polycaprolactone (PCL) lattice scaffolds that supported the growth of a tri-culture of primary rat hepatocytes, HUVECs, and human lung fibroblasts to study liver tissue engineering in a mechanically robust 3D environment Lee *et al.* [155]. Seeding the tri-culture in a collagen solution into alternating canals in the bioprinted scaffold allowed for the formation of a dense, interconnected capillary-like network that spanned 36.9% of the $10.2 \times 10.2 \times 1.2$ mm hydrogel within 14 days.

Similar to inkjet printing is pressure-based bioprinters, which act by inducing pneumatic pulses that extrude solutions onto a substrate [10]. Using an air pressure-based 3D bioprinting process, Kolesky et al. were able to simultaneously extrude a cell-laden (HUVECs and human MSCs) fibrinogen-gelatin solution alongside a vascular ink made of Pluronic and thrombin into a 3D perfusable chip to create bone tissue with thicknesses greater than 1 cm [156]. ECM-native proteins were then gelled on top of the inks and the vascular ink was evacuated, forming a perfusable channel network in the gel which was endothelialized by the vascular cells. Perfusion of an osteogenic differentiation media containing BMP-2 enhanced mature osteogenic markers such as mineral deposition in the vascularized tissues, but not in the avascular controls [156]. In a study combining both 3D bioprinting and encapsulation techniques, Poldervaart et al. demonstrated the release of VEGF from gelatin microparticles in a pressure-based, 3D-bioprinted Matrigel-alginate lattice scaffold for localized angiogenesis [157]. *In vitro* studies with human late outgrowth EPCs showed

peak migration between 50-100 ng/mL of VEGF, and *in vivo* studies with subcutaneously implanted scaffolds in mice showed that despite quick scaffold degradation, the localized release of VEGF (25 µg/mL in 5 mg microparticles in scaffolds) into the surrounding tissue resulted in the formation of an average of 18 erythrocyte-perfused vessels/100 µm² in the encapsulated VEGF group at one week as determined by Goldner's trichrome stains. In comparison, the control and free (not encapsulated) VEGF groups had approximately 5 vessels/100 µm² ($p < 0.01$) [157].

Inkjet and pressure-based bioprinting techniques have also been used to directly fabricate tubes to act as vasculature, such as in Zhang et al. who demonstrated the ability to create, perfuse, and embed chitosan-alginate tubes with diameters as low as ~200 µm and variable pathways in alginate hydrogels using a pressure-assisted bioprinter [158]. In another study, Miller et al. used a thermal bioprinter to create a sacrificial glass lattice made of sucrose, glucose, and dextran, which was then embedded into either a Matrigel, alginate, agarose, fibrin, or PEG-based gel Miller *et al.* [159]. The lattice was then sacrificed, generating channels with diameters as low as 150 µm and perfused with HUVECs, generating a confluent vascular wall within 1 day. In a similar approach derived from microfluidics, DiVito et al. utilized a hydrodynamic shaping device to directly fabricate 125 µm vessels composed of either HUVECs or a combination of HUVECS, SMCs, and vascular pericytes in a complex matrix of PEG, methacrylated gelatin, fibronectin, hyaluronic acid, and collagen [59]. These capillary and arteriole mimics developed a basement membrane within 12 days in culture as quantified by immunofluorescence staining of collagen IV and laminin. When embedded in Matrigel or gelatin hydrogels containing human dermal fibroblasts, fabricated channels showed prolific sprouting angiogenesis into the gel and remained viable during perfusion with pressures of up to 2500 Pa and shear stresses up to 25 dyn/cm².

A final class of 3D bioprinting is laser-based bioprinting, a nozzle-free method that is performed by pulsing a laser on a bioink coated with an energy-absorbing layer (often a

metal like titanium), and collecting the resulting droplets on a substrate below [154, 160]. The absence of a nozzle eschews the issues of clogging and shear stresses on cells, and the technique can even deposit individual cells [152]. However it is extremely expensive, relatively low throughput, and prone to contamination from the metallic film [153]. It has however shown success in patterning vascular networks, such as in a study in which Wu et al. were able to direct the growth of HUVECs and SMCs into branch and stem structures using laser-assisted bioprinting [161]. The deposited HUVECs formed interconnected lumens at droplet spacings of 50 and 100 μm within 20 hours, and deposition of SMCs near these lumens appeared to encourage both SMC proliferation and stabilization of the HUVEC lumens. Of interest to note in this subclass of bioprinting is the multiphoton excitation-based technique of laser bioprinting, which uses similar principles to multi-photon microscopy to print biological materials into 3D scaffolds [160]. This technique enables resolutions as high as 3 μm , but currently cannot support the printing of cells and limits scaffold size to the millimeter length scale. Gao et al. demonstrated the use of this technique to print gelatin methacrylate into a 2.4×2.4 mm scaffold with a submicron topography that mimicked the submicron features of fibronectin distribution in adult murine myocardium, using a 100×15 μm grid of patterned scaffold for cellular alignment [162]. The scaffolds were seeded with 50,000 hiPSC-cardiomyocytes, hiPSC-SMCs, and hiPSC-ECs (all differentiated from a human cardiac lineage stem cell) and implanted in a mouse MI model, and by 4 weeks the implants improved MI recovery shown by reduced scar size and increased fractional shortening (from 35% to 45% at 4 weeks), likely through reduced apoptosis and increased vascular area density (from 400/ mm^2 for acellular scaffold implants up to 1200/ mm^2 for cell-laden scaffolds) in the infarct border zone.

3D bioprinting is relatively new and underdeveloped, thus its adoption is limited to those groups able to develop the technology or utilize commercially available bioprinters. However, continued innovations and technical developments in the coming decade are certain to increase the popularity and adoption of bioprinting, such as advances in instru-

mentation and biomaterials to better accommodate cells for printing at higher resolution and in customized microenvironments with reasonable cost and time. Current studies have demonstrated progress in creating vascularized constructs using 3D bioprinting, and the compatibility of multiple cell types means that this technique is feasible for generating vascularized cardiac tissues as well, such as a heart valve and myocardium [150, 153]. Combinatorial approaches to vascularization utilizing multiple techniques such as bioprinting and encapsulation in one construct have also been demonstrated and show promise in providing hierarchical structure to bioprinted tissues [157, 163]. Yet there is still significant work to be done to create large, complex tissues with deeply penetrating microvascular networks for organ engineering [154].

2.7 Beyond *in vitro* analysis of combinatorial angiogenesis strategies

Developing a perfusable angiogenic network *in vivo* presents a significantly greater challenge than *in vitro*, owing to the great multitude of known and unknown biological processes that impact the effectiveness of a therapeutic. However, 2D *in vitro* cultures cannot accurately mimic the complex 3D *in vivo* environment, whether that be the topography, paracrine cues, or host cell recruitment [164]. Further studies are thus necessary to understand how these controlled systems behave in more dynamic environments. **Table 2.1.** provides a succinct summary of the studies discussed below, with additional articles included that help illustrate the progress of *in vivo* vascularization efforts.

2.7.1 *Ex vivo* models of angiogenesis

Ex vivo assays are commonly used to bridge the gap between *in vitro* and *in vivo* studies as they are often relatively cheap and provide a more physiologically relevant environment than *in vitro* studies. The chicken chorioallantoic membrane (CAM) assay is a standard model for determining vascular growth due to the relative ease of manipulating the model and visually assessing results. The CAM acts as a respiratory organ for chicken embryos that grows and matures rapidly, quickly generating a densely packed vascular plexus within 7 days [165]. Intervention via the addition of reagents allows for quick and inexpensive determination of angiogenic efficacy, although it can be difficult to differentiate between new and existing capillary formation. The CAM assay is commonly used as a precursor to trials with traditional small animal models, such as in the study by Chiu et al. that examined collagen scaffolds with immobilized VEGF and Ang-1 [127]. Dashnyam et al. used the CAM assay to create vascular maps and quantitate total length, size and junctions that developed as a result of their silicon/VEGF-encapsulated microspheres [139]. Wu et al. found that heparinized, VEGF-bound liver scaffolds had increased vessel number and density in the CAM assay, and were able to then move forward with an *in vivo* model [129].

Another common assay used to determine angiogenic efficacy is the aortic ring assay, in which an animal aorta is dissected, cut into thin slices, embedded in a collagen or fibrin matrix, and then cultured with a therapeutic of interest and examined over a period of time [166, 167]. Slicing the aorta initiates an angiogenic response from the native cells which can be compared to the response from the therapeutic treatment via quantifiable angiogenic metrics such as EC proliferation, migration, tubulogenesis, and branching. The assay is an excellent *ex vivo* procedure for determining sprouting angiogenesis as it is inexpensive, high-throughput, and requires relatively little expertise to perform in comparison to *in vivo* procedures, but fresh tissues must be prepared for each experiment [168]. Further, the common practice of using Matrigel and serum must be carefully considered when

interpreting results or may be eliminated by using a completely chemically defined culture system. The microvessels that develop are physiologically similar to those generated *in vivo* and paracrine interactions are more apparent due to the presence of multiple cell types. For example, Izadifar et al. cultured rat aorta slices with their bilayer nanoparticles and showed that co-release of VEGF and bFGF followed by sequential release of PDGF generated microvessels with significantly greater average diameter, sprout length, and number of sprouts versus those generated with VEGF alone ($p < 0.05$) [138]. Pericyte recruitment and anastomosis by day 9 indicated that the vessels had stabilized and matured due to the growth-factor nanoparticles.

2.7.2 *In vivo* models of angiogenesis

In vivo research models for tissue engineering are paramount for investigation into a scaffold's angiogenic and inoscultative efficacy in a physiologically relevant environment. The choice of animal is often dependent on the results of interest. Small animal models such as rodents are often preferred over large animal models due to ease in handling, gestation period, costs, and particularly the ability to genetically engineer strains for studying diseases [169, 170]. Small animal models are often used for a general understanding of the host response to the implant, whether that be immune response, vascularization, degradation, or other factors that can be dependent on the specific location of the implant [171]. In studying organ-specific phenomena, it becomes more important to select animals with the most relevant physiology to humans. For example, pigs are a common choice of large animal model for preclinical cardiovascular studies due to their similarity in excitation coupling, excitation-relaxation kinetics, and heart rate in comparison to other models [170].

The subcutaneous site is prevalent in the literature for assessing tissue invasion and vascularization because it is a relatively easy operation to perform which provides a suitable wound bed to initiate angiogenesis with multiple implant sites in one animal. Jiang et al.

implanted PEG-PLLA scaffolds loaded with bFGF and PDGF-BB subcutaneously in rats and demonstrated sequential growth factor release that encouraged mature vessel formation with SMCs, although vessel density peaked at 1 week after implantation and decreased afterwards, suggesting pruning of the new vascular bed as one might expect in the absence of an oxygen sink [123]. Kang et al. utilized the principles of pressure-assisted 3D bioprinting combined with topographical engineering to develop a multi-cartridge extrusion machine that could fabricate tissue constructs as large as $3.6 \times 3.0 \times 1.6$ cm [172]. Dubbed an integrated tissue-organ printer (ITOP), the machine extruded PCL fibers, sacrificial Pluronic hydrogels, and various cell types to create porous tissue constructs with incorporated channels as small as $500 \times 300 \mu\text{m}^2$ to facilitate nutrient diffusion. In demonstration of the ITOP, constructs were fabricated for human mandible bone, rat calvarial bone, human ear, and mouse muscle tissue reconstructions based on clinical imaging data. In the case of a skeletal muscle reconstruction, Kang et al. showed that printed mouse myoblast constructs implanted subcutaneously in rats along with a section of the common peroneal nerve developed aligned muscle fibers, acetylcholine receptors and neurofilament contacts indicative of nerve integration, electrical activation when stimulated, and vascularization as determined by staining for von Willebrand factor throughout the implant (density not quantified) after 2 weeks.

To demonstrate the use of physical cues for *in vivo* vascularization, Jang et al. fabricated a 3D-printed lattice scaffold using a bioink composed of decellularized porcine heart ECM and printed on top of a PCL mesh layer [173]. Human cardiac progenitor cells and human turbinate MSCs with exogenous VEGF were additionally printed with the bioink in alternating strips. In a BALB/c mouse subcutaneous model, the striped scaffolds induced the formation of blood vessels with $50 \mu\text{m}$ diameter and 25 vessels/ mm^2 in density, whereas scaffolds with mixed cell types and no stripes generated vessels $< 22 \mu\text{m}$ in diameter and a density of 16 vessels/ mm^2 . In a rat MI model, fluorescent staining of CD31 at 8 weeks revealed that the MSCs differentiated into endothelial cells and penetrated the

infarct area. The patterned scaffolds had ~ 175 CD31-positive vessels/ mm^2 versus 110 and 100 in the mixed cell and cardiac progenitor-only scaffolds, respectively. In another example, Fleischer et al. developed a modular, multi-layer scaffold in which each 5×5 mm sheet of electrospun albumin was topographically engineered for a specific function and individually cultured before being glued together and implanted in a rodent MI model [163]. The cardiomyocyte layer was comprised of NRVMs seeded to alternating grooves and ridges spaced 115 and 120 μm apart, respectively, with transversely oriented 40 μm micro-holes placed on the ridges to facilitate nutrient exchange. The vascular layer consisted of 450 μm micro-channels seeded with HUVECs between cage-like structures that housed VEGF-loaded PLGA particles, and the final unique layer consisted entirely of cage structures housing dexamethasone-loaded PLGA particles. These unique layers enabled alignment and electromechanical coupling of cardiomyocytes, vascular patterning within the predefined channels, and extended release of both VEGF and dexamethasone up to 14 days. Analysis of 5 mm thick scaffolds consisting of six cardiomyocyte layers and six vascular layers flanked on either side by dexamethasone layers implanted onto post-MI rat hearts revealed an increased capillary density in the patch (approximately 110 vessels/ mm^2 up from 60/ mm^2 , $p < 0.007$) and significantly increased area occupied by vessels (from 2% to 4.5%, $p < 0.0004$) due to the presence of the VEGF-loaded microparticles.

Other implant locations can be utilized, which can be chosen depending on the application or intended data collection. As a proof-of-concept demonstration of immediate surgical anastomosis *in vivo*, Zhang et al. showed rapid perfusion through surgical anastomosis of their fabricated Angiochip with the rat femoral artery in both artery-to-artery and artery-to-vein configurations [150]. Perfusion occurred immediately regardless of the presence of ECs lining the lumens with apparent pulsatile flow, and after one week there was minimal clotting and angiogenic sprouting was evident surrounding the implant. The mesentery is an example of a highly vascularized membrane that has been used to demonstrate the angiogenic efficacy of therapeutics, such as in Benest et al. (see Section 2.5.4) [118]. In the

same vein, implantation on the greater omentum acts as another pro-angiogenic implant site. Wu et al. illustrated the angiogenic potential of their decellularized liver scaffolds with heparin-bound VEGF using this rodent implant model, which resulted in increased blood vessel formation within the scaffold at 28 days versus controls [129]. The scaffolds with heparin-bound VEGF had higher vessel penetration into scaffolds at approximately 28 vessels/visual field in comparison to 7 vessels/visual field in control liver scaffolds.

While these types of implants are useful in demonstrating *in vivo* effectiveness of a therapeutic, direct implantation onto the organ or tissue of interest is necessary to acquire the most clinically relevant information. In the case of cardiac applications, implantation onto an infarcted heart and subsequent analysis can provide valuable information regarding the therapeutics' ability to modulate coronary perfusion, sprouting angiogenesis, cardiac sparing and remodeling, and heart function post-MI. Dvir et al. implanted alginate scaffolds composed of NRVMs along with 100 ng of VEGF, IGF-1 and SDF-1 on the greater omentum of rats for 7 days as a pre-vascularization medium before transplanting the scaffolds onto rat hearts 7 days after infarction for 28 days [106]. Analysis showed that the omentum-vascularized tissues were able to successfully engraft on top of existing scar tissue, thus thickening the ventricular wall to values close to control and maintaining fractional shortening as well as fractional area change values in comparison to baseline. The tissues further demonstrated greater electrical activity between the patches and the myocardium and contained networks of 60 vessels/mm² versus 30 vessels/mm² in controls. In another study, Song et al. implanted acrylated hyaluronic acid hydrogels that contained immobilized SDF-1 as well as the angiogenic peptide Ac-SDKP derived from the protein thymosin beta 4 on an rat heart 4 weeks after MI induction to demonstrate the effectiveness of their constructs in a chronic heart failure model [113]. Histological analysis revealed an increase in arteriole number comparable to that of the sham group and a ~141.5% increase in capillary number relative to the infarct group. Heart functionality determined by intraluminal pressure-volume measurements showed significant increases in stroke volume,

cardiac output, and ejection fraction, although none of these values reached the respective levels in negative controls. In a slightly larger animal model, Lakshmanan et al. implanted their VEGF- and bFGF-incorporated PLCL-PEOz electrospun scaffolds onto infarcted rabbit hearts and demonstrated preserved left ventricular wall diameter and increased ejection fraction and fractional shortening without causing unwanted surgical complications like adhesions or edemas that may disrupt implant integration with the host [132]. Uniform and α -SMA-positive blood vessels (~ 20 vessels in the patch region compared to < 10 in all other groups) formed in the region of the implanted patch, indicative of angiogenic processes initiated by the growth factors which contributed to the increased viable myocardium and reduced necrotic regions. However, the authors did not report any vascular or cardiomyocyte alignment *in vivo* in response to the presence of the electrospun fibers. Riemenschneider et al. developed fibrin scaffolds seeded with human outgrowth EPCs, human brain pericytes, and an incorporated growth factor cocktail containing stem cell factor, interleukin-3, and SDF-1 α , and cultured the constructs for 8-9 days to develop an aligned vascular bed prior to implantation on infarcted rat hearts for 6 days [8]. The authors reported a total human and rat vessel density of 435 vessels/mm², but only 40% (173 human and 93 rat vessels/mm²) were perfused, and mainly around the border zone. In comparison, patches lacking EPCs contained approximately 100 vessels/mm², all originating from the host. Furthermore, capillary diameter increased from 6 μ m pre-implantation to 8 μ m at explant. The patches were not found to significantly improve functional cardiac output by 6 days despite a noted increase in ejection fraction and fractional shortening across all treatment groups by 10%.

2.7.3 Analysis of *in vivo* progress of vascularization

Discussed above are just a few of the studies that have demonstrated the progressive steps taken to better understand the interplay of physical and chemical cues that dictate the

formation of microvascular networks, with an emphasis on cardiac regeneration. Of interest to note is the difficulty of preserving and assessing the benefits of physical cues *in vivo*, particularly on the micron scale, because geometry can be easily lost during the implantation procedure, over time from scaffold degradation, and during histological processing. Intentional micron- and submicron-level cues for vascularization are thus a significant challenge to maintain *in vivo*, and their necessity is debatable still [71, 137]. This is a prime opportunity for applications in topographical engineering to transition into *in vivo* applications, but this requires a re-envisioning of system design and thoughtful material choice to facilitate incorporation into animal models at the same micron scale. Current state of the art scaffold implants often rely on pro-angiogenic molecules (mainly growth factors), hydrogels, and relatively macro-scale physical cues to promote vascularization and overall cellular orientation.

Methods for characterizing vascularization are often inadequate, and angiogenic analysis is often relegated to spatially- and temporally-vague metrics such as number of vessels present in the scaffold. One basic characterization of vasculature that is often overlooked is the presence of lumens within an endothelial network (as opposed to simple EC aggregates), which can be clearly distinguished as shown by Davis and colleagues [174]. Capillaries and arterioles are differentiated through quantification of either vessel size or staining for vascular SMCs, but venule development is altogether neglected [141]. This is a prime opportunity to utilize other molecular markers to differentiate endothelium, such as NRP1 and NRP2 expression for arterial and venous specification, respectively [54]. Quantification of vascular geometry can also be improved through metrics like branch points, branch angles, branch lengths, vessel alignment, and tortuosity and comparisons to native, healthy tissue vascular geometries. These metrics should stabilize in time to provide evidence for vascular bed maturation and persistent function, and although these types of vascular characterizations are challenging to implement, they need to be more widely adopted *in vivo*. It is a natural next step to analyze normal vessel functions like perfusion and mod-

ulation of vascular tone, followed by organ-specific function of implants like filtration via permeability in liver regeneration applications, or barrier function in blood-brain barrier recapitulation.

Temporal metrics to assess vessel stability and regression are critically important but extremely difficult to capture. Indeed, it is a fundamental challenge in vascular tissue engineering to accurately assess vascularization *in vivo* (particularly in deep tissues like the heart) at high resolution with a non-invasive and longitudinal (non-terminal) imaging technique. The dynamic nature of angiogenesis necessitates a dynamic imaging modality or examination at multiple time points, but the current gold standard of histology is a static 2D image often assessed at a single time point and not wholly representative of the underlying processes that occur during wound healing. Techniques that enable the 3D reconstruction and skeletonization of vascular networks are desirable, such as methods to label cardiac vasculature via retrograde coronary perfusion of isolectin or a radiopaque dye [12, 35]. However, this is also a terminal procedure, precluding longitudinal studies, whereas an ideal imaging technique should be able to provide live flow imaging at the capillary scale in a non-invasive manner. High spatial and temporal resolution of vascularization *in situ* may be possible using combinations of imaging techniques, such as a study by Liu et al. that utilized optical coherence tomography (OCT) and photoacoustic tomography to noninvasively view the microvasculature in various regions of the skin [175]. Another study by Qin et al. utilized OCT angiography to image coronary vasculature at an axial resolution of 3 μm via *ex vivo* retrograde perfusion of Intralipid solution [176]. The dorsal skinfold chamber is a device that has been used for decades that enables examination of implant vascularization in rodents via non-invasive intravital microscopy, but the device is large, unwieldy, and cannot be used outside of subcutaneous studies [177]. For applications deeper than superficial imaging, inspiration and innovation is necessary to improve the resolution of techniques like ultrasound and angiography. A major challenge today is the lack of standardization in vascular bed quantification, which complicates comparison

between studies and thus communication within the scientific community. The field must determine what the necessary parameters are to measure for a given vascular application, and how to best assess them.

Progress in developing a vascular bed *in vivo* has been aided by improved prevascularization of tissue *in vitro* (as opposed to fabrication and implantation around or at the same time, which can reduce the amount of time an implant must endure hypoxic conditions before vascular ingrowth and anastomosis occurs [178]. Vessel stabilization due to co-culture has enabled studies of *in vitro* prevascularization, such as in Riemenschneider et al. and Fleischer et al. described above [8, 163]. In another example, Kreutziger et al. formed scaffold-free patches containing various ratios of hESC-derived cardiomyocytes, HUVECs, and human MSCs, and cultured them for 2 days prior to implantation on uninjured nude rat hearts for 1 week [65]. Upon explant, tri-cell patches supported higher (~ 175 vessels/mm²) vessel formation than other cell formulations with either no stromal support cell, mouse embryonic fibroblasts, or neonatal human dermal fibroblasts (approximately 35, 60, and 125 vessels/mm², respectively). *In situ* prevascularization is another option, where the implant is placed on a highly vascular bed (such as the greater omentum of rodents) prior to transplantation to the site of interest, which has been used with some success in the heart [106]. The arterio-venous (AV) loop model is another example of an *in situ* prevascularization technique that is accomplished by surgically connecting an artery and vein (often the saphenous vein and artery) into a loop fistula and isolating it in a protected chamber within the animal [179]. Constructs housed within the chamber are then vascularized from angiogenic sprouts off the main loop. Both Tee et al. and Morritt et al. have demonstrated the use of this technique to generate vascularized, beating cardiac tissues by seeding NRVMs in Matrigel within the chamber, and the AV loop has even been used in a clinical setting, which indicates translational feasibility [180–182]. Prevascularization may be necessary in the near future, as a study by Utzinger et al. showed that neovessel growth (of rodent microvessel fragments in a 3D collagen gel) averages only 5

$\mu\text{m/hr}$, which will not establish vasculature fast enough to prevent necrosis in larger and physiologically dense tissues [183].

Additionally, multiple studies suggest that prevascularization enables faster functional anastomoses once implanted, with anastomoses occurring within as little as three days when prevascularized, as opposed to at least 8 days without prevascular support [65, 184, 185]. The exact molecular mechanisms of anastomosis in mammals are not fully known yet, but studies have also found evidence of vessel chimerism, in which engineered tissues with prevascularized human vessels anastomose with host rodent vessels upon implantation [65]. In examining the dorsal longitudinal anastomotic vessel of zebrafish embryos, Herwig et al. determined that anastomosis occurred through two distinct mechanisms [186]. After primordial ECs made contact, junctional proteins were expressed in a ring-like fashion, causing apical polarization and the formation of membrane compartments. The cells then either rearranged themselves into a multicellular tube through the merging of distinct membrane compartment, or generated a unicellular tube via progressive invagination of the membrane compartments. Whether this is a stochastic process has yet to be determined, and further studies are necessary to understand how this occurs in mammals, and whether mechanisms differ between tissues.

Despite these complications, combinatorial growth factor approaches to vascularization of host tissue and implanted engineered tissues have shown *in vivo* success with incorporation of sophisticated spatiotemporal cues in the experimental design of the therapeutic. Application of these principles in the field of cardiac tissue engineering has demonstrated the ability of implanted tissues to anastomose with host vasculature and improve post-MI cardiac remodeling and function. Yet there is more to be done, as the goal of the field is to create a bed of capillaries with densities that are tailored to the needs of the host and engineered tissues to regenerate the native structure and function, whether it is myocardium, brain, liver, or another organ. To reach that goal, novel technologies and customized bio-

materials must continue to be pursued, as well as a deeper understanding of material-cell interactions, physical and chemical cues to drive cellular signaling, and the host inflammatory and wound healing response after injury, disease, and therapeutic intervention.

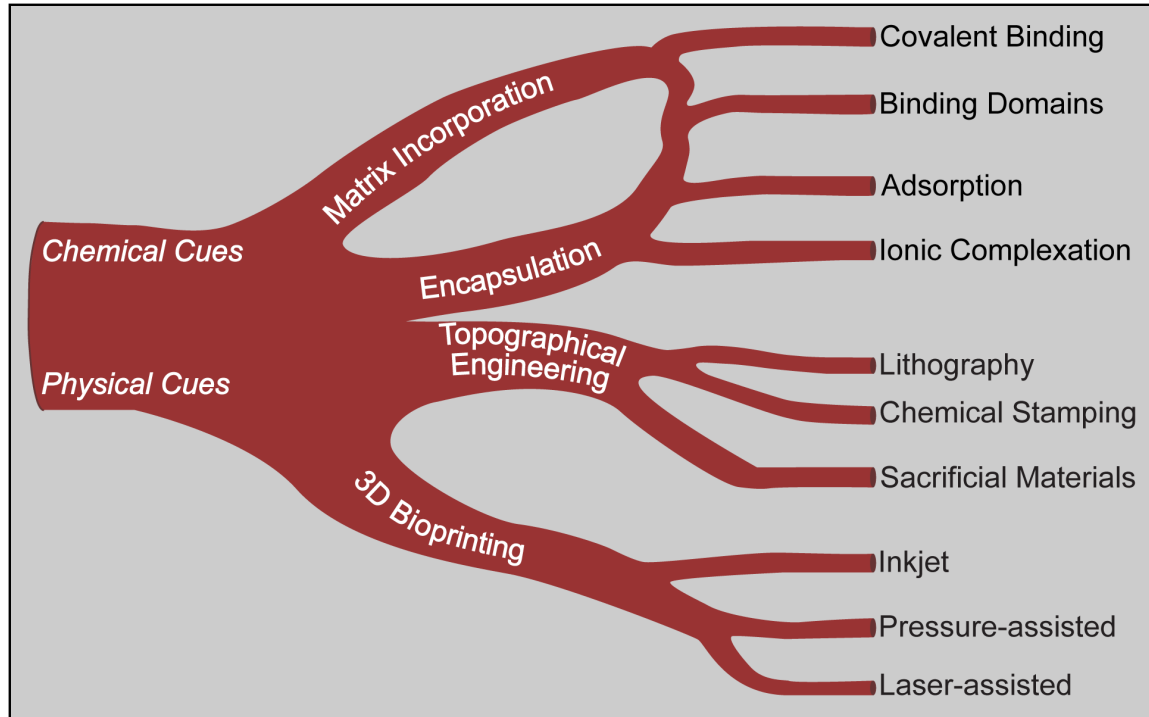


Figure 2.3: Spatiotemporal control of angiogenesis can be achieved through the presentation of chemical and physical cues. This can be categorized into four distinct categories, which is further differentiated by the techniques utilized to present spatiotemporal cues within each category. Combinations of these techniques are often utilized to precisely engineer vessel network formation using chemical and physical cues integrated in one system. Of interest to note is the similarity in techniques used to present chemical cues in the matrix incorporation and encapsulation methods, the distinction being one related to material choice and design.

2.8 Conclusions

As the development of replacement tissues has become a reality and feasible therapeutic option, research has turned towards understanding the mechanisms behind vascularization as a necessity for building organs from biomaterials, cells, and signaling molecules. Replicating the complex microvasculature of an engineered tissue in a way such that it can

perfuse the entire implant and inosculate with host tissue has proven to be a complex undertaking. It is now understood that single growth factor systems may initiate angiogenesis, but are too simple to mimic the complete vascularization process, and as a result, vascularization research has turned to understanding the interplay of physical and biological cues involved in establishing a mature, hierarchical, and perfused vessel network. *In vitro* work has embraced spatiotemporal mediation of these cues, and a deeper understanding of these processes through *in vivo* application is currently unfolding. Ongoing work is required to identify pathways that promote increased capillary density, appropriate hierarchical vessel remodeling, and permanence of new vessels.

As the field of neovascularization advances, the broader issue of building tissues with sufficient architecture and signaling cues to be able to unite with the biology of the target host tissue to develop a sufficient vasculature must be considered. The field has relied on powerful growth factors like VEGF and bFGF, which have shown to be insufficient clinically when administered alone without spatial or temporal control, and is now slowly adopting an approach that recognizes that there are nuanced inter-related pathways for vascular development depending on the tissue type and disease context [187]. Identifying an optimal cocktail of growth factors and the appropriate spatiotemporal presentation of them via a biomaterial scaffold is essential in creating a highly vascularized network within dense engineered tissues, and this must be tailored to the application. An ongoing challenge is to design systems that are going to be adopted broadly for translational applications. Thus, using materials that are already FDA-approved is appealing to fast-track clinical use, but is not suited for all applications. Development of novel materials or new methods that utilize currently available materials will be required as technologies advance for designing capillaries (with < 10 microns diameter). Technologies that allow for precise, nanoscale fabrication of vessel architecture could be invaluable in spatially controlling vascular bed formation. Prospective studies should examine the ability of engineered vessel networks to handle physiological characteristics of organ-specific vasculature, such as flow rates,

homeostasis, permeability, vascular tone, and immune response upon implantation in the host. These studies should further seek to push the boundaries of current characterization practices to develop techniques that provide temporal vascularization information with high spatial fidelity. The continued development of these parallel and complex challenges will surely advance the field towards the generation of mature vessel networks in host tissue and engineered tissues to restore organ function.

2.9 References

1. Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H. & Tomic-Canic, M. Growth factors and cytokines in wound healing. *Wound Repair and Regeneration* **16**, 585–601. ISSN: 10671927. <http://doi.wiley.com/10.1111/j.1524-475X.2008.00410.x> (Sept. 2008).
2. Laflamme, M. A. & Murry, C. E. Heart regeneration. *Nature* **473**, 326–335. ISSN: 0028-0836. <http://www.nature.com/doifinder/10.1038/nature10147> (2011).
3. Reis, L. A., Chiu, L. L. Y., Feric, N., Fu, L. & Radisic, M. Biomaterials in myocardial tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine* **10**, 11–28. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.1944> (Jan. 2016).
4. Kaneko, N., Matsuda, R., Toda, M. & Shimamoto, K. Three-dimensional reconstruction of the human capillary network and the intramyocardial micronecrosis. *AJP: Heart and Circulatory Physiology* **300**, H754–H761. ISSN: 0363-6135. <http://ajpheart.physiology.org/cgi/doi/10.1152/ajpheart.00486.2010> (2011).

5. Rawal, S. *et al.* Down-regulation of proangiogenic microRNA-126 and microRNA-132 are early modulators of diabetic cardiac microangiopathy. *Cardiovascular Research* **113**, 90–101. ISSN: 0008-6363. <https://academic.oup.com/cardiovascres/article-lookup/doi/10.1093/cvr/cvw235> (2017).
6. Ertl, G. & Frantz, S. Healing after myocardial infarction. *Cardiovascular Research* **66**, 22–32. ISSN: 00086363 (2005).
7. Bui Anh, L., Horwisch Tamara, B. & Fonarow Gregg, C. Epidemiology and risk profile of heart failure. *Nature Publishing Group* **8**, 1–25. ISSN: 2164-8344 (2012).
8. Riemenschneider, S. B. *et al.* Inosculation and Perfusion of Pre-Vascularized Tissue Patches Containing Aligned Human Microvessels after Myocardial Infarction. *Biomaterials* **97**, 51–61. ISSN: 1527-5418. <https://pubmed.ncbi.nlm.nih.gov/27162074/> (2016).
9. Kaiser, N. J. & Coulombe, K. L. K. Physiologically inspired cardiac scaffolds for tailored in vivo function and heart regeneration. *Biomedical materials (Bristol, England)* **10**, 034003. ISSN: 1748-605X. <http://www.ncbi.nlm.nih.gov/pubmed/25970645> %5Cn<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4696555> (2015).
10. Kim, J. J., Hou, L. & Huang, N. F. Vascularization of three-dimensional engineered tissues for regenerative medicine applications. *Acta Biomaterialia* **41**, 17–26. ISSN: 18787568. <http://dx.doi.org/10.1016/j.actbio.2016.06.001> (2016).
11. Kannan, R. Y., Salacinski, H. J., Sales, K., Butler, P. & Seifalian, A. M. The roles of tissue engineering and vascularisation in the development of micro-vascular networks: A review. *Biomaterials* **26**, 1857–1875. ISSN: 01429612 (2005).

12. Coulombe, K. L. K. & Murry, C. E. Vascular perfusion of implanted human engineered cardiac tissue. *Proceedings of the IEEE Annual Northeast Bioengineering Conference, NEBEC 2014-Decem*, 4–5. ISSN: 21607001 (2014).
13. Cochain, C., Channon, K. M. & Silvestre, J.-S. Angiogenesis in the Infarcted Myocardium. *Antioxidants & Redox Signaling* **18**, 1100–1113. ISSN: 1523-0864. <http://online.liebertpub.com/doi/abs/10.1089/ars.2012.4849> (2013).
14. Santo, V. E., Gomes, M. E., Mano, J. F. & Reis, R. L. Controlled Release Strategies for Bone, Cartilage, and Osteochondral Engineering—Part II: Challenges on the Evolution from Single to Multiple Bioactive Factor Delivery. *Tissue Engineering Part B: Reviews* **19**, 327–352. ISSN: 1937-3368. <http://online.liebertpub.com/doi/abs/10.1089/ten.teb.2012.0727> (2013).
15. Malafaya, P. B., Silva, G. A. & Reis, R. L. Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. *Advanced Drug Delivery Reviews* **59**, 207–233. ISSN: 0169409X (2007).
16. Drury, J. L. & Mooney, D. J. Hydrogels for tissue engineering: Scaffold design variables and applications. *Biomaterials* **24**, 4337–4351. ISSN: 01429612 (2003).
17. Pedde, R. D. *et al.* Emerging Biofabrication Strategies for Engineering Complex Tissue Constructs. *Advanced Materials* **29**, 1–27. ISSN: 15214095 (2017).
18. Flamme, I., Frölich, T. & Risau, W. Molecular mechanisms of vasculogenesis and embryonic angiogenesis. *Journal of Cellular Physiology* **173**, 206–210. ISSN: 0021-9541. <http://doi.wiley.com/10.1002/%28SICI%291097-4652%28199711%29173%3A2%3C206%3A%3AAID-JCP22%3E3.0.CO%3B2-C> (Nov. 1997).
19. Ribatti, D., Vacca, A., Nico, B., Roncali, L. & Dammacco, F. Postnatal vasculogenesis. *Mechanisms of Development* **100**, 157–163. ISSN: 09254773 (2001).

20. Czirok, A. & Little, C. D. Pattern formation during vasculogenesis. *Birth Defects Research Part C - Embryo Today: Reviews* **96**, 153–162. ISSN: 1542975X (2012).
21. Hogan, B. M. & Schulte-Merker, S. How to Plumb a Pisces: Understanding Vascular Development and Disease Using Zebrafish Embryos. *Developmental cell* **42**, 567–583. ISSN: 18781551. <http://dx.doi.org/10.1016/j.devcel.2017.08.015> (2017).
22. Balaji, S., King, A., Crombleholme, T. M. & Keswani, S. G. The Role of Endothelial Progenitor Cells in Postnatal Vasculogenesis: Implications for Therapeutic Neovascularization and Wound Healing. *Advances in Wound Care* **2**, 283–295. ISSN: 2162-1918. <http://online.liebertpub.com/doi/abs/10.1089/wound.2012.0398> (2013).
23. Asahara, T. *et al.* Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**, 221–228. ISSN: 0009-7330. <http://www.ncbi.nlm.nih.gov/pubmed/10436164> (1999).
24. Valarmathi, M. T., Fuseler, J. W., Davis, J. M. & Price, R. L. A Novel Human Tissue-Engineered 3-D Functional Vascularized Cardiac Muscle Construct. *Frontiers in Cell and Developmental Biology* **5**, 2. ISSN: 2296-634X. <http://journal.frontiersin.org/article/10.3389/fcell.2017.00002/full> (Jan. 2017).
25. Himeno, T. *et al.* Angioblast derived from ES cells construct blood vessels and ameliorate diabetic polyneuropathy in mice. *Journal of Diabetes Research* **2015**. ISSN: 23146753 (2015).
26. Tung, J. J., Tattersall, I. W. & Kitajewski, J. Tips, Stalks, Tubes: Notch-Mediated Cell Fate Determination and Mechanisms of Tubulogenesis during Angiogenesis. *Cold Spring Harbor Perspectives in Medicine* **2**, a006601. ISSN: 2157-1422. <http://www.cshperspectives.org/>

- //perspectivesinmedicine.cshlp.org/lookup/doi/10.1101/cshperspect.a006601 (Feb. 2012).
27. Moon, J. J. & West, J. L. Vascularization of engineered tissues: approaches to promote angio-genesis in biomaterials. *Current topics in medicinal chemistry* **8**, 300–10. ISSN: 1873-4294. <http://www.ncbi.nlm.nih.gov/pubmed/18393893>5Cn<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3929210> (2008).
 28. Senger, D. R. & Davis, G. E. Angiogenesis. *Cold Spring Harbor perspectives in biology* **3**, a005090. ISSN: 1943-0264. <http://www.ncbi.nlm.nih.gov/pubmed/21807843>%0A<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC3140681> (2011).
 29. Burri, P. H., Hlushchuk, R. & Djonov, V. Intussusceptive angiogenesis: Its emergence, its characteristics, and its significance. *Developmental Dynamics* **231**, 474–488. ISSN: 10588388 (2004).
 30. Watson, E. C., Grant, Z. L. & Coultas, L. Endothelial cell apoptosis in angiogenesis and vessel regression. *Cellular and Molecular Life Sciences*, 1–17. ISSN: 14209071 (2017).
 31. Heil, M., Eitenmüller, I., Schmitz-Rixen, T. & Schaper, W. Arteriogenesis versus angiogenesis: Similarities and differences. *Journal of Cellular and Molecular Medicine* **10**, 45–55. ISSN: 15821838 (2006).
 32. Nakayama, K. H. *et al.* Aligned-Braided Nanofibrillar Scaffold with Endothelial Cells Enhances Arteriogenesis. *ACS Nano* **9**, 6900–6908 (2015).
 33. Hershey, J. C. *et al.* Revascularization in the rabbit hindlimb: Dissociation between capillary sprouting and arteriogenesis. *Cardiovascular Research* **49**, 618–625. ISSN: 00086363 (2001).

34. Troidl, K. & Schaper, W. Arteriogenesis versus angiogenesis in peripheral artery disease. *Diabetes/metabolism research and reviews* **28 Suppl 1**, 27–9. ISSN: 1520-7560. <http://libweb.anglia.ac.uk/%20http://doi.wiley.com/10.1002/dmrr.2232%20http://www.ncbi.nlm.nih.gov/pubmed/19145587%20http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4153414%20http://www.ncbi.nlm.nih.gov/pubmed/22271719> (Feb. 2012).
35. Weyers, J. J. *et al.* Effects of cell grafting on coronary remodeling after myocardial infarction. *Journal of the American Heart Association* **2**, 1–13. ISSN: 20479980 (2013).
36. Aspelund, A., Robciuc, M. R., Karaman, S., Makinen, T. & Alitalo, K. Lymphatic System in Cardiovascular Medicine. *Circulation Research* **118**, 515–530. ISSN: 0009-7330. <http://circres.ahajournals.org/lookup/doi/10.1161/CIRCRESAHA.115.306544> (Feb. 2016).
37. Corliss, B. A., Azimi, M. S., Munson, J. M., Peirce, S. M. & Murfee, W. L. Macrophages: An Inflammatory Link Between Angiogenesis and Lymphangiogenesis. *Microcirculation* **23**, 95–121. ISSN: 10739688. <http://doi.wiley.com/10.1111/micc.12259> (Feb. 2016).
38. Peters, E. B. Endothelial Progenitor Cells for the Vascularization of Engineered Tissues. *Tissue Engineering Part B: Reviews*, ten.teb.2017.0127. ISSN: 1937-3368. <http://online.liebertpub.com/doi/10.1089/ten.teb.2017.0127> (2017).
39. Weitman, E., Cuzzzone, D. & Mehrara, B. J. Tissue engineering and regeneration of lymphatic structures. *Future Oncology* **9**, 1365–1374. ISSN: 1479-6694. <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4095806/%20http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4095806/>

- //www.futuremedicine.com/doi/10.2217/fon.13.110 (Sept. 2013).
40. Klotz, L. *et al.* Cardiac lymphatics are heterogeneous in origin and respond to injury. *Nature* **522**, 62–67. ISSN: 0028-0836. <http://www.nature.com/doi/10.1038/nature14483> (May 2015).
 41. Güç, E. *et al.* Local induction of lymphangiogenesis with engineered fibrin-binding VEGF-C promotes wound healing by increasing immune cell trafficking and matrix remodeling. *Biomaterials* **131**, 160–175. ISSN: 18785905. <http://dx.doi.org/10.1016/j.biomaterials.2017.03.033> (2017).
 42. Park, S., Sorenson, C. M. & Sheibani, N. PECAM-1 isoforms, eNOS and endoglin axis in regulation of angiogenesis. *Clinical Science* **129**, 217–234. ISSN: 0143-5221. <http://clinsci.org/lookup/doi/10.1042/CS20140714> (2015).
 43. Staton, C. A., Reed, M. W. R. & Brown, N. J. A critical analysis of current in vitro and in vivo angiogenesis assays. *International Journal of Experimental Pathology* **90**, 195–221. ISSN: 09599673 (2009).
 44. Chiu, L. L. Y., Weisel, R. D., Li, R.-K. K. & Radisic, M. Defining conditions for covalent immobilization of angiogenic growth factors onto scaffolds for tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine* **5**, 69–84. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.292> (Jan. 2011).
 45. Lin, Y., Gil, C.-H. & Yoder, M. C. Differentiation, Evaluation, and Application of Human Induced Pluripotent Stem Cell–Derived Endothelial Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology* **37**, 2014–2025. ISSN: 1079-5642. <http://atvb.ahajournals.org/lookup/doi/10.1161/ATVBAHA.117.309962> (Nov. 2017).
 46. Quade, M. *et al.* Central Growth Factor Loaded Depots in Bone Tissue Engineering Scaffolds for Enhanced Cell Attraction. *Tissue Engineering Part A* **00**, ten.tea.2016.0483.

- ISSN: 1937-3341. <http://online.liebertpub.com/doi/10.1089/ten.tea.2016.0483> (2017).
47. Pross, C. *et al.* Rat and human aortic smooth muscle cells display differing migration and matrix metalloproteinase activities in response to dexamethasone. *Journal of Vascular Surgery* **35**, 1253–1259. ISSN: 07415214 (2002).
 48. Clayton, Z., Sadeghipour, S. & Patel, S. Generating induced pluripotent stem cell derived endothelial cells and induced endothelial cells for cardiovascular disease modelling and therapeutic angiogenesis. *International Journal of Cardiology* **197**, 116–122. ISSN: 01675273. <http://dx.doi.org/10.1016/j.ijcard.2015.06.038>
<http://linkinghub.elsevier.com/retrieve/pii/S016752731501339X> (Oct. 2015).
 49. Lowenthal, J. & Gerecht, S. Stem cell-derived vasculature: A potent and multidimensional technology for basic research, disease modeling, and tissue engineering. *Biochemical and Biophysical Research Communications* **473**, 733–742. ISSN: 0006291X. <http://linkinghub.elsevier.com/retrieve/pii/S0006291X15306446> (May 2016).
 50. Sieveking, D. P., Buckle, A., Celermajer, D. S. & Ng, M. K. C. Strikingly Different Angiogenic Properties of Endothelial Progenitor Cell Subpopulations. Insights From a Novel Human Angiogenesis Assay. *Journal of the American College of Cardiology* **51**, 660–668. ISSN: 07351097 (2008).
 51. Tura, O. *et al.* Late outgrowth endothelial cells resemble mature endothelial cells and are not derived from bone marrow. *Stem Cells* **31**, 338–348. ISSN: 10665099 (2013).
 52. Hall, K. L., Volk-Draper, L. D., Flister, M. J. & Ran, S. New model of macrophage acquisition of the lymphatic endothelial phenotype. *PLoS ONE* **7**. ISSN: 19326203 (2012).

53. Chi, J.-T. *et al.* Endothelial cell diversity revealed by global expression profiling. *Proceedings of the National Academy of Sciences* **100**, 10623–10628. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.1434429100> (Sept. 2003).
54. Rocha, S. F. & Adams, R. H. Molecular differentiation and specialization of vascular beds. *Angiogenesis* **12**, 139–147. ISSN: 09696970 (2009).
55. Cross, M. J. & Claesson-Welsh, L. FGF and VEGF function in angiogenesis: Signalling pathways, biological responses and therapeutic inhibition. *Trends in Pharmacological Sciences* **22**, 201–207. ISSN: 01656147 (2001).
56. Avolio, E., Alvino, V. V., Ghorbel, M. T. & Campagnolo, P. Perivascular cells and tissue engineering: Current applications and untapped potential. *Pharmacology & Therapeutics* **171**, 83–92. ISSN: 01637258. <http://linkinghub.elsevier.com/retrieve/pii/S016372581630211X> (2017).
57. Pill, K., Hofmann, S., Redl, H. & Holnthoner, W. Vascularization mediated by mesenchymal stem cells from bone marrow and adipose tissue: a comparison. *Cell Regeneration* **4**, 4:8. ISSN: 20459769. <http://linkinghub.elsevier.com/retrieve/pii/S2045976917300135> (2015).
58. Baldwin, J. *et al.* In vitro pre-vascularisation of tissue-engineered constructs A co-culture perspective. *Vascular Cell* **6**, 13. ISSN: 2045-824X. <http://vascularcell.biomedcentral.com/articles/10.1186/2045-824X-6-13> %20<http://vascularcell.com/index.php/vc/article/view/10.1186-2045-824X-6-13> (2014).
59. DiVito, K. A., Daniele, M. A., Roberts, S. A., Ligler, F. S. & Adams, A. A. Microfabricated blood vessels undergo neoangiogenesis. *Biomaterials* **138**, 142–152. ISSN: 18785905 (2017).

60. Orlidge, A. Inhibition of capillary endothelial cell growth by pericytes and smooth muscle cells. *The Journal of Cell Biology* **105**, 1455–1462. ISSN: 0021-9525. <http://www.jcb.org/cgi/doi/10.1083/jcb.105.3.1455> (Sept. 1987).
61. Koike, N. *et al.* Tissue engineering: Creation of long-lasting blood vessels. *Nature* **428**, 138–139. ISSN: 0028-0836. <http://www.nature.com/doi/10.1038/428138a> (Mar. 2004).
62. Sekine, H. *et al.* Endothelial cell coculture within tissue-engineered cardiomyocyte sheets enhances neovascularization and improves cardiac function of ischemic hearts. *Circulation* **118**, 145–152. ISSN: 15244539 (2008).
63. Santos, M. I., Unger, R. E., Sousa, R. A., Reis, R. L. & Kirkpatrick, C. J. Crosstalk between osteoblasts and endothelial cells co-cultured on a polycaprolactone-starch scaffold and the in vitro development of vascularization. *Biomaterials* **30**, 4407–4415. ISSN: 01429612. <http://dx.doi.org/10.1016/j.biomaterials.2009.05.004> (2009).
64. Tulloch, N. L. *et al.* Growth of engineered human myocardium with mechanical loading and vascular coculture. *Circulation Research* **109**, 47–59. ISSN: 00097330 (2011).
65. Kreutziger, K. L. *et al.* Developing Vasculature and Stroma in Engineered Human Myocardium. *Tissue Engineering Part A* **17**, 1219–1228. ISSN: 1937-3341. <http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2010.0557> (2011).
66. Goers, L., Freemont, P. & Polizzi, K. M. Co-culture systems and technologies: taking synthetic biology to the next level. *Journal of The Royal Society Interface* **11**, 20140065–20140065. ISSN: 1742-5689. <http://rsif.royalsocietypublishing.org/cgi/doi/10.1098/rsif.2014.0065> (2014).

67. Iyer, R. K., Chiu, L. L. Y. & Radisic, M. Microfabricated poly(ethylene glycol) templates enable rapid screening of triculture conditions for cardiac tissue engineering. *Journal of Biomedical Materials Research - Part A* **89**, 616–631. ISSN: 15493296 (2009).
68. Thomson, K. S. *et al.* Prevascularized microtemplated fibrin scaffolds for cardiac tissue engineering applications. *Tissue Engineering Part A* **19**, 967–77. ISSN: 1937-335X. http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2012.0286?url_ver=Z39.88-2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub=pubmed (2013).
69. Mitchell, A. C., Briquez, P. S., Hubbell, J. A. & Cochran, J. R. Engineering growth factors for regenerative medicine applications. *Acta Biomaterialia* **30**, 1–12. ISSN: 18787568. <http://dx.doi.org/10.1016/j.actbio.2015.11.007> (2016).
70. Ferrara, N. & Alitalo, K. Clinical applications of angiogenic growth factors and their inhibitors. *Nature Medicine* **5**, 1359–1364. ISSN: 1078-8956. <http://www.ncbi.nlm.nih.gov/pubmed/10581076> http://www.nature.com/articles/nm1299_1359 (Dec. 1999).
71. Yuen, W. W., Du, N. R., Chan, C. H., Silva, E. A. & Mooney, D. J. Mimicking nature by codelivery of stimulant and inhibitor to create temporally stable and spatially restricted angiogenic zones. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 17933–8. ISSN: 1091-6490. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2964247&tool=pmcentrez&rendertype=abstract> (2010).
72. Potz, B. A. *et al.* Glycogen Synthase Kinase 3 β Inhibition Improves Myocardial Angiogenesis and Perfusion in a Swine Model of Metabolic Syndrome. *Journal of the American Heart Association* **5**, 1–10. ISSN: 20479980 (2016).

73. Räsänen, M. *et al.* VEGF-B gene therapy inhibits doxorubicin-induced cardiotoxicity by endothelial protection. *Proceedings of the National Academy of Sciences* **113**, 13144–13149. ISSN: 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.1616168113> (2016).
74. Henning, R. J. Therapeutic angiogenesis: angiogenic growth factors for ischemic heart disease. *Future Cardiology* **12**, 585–599. ISSN: 1479-6678. <http://www.futuremedicine.com/doi/10.2217/fca-2016-0006> (Sept. 2016).
75. Dashkevich, A., Hagl, C., Beyersdorf, F., Nykänen, A. I. & Lemström, K. B. VEGF Pathways in the Lymphatics of Healthy and Diseased Heart. *Microcirculation* **23**, 5–14. ISSN: 15498719 (2016).
76. Koch, S. & Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Cold Spring Harbor Perspectives in Medicine* **2**, 1–21. ISSN: 21571422 (2012).
77. Yang, L. *et al.* Resveratrol attenuates myocardial ischemia/reperfusion injury through up-regulation of vascular endothelial growth factor B. *Free Radical Biology and Medicine* **101**, 1–9. ISSN: 18734596. <http://dx.doi.org/10.1016/j.freeradbiomed.2016.09.016> (2016).
78. Pepe, M. *et al.* Intramyocardial VEGF-B167 gene delivery delays the progression towards congestive failure in dogs with pacing-induced dilated cardiomyopathy. *Circulation Research* **106**, 1893–1903. ISSN: 00097330 (2010).
79. Shibuya, M. Vascular Endothelial Growth Factor (VEGF) and Its Receptor (VEGFR) Signaling in Angiogenesis: A Crucial Target for Anti- and Pro-Angiogenic Therapies. *Genes & Cancer* **2**, 1097–1105. ISSN: 1947-6019. <http://gan.sagepub.com/lookup/doi/10.1177/1947601911423031> (2011).
80. Stone, O. A. *et al.* Differential regulation of blood flow-induced neovascularization and mural cell recruitment by vascular endothelial growth factor and angiopoietin

- signalling. *The Journal of physiology* **5**, 1575–1591. ISSN: 00223751. <http://doi.wiley.com/10.1113/JP273430><http://www.ncbi.nlm.nih.gov/pubmed/27868196> (Mar. 2016).
81. Jain, R. K., Au, P., Tam, J., Duda, D. G. & Fukumura, D. Engineering vascularized tissue. *Nature Biotechnology* **23**, 821–823. ISSN: 1087-0156. <http://www.nature.com/doifinder/10.1038/nbt0705-821> (2005).
82. Zhang, J. & Li, Y. Therapeutic uses of FGFs. *Seminars in Cell and Developmental Biology* **53**, 144–154. ISSN: 10963634. <http://dx.doi.org/10.1016/j.semcd.2015.09.007> (2016).
83. Faul, C. Cardiac actions of fibroblast growth factor 23. *Bone* **100**, 69–79. ISSN: 87563282. <http://dx.doi.org/10.1016/j.bone.2016.10.001> (2017).
84. Ucuzian, A. A., Gassman, A. A., East, A. T., Greisler, P. & Greisler, H. P. Molecular Mediators of Angiogenesis. *Journal of Burn Care & Research* **31**, 1–28. ISSN: 1559-047X. <http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=01253092-201001000-00022> (Jan. 2011).
85. Litwin, M. *et al.* The role of FGF2 in migration and tubulogenesis of endothelial progenitor cells in relation to pro-angiogenic growth factor production. *Molecular and Cellular Biochemistry* **410**, 131–142. ISSN: 15734919 (2015).
86. Raz, O., Lev, D. L., Battler, A. & Lev, E. I. Pathways mediating the interaction between endothelial progenitor cells (EPCs) and platelets. *PLoS ONE* **9**, 1–8. ISSN: 19326203 (2014).
87. Lee, K. Y., Peters, M. C. & Mooney, D. J. Comparison of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in SCID mice. *Journal of Controlled Release* **87**, 49–56. ISSN: 01683659 (2003).

88. Antoniades, H. N. & Williams, L. T. Human platelet-derived growth factor: structure and function. *Federation proceedings* **42**, 2630–4. ISSN: 0014-9446. <http://www.ncbi.nlm.nih.gov/pubmed/6303866> (June 1983).
89. Heldin, C.-H. Targeting the PDGF signaling pathway in tumor treatment. *Cell Communication and Signaling* **11**, 97. ISSN: 1478-811X. <http://biosignaling.biomedcentral.com/articles/10.1186/1478-811X-11-97> (2013).
90. Herbert, S. P. & Stainier, D. Y. R. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nature Reviews Molecular Cell Biology* **12**, 551–564. ISSN: 1471-0072. <http://www.nature.com/articles/nrm3176> (Sept. 2011).
91. Hellström, M., Kalén, M., Lindahl, P., Abramsson, A. & Betsholtz, C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development (Cambridge, England)* **126**, 3047–55. ISSN: 0950-1991. <http://www.ncbi.nlm.nih.gov/pubmed/10375497> (June 1999).
92. Li, X. *et al.* VEGF-independent angiogenic pathways induced by PDGF-C. *Oncotarget* **1**, 309–314. ISSN: 1949-2553. <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2944232&tool=pmcentrez&rendertype=abstract> (2010).
93. Benjamin, L. E., Hemo, I. & Keshet, E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development (Cambridge, England)* **125**, 1591–1598. ISSN: 0950-1991 (1998).
94. Brudno, Y., Ennett-Shepard, A. B., Chen, R. R., Aizenberg, M. & Mooney, D. J. Enhancing microvascular formation and vessel maturation through temporal control over multiple pro-angiogenic and pro-maturation factors. *Biomaterials* **34**, 9201–

9209. ISSN: 01429612. <http://linkinghub.elsevier.com/retrieve/pii/S0142961213009381> (Dec. 2013).
95. Saharinen, P. & Alitalo, K. The yin, the yang, and the Angiopoietin-1. *Journal of Clinical Investigation* **121**, 2157–2159. ISSN: 0021-9738. <http://www.jci.org/articles/view/58196> (June 2011).
96. Soleti, R. & Martinez, M. C. in *Vitamins and Hormones* 1st ed., 395–438 (Elsevier Inc., 2012). ISBN: 0083-6729 (Print)\r0083-6729 (Linking). <http://dx.doi.org/10.1016/B978-0-12-394622-5.00018-3><http://linkinghub.elsevier.com/retrieve/pii/B9780123946225000183>.
97. Pola, R. *et al.* The morphogen Sonic hedgehog is an indirect angiogenic agent up-regulating two families of angiogenic growth factors. *Nature medicine* **7**, 706–711. ISSN: 1078-8956 (2001).
98. Fuchs, S., Dohle, E. & Kirkpatrick, C. J. *Sonic Hedgehog-Mediated Synergistic Effects Guiding Angiogenesis and Osteogenesis* 1st ed., 491–506. ISBN: 9780123946225. <http://dx.doi.org/10.1016/B978-0-12-394622-5.00022-5> (Elsevier Inc., 2012).
99. Asai, J. *et al.* Topical sonic hedgehog gene therapy accelerates wound healing in diabetes by enhancing endothelial progenitor cell-mediated microvascular remodeling. *Circulation* **113**, 2413–2424. ISSN: 00097322 (2006).
100. Kolesová, H. *et al.* Sonic hedgehog is required for the assembly and remodeling of branchial arch blood vessels. *Developmental Dynamics* **237**, 1923–1934. ISSN: 10588388. <http://doi.wiley.com/10.1002/dvdy.21608> (July 2008).
101. Lawson, N. D., Vogel, A. M. & Weinstein, B. M. Sonic hedgehog and vascular endothelial growth factor act upstream of the Notch pathway during arterial endothelial differentiation. *Developmental Cell* **3**, 127–136. ISSN: 15345807 (2002).

102. Johnson, N. R. & Wang, Y. Controlled Delivery of Sonic Hedgehog Morphogen and Its Potential for Cardiac Repair. *PLoS ONE* **8**, 4–9. ISSN: 19326203 (2013).
103. Kusano, K. F. *et al.* Sonic hedgehog myocardial gene therapy: tissue repair through transient reconstitution of embryonic signaling. *Nature Medicine* **11**, 1197–1204. ISSN: 1078-8956. <http://www.nature.com/doi/10.1038/nm1313> (2005).
104. Ghajar, C. M. *et al.* Mesenchymal Stem Cells Enhance Angiogenesis in Mechanically Viable Prevascularized Tissues via Early Matrix Metalloproteinase Upregulation. *Tissue engineering* **12**, 2875–2888. ISSN: 1076-3279. <http://www.liebertonline.com/doi/abs/10.1089/ten.2006.12.2875> (Oct. 2006).
105. Stetler-Stevenson, W. G. Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *Journal of Clinical Investigation* **103**, 1237–1241. ISSN: 0021-9738. <http://www.jci.org/articles/view/6870> (May 1999).
106. Dvir, T. *et al.* Prevascularization of cardiac patch on the omentum improves its therapeutic outcome. *Proceedings of the National Academy of Sciences* **106**, 14990–14995. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.0812242106> (2009).
107. Meloni, M. *et al.* Nerve Growth Factor Promotes Cardiac Repair following Myocardial Infarction. *Circ Res* **106**, 1275–1284 (2010).
108. Hastings, C. L. *et al.* Drug and cell delivery for cardiac regeneration. *Advanced Drug Delivery Reviews* **84**, 85–106. ISSN: 18728294. <http://dx.doi.org/10.1016/j.addr.2014.08.006> (2015).
109. Ho, T. K. *et al.* Angiogenic effects of stromal cell-derived factor-1 (SDF-1/CXCL12) variants in vitro and the in vivo expressions of CXCL12 variants and CXCR4 in hu-

- man critical leg ischemia. *Journal of Vascular Surgery* **51**, 689–699. ISSN: 07415214. <http://dx.doi.org/10.1016/j.jvs.2009.10.044> (2010).
110. Liu, Y. *et al.* Effect of stromal cell-derived factor-1 on myocardial apoptosis and cardiac function recovery in rats with acute myocardial infarction. *Experimental and Therapeutic Medicine* **12**, 3282–3286. ISSN: 17921015 (2016).
 111. Devalliere, J. *et al.* Co-delivery of a growth factor and a tissue-protective molecule using elastin biopolymers accelerates wound healing in diabetic mice. *Biomaterials* **141**, 149–160. ISSN: 18785905. <http://dx.doi.org/10.1016/j.biomaterials.2017.06.043> (2017).
 112. Ross, J. *et al.* Protein engineered variants of hepatocyte growth factor/scatter factor promote proliferation of primary human hepatocytes and in rodent liver. *Gastroenterology* **142**, 897–906. ISSN: 00165085. <http://dx.doi.org/10.1053/j.gastro.2011.12.006> (2012).
 113. Song, M. *et al.* Regeneration of chronic myocardial infarction by injectable hydrogels containing stem cell homing factor SDF-1 and angiogenic peptide Ac-SDKP. *Biomaterials* **35**, 2436–2445. ISSN: 01429612. <http://dx.doi.org/10.1016/j.biomaterials.2013.12.011> <https://linkinghub.elsevier.com/retrieve/pii/S0142961213014804> (Mar. 2014).
 114. Sonnenberg, S. B. *et al.* Delivery of an engineered HGF fragment in an extracellular matrix-derived hydrogel prevents negative LV remodeling post-myocardial infarction. *Biomaterials* **45**, 56–63. ISSN: 18785905. <http://linkinghub.elsevier.com/retrieve/pii/S014296121401271X> (Mar. 2015).
 115. Boopathy, A. V. *et al.* Intramyocardial Delivery of μ Notch μ Ligand-Containing Hydrogels Improves Cardiac Function and Angiogenesis Following Infarction. *Tissue Engineering Part A* **21**, 2315–2322. ISSN: 1937-3341. <http://online.liebertpub.com/doi/10.1089/ten.tea.2014.0622> (2015).

116. Frueh, F. S. *et al.* Adipose Tissue-Derived Microvascular Fragments Improve Vascularization, Lymphangiogenesis, and Integration of Dermal Skin Substitutes. *Journal of Investigative Dermatology* **137**, 217–227. ISSN: 0022202X. <http://linkinghub.elsevier.com/retrieve/pii/S0022202X16322758> (Jan. 2017).
117. Laschke, M. W. & Menger, M. D. Adipose tissue-derived microvascular fragments: natural vascularization units for regenerative medicine. *Trends in Biotechnology* **33**, 442–448. ISSN: 01677799. <http://dx.doi.org/10.1016/j.tibtech.2015.06.001> <http://linkinghub.elsevier.com/retrieve/pii/S0167779915001237> (Aug. 2015).
118. Benest, A. V. *et al.* VEGF and angiopoietin-1 stimulate different angiogenic phenotypes that combine to enhance functional neovascularization in adult tissue. *Microcirculation* **13**, 423–437. ISSN: 10739688 (2006).
119. Odent (Grigorescu), G. *et al.* Synergic effects of VEGF-A and SDF-1 on the angiogenic properties of endothelial progenitor cells. *Journal of Tissue Engineering and Regenerative Medicine*, 1–12. ISSN: 19327005 (2016).
120. Lai, H. J. *et al.* Tailored design of electrospun composite nanofibers with staged release of multiple angiogenic growth factors for chronic wound healing. *Acta Biomaterialia* **10**, 4156–4166. ISSN: 18787568. <http://dx.doi.org/10.1016/j.actbio.2014.05.001> (2014).
121. Deckers, M. M. *et al.* Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A. *Endocrinology* **143**, 1545–1553. ISSN: 0013-7227. <http://www.ncbi.nlm.nih.gov/pubmed/11897714> (2002).
122. Bagdadi, A. V. *et al.* Poly(3-hydroxyoctanoate), a promising new material for cardiac tissue engineering. *Journal of Tissue Engineering and Regenerative Medicine*. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.2318> (2016).

123. Jiang, B. *et al.* Design of a composite biomaterial system for tissue engineering applications. *Acta Biomaterialia* **10**, 1177–1186. ISSN: 17427061. <http://dx.doi.org/10.1016/j.actbio.2013.11.029> (2014).
124. Justin, A. W., Brooks, R. A. & Markaki, A. E. Multi-casting approach for vascular networks in cellularized hydrogels. *Journal of The Royal Society Interface* **13**, 20160768. ISSN: 1742-5689. <http://rsif.royalsocietypublishing.org/lookup/doi/10.1098/rsif.2016.0768> (2016).
125. Vo, T. N., Kasper, F. K. & Mikos, A. G. Strategies for controlled delivery of growth factors and cells for bone regeneration. *Advanced Drug Delivery Reviews* **64**, 1292–1309. ISSN: 0169409X. <http://www.sciencedirect.com/science/article/pii/S0169409X12000191%5Cnhttp://linkinghub.elsevier.com/retrieve/pii/S0169409X12000191%20http://linkinghub.elsevier.com/retrieve/pii/S0169409X12000191> (Sept. 2012).
126. Chen, F. M., Zhang, M. & Wu, Z. F. Toward delivery of multiple growth factors in tissue engineering. *Biomaterials* **31**, 6279–6308. ISSN: 01429612. <http://dx.doi.org/10.1016/j.biomaterials.2010.04.053> (2010).
127. Chiu, L. L. Y. & Radisic, M. Scaffolds with covalently immobilized VEGF and Angiopoietin-1 for vascularization of engineered tissues. *Biomaterials* **31**, 226–241. ISSN: 01429612. <http://dx.doi.org/10.1016/j.biomaterials.2009.09.039> (2010).
128. Odedra, D., Chiu, L. L. Y., Shoichet, M. & Radisic, M. Endothelial cells guided by immobilized gradients of vascular endothelial growth factor on porous collagen scaffolds. *Acta Biomaterialia* **7**, 3027–3035. ISSN: 17427061. <http://dx.doi.org/10.1016/j.actbio.2011.05.002> (2011).

129. Wu, Q. *et al.* The effect of heparinized decellularized scaffolds on angiogenic capability. *Journal of Biomedical Materials Research - Part A* **104**, 3021–3030. ISSN: 15524965 (2016).
130. Lafrance, L. K. & Dapron, J. End Point Attached Heparin Affinity Matrix. *Journal of Molecular Recognition* **10**, 748–751. ISSN: 0952-3499 (1997).
131. Dick, B., Schmidt, K. G., Eisenmann, D. & Pfeiffer, N. A new method for direct detection of heparin on surface-modified intraocular lenses. A modification of Jaques' toluidine blue staining method. *Ophthalmologica. Journal internationale d'ophtalmologie. International journal of ophthalmology. Zeitschrift fur Augenheilkunde* **211**, 75–8. ISSN: 0030-3755. <http://www.ncbi.nlm.nih.gov/pubmed/9097308> (1997).
132. Lakshmanan, R., Kumaraswamy, P., Krishnan, U. M. & Sethuraman, S. Engineering a growth factor embedded nanofiber matrix niche to promote vascularization for functional cardiac regeneration. *Biomaterials* **97**, 176–195. ISSN: 18785905. <http://dx.doi.org/10.1016/j.biomaterials.2016.02.033> (2016).
133. Freitas, S., Merkle, H. P. & Gander, B. Microencapsulation by solvent extraction/evaporation: Reviewing the state of the art of microsphere preparation process technology. *Journal of Controlled Release* **102**, 313–332. ISSN: 01683659 (2005).
134. Nyberg, E., Holmes, C., Witham, T. & Grayson, W. L. Growth factor-eluting technologies for bone tissue engineering. *Drug Delivery and Translational Research* **6**, 184–194. ISSN: 21903948 (2016).
135. Roberts, J. J., Farrugia, B. L., Green, R. A., Rnjak-Kovacina, J. & Martens, P. J. In situ formation of poly(vinyl alcohol)–heparin hydrogels for mild encapsulation and prolonged release of basic fibroblast growth factor and vascular endothelial growth factor. *Journal of Tissue Engineering* **7**, 204173141667713. ISSN: 2041-7314. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5117248/>

- %20http://journals.sagepub.com/doi/10.1177/2041731416677132 (Jan. 2016).
136. Munarin, F. & Coulombe, K. L. K. *A novel 3-dimensional approach for cardiac regeneration in 2015 37th Annual International Conference of the IEEE Engineering in Medicine and Biology Society (EMBC) 2015* (IEEE, Aug. 2015), 1741–1744. ISBN: 978-1-4244-9271-8. <http://ieeexplore.ieee.org/document/7318714/>.
 137. Akar, B. *et al.* Biomaterials with persistent growth factor gradients in vivo accelerate vascularized tissue formation. *Biomaterials* **72**, 61–73. ISSN: 18785905. <http://dx.doi.org/10.1016/j.biomaterials.2015.08.049> (2015).
 138. Izadifar, M., Kelly, M. E. & Chen, X. Regulation of sequential release of growth factors using bilayer polymeric nanoparticles for cardiac tissue engineering. *Nanomedicine* **11**, 3237–3259. ISSN: 1743-5889 (2016).
 139. Dashnyam, K. *et al.* Promoting angiogenesis with mesoporous microcarriers through a synergistic action of delivered silicon ion and VEGF. *Biomaterials* **116**, 145–157. ISSN: 18785905. <http://dx.doi.org/10.1016/j.biomaterials.2016.11.053> (2017).
 140. Li, H. & Chang, J. Bioactive silicate materials stimulate angiogenesis in fibroblast and endothelial cell co-culture system through paracrine effect. *Acta Biomaterialia* **9**, 6981–6991. ISSN: 17427061 (2013).
 141. Garbern, J. C., Minami, E., Stayton, P. S. & Murry, C. E. Delivery of basic fibroblast growth factor with a pH-responsive, injectable hydrogel to improve angiogenesis in infarcted myocardium. *Biomaterials* **32**, 2407–2416. ISSN: 01429612. <http://linkinghub.elsevier.com/retrieve/pii/S0142961210015309> (Mar. 2011).

142. Chong, D. S. T. *et al.* Luminal surface engineering, 'micro and nanopatterning': Potential for self endothelialising vascular grafts? *European Journal of Vascular and Endovascular Surgery* **47**, 566–576. ISSN: 15322165. <http://dx.doi.org/10.1016/j.ejvs.2014.02.007> (2014).
143. Zorlutuna, P., Vadgama, P. & Hasirci, V. Both sides nanopatterned tubular collagen scaffolds as tissue-engineered vascular grafts. *Journal of Tissue Engineering and Regenerative Medicine* **4**, 628–637. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.278> (Dec. 2010).
144. Zheng, Y. *et al.* In vitro microvessels for the study of angiogenesis and thrombosis. *Proceedings of the National Academy of Sciences* **109**, 9342–9347. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.1201240109> (June 2012).
145. Kurokawa, Y. K. *et al.* Human Induced Pluripotent Stem Cell-Derived Endothelial Cells for Three-Dimensional Microphysiological Systems. *Tissue Engineering Part C: Methods* **23**, 474–484. ISSN: 1937-3384. <http://online.liebertpub.com/doi/10.1089/ten.tec.2017.0133> (2017).
146. Gerberich, B. G. & Bhatia, S. K. Tissue scaffold surface patterning for clinical applications. *Biotechnology Journal* **8**, 73–84. ISSN: 18606768 (2013).
147. Lima, M. J., Correlo, V. M. & Reis, R. L. Micro/nano replication and 3D assembling techniques for scaffold fabrication. *Materials Science and Engineering C* **42**, 615–621. ISSN: 09284931. <http://dx.doi.org/10.1016/j.msec.2014.05.064> (2014).
148. Lei, Y., Zouani, O. F., Rémy, M., Ayela, C. & Durrieu, M. C. Geometrical micro-feature cues for directing tubulogenesis of endothelial cells. *PLoS ONE* **7**. ISSN: 19326203 (2012).

149. Vollert, I. *et al.* In vitro perfusion of engineered heart tissue through endothelialized channels. *Tissue engineering. Part A* **20**, 854–63. ISSN: 1937-335X. <http://www.ncbi.nlm.nih.gov/pubmed/24156346> (2014).
150. Zhang, B. *et al.* Biodegradable scaffold with built-in vasculature for organ-on-a-chip engineering and direct surgical anastomosis. *Nature Materials* **15**, 669–678. ISSN: 1476-1122. <http://www.nature.com/doifinder/10.1038/nmat4570> <http://www.nature.com/articles/nmat4570> (June 2016).
151. Li, J., Chen, M., Fan, X. & Zhou, H. Recent advances in bioprinting techniques: approaches, applications and future prospects. *Journal of Translational Medicine* **14**, 271. ISSN: 1479-5876. <http://translational-medicine.biomedcentral.com/articles/10.1186/s12967-016-1028-0> (2016).
152. Hoch, E., Tovar, G. E. & Borchers, K. Bioprinting of artificial blood vessels: Current approaches towards a demanding goal. *European Journal of Cardio-thoracic Surgery* **46**, 767–778. ISSN: 1873734X (2014).
153. Jana, S. & Lerman, A. Bioprinting a cardiac valve. *Biotechnology Advances* **33**, 1503–1521. ISSN: 07349750. <http://dx.doi.org/10.1016/j.biotechadv.2015.07.006> (2015).
154. Patra, S. & Young, V. A Review of 3D Printing Techniques and the Future in Bio-fabrication of Bioprinted Tissue. *Cell Biochemistry and Biophysics* **74**, 93–98. ISSN: 10859195 (2016).
155. Lee, J. W. *et al.* Development of a 3D cell printed construct considering angiogenesis for liver tissue engineering. *Biofabrication* **8**, 015007. ISSN: 1758-5090. <http://stacks.iop.org/1758-5090/8/i=1/a=015007?key=crossref.6bb9f75f3315935761741ef3aa62a8a3> (2016).

156. Kolesky, D. B., Homan, K. A., Skylar-Scott, M. A. & Lewis, J. A. Three-dimensional bioprinting of thick vascularized tissues. *Proceedings of the National Academy of Sciences* **113**, 3179–3184. ISSN: 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.1521342113> (2016).
157. Poldervaart, M. T. *et al.* Prolonged presence of VEGF promotes vascularization in 3D bioprinted scaffolds with defined architecture. *Journal of Controlled Release* **184**, 58–66. ISSN: 18734995. <http://dx.doi.org/10.1016/j.jconrel.2014.04.007> (2014).
158. Zhang, Y., Yu, Y. & Ozbolat, I. T. Direct Bioprinting of Vessel-Like Tubular Microfluidic Channels. *Journal of Nanotechnology in Engineering and Medicine* **4**, 020902. ISSN: 1949-2944. <http://nanoengineeringmedical.asmedigitalcollection.asme.org/article.aspx?doi=10.1115/1.4024398> (2013).
159. Miller, J. S. *et al.* Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials* **11**, 768–774. ISSN: 1476-1122. <http://www.nature.com/doifinder/10.1038/nmat3357> (July 2012).
160. Borovjagin, A. V., Ogle, B. M., Berry, J. L. & Zhang, J. From Microscale Devices to 3D Printing: Advances in Fabrication of 3D Cardiovascular Tissues. *Circulation Research* **120**, 150–165. ISSN: 15244571 (2017).
161. Wu, P. K. & Ringeisen, B. R. Development of human umbilical vein endothelial cell (HUVEC) and human umbilical vein smooth muscle cell (HUVSMC) branch/stem structures on hydrogel layers via biological laser printing (BioLP). *Biofabrication* **2**, 014111. ISSN: 1758-5082. <http://stacks.iop.org/1758-5090/2/i=1/a=014111?key=crossref.6a5246d1a7af601c17569dc695797706> (2010).

162. Gao, L. *et al.* Myocardial Tissue Engineering with Cells Derived from Human-Induced Pluripotent Stem Cells and a Native-Like, High-Resolution, 3-Dimensionally Printed Scaffold. *Circulation Research* **120**, 1318–1325. ISSN: 15244571 (2017).
163. Fleischer, S., Shapira, A., Feiner, R. & Dvir, T. Modular assembly of thick multi-functional cardiac patches. *Proceedings of the National Academy of Sciences* **114**, 1898–1903. ISSN: 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.1615728114> (2017).
164. Vunjak Novakovic, G., Eschenhagen, T. & Mummery, C. Myocardial Tissue Engineering: In Vitro Models. *Cold Spring Harbor Perspectives in Medicine* **4**, 1–15. ISSN: 2157-1422. <http://perspectivesinmedicine.cshlp.org/lookup/doi/10.1101/cshperspect.a014076> (2014).
165. Nowak-sliwinska, P., Segura, T., Iruela-Arispe, M. L. & Angeles, L. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. *Angiogenesis* **17**, 779–804. ISSN: 0969-6970. <http://link.springer.com/10.1007/s10456-014-9440-7> (Oct. 2014).
166. Baker, M. *et al.* Use of the mouse aortic ring assay to study angiogenesis. *Nature Protocols* **7**, 89–104. ISSN: 1754-2189. 10.1038/nprot.2011.435%20<http://www.nature.com/articles/nprot.2011.435> (Jan. 2012).
167. Go, R. S. & Owen, W. G. The rat aortic ring assay for in vitro study of angiogenesis. *Methods in molecular medicine* **85**, 59–64. ISSN: 1543-1894. <http://www.ncbi.nlm.nih.gov/pubmed/12710197> (2003).
168. Goodwin, A. M. *In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents* 2007.
169. Gomes, A. C., Falcão-Pires, I., Pires, A. L., Brás-Silva, C. & Leite-Moreira, A. F. Rodent models of heart failure: An updated review. *Heart Failure Reviews* **18**, 219–249. ISSN: 13824147 (2013).

170. Milani-Nejad, N. & Janssen, P. M. Small and large animal models in cardiac contraction research: Advantages and disadvantages. *Pharmacology & Therapeutics* **141**, 235–249. ISSN: 01637258. <http://linkinghub.elsevier.com/retrieve/pii/S016372581300212X> (Mar. 2014).
171. Moran, C. J. *et al.* The benefits and limitations of animal models for translational research in cartilage repair. *Journal of Experimental Orthopaedics* **3**, 1. ISSN: 2197-1153. <http://www.jeo-esska.com/content/3/1/1> (Dec. 2016).
172. Kang, H.-W. *et al.* A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology* **34**, 312–319. ISSN: 1087-0156. <http://www.nature.com/doi/10.1038/nbt.3413> (2016).
173. Jang, J. *et al.* 3D printed complex tissue construct using stem cell-laden decellularized extracellular matrix bioinks for cardiac repair. *Biomaterials* **112**, 264–274. ISSN: 01429612. <http://dx.doi.org/10.1016/j.biomaterials.2016.10.026> <http://linkinghub.elsevier.com/retrieve/pii/S0142961216305695> (Jan. 2017).
174. Bowers, S., Norden, P. & Davis, G. in *Advances in Pharmacology* 1st ed., 241–280 (Elsevier Inc., 2016). ISBN: 9780128043967. <http://dx.doi.org/10.1016/bs.apha.2016.04.005> <http://linkinghub.elsevier.com/retrieve/pii/S105435891630028X>.
175. Liu, M. *et al.* Combined multi-modal photoacoustic tomography, optical coherence tomography (OCT) and OCT angiography system with an articulated probe for in vivo human skin structure and vasculature imaging. *Biomedical Optics Express* **7**, 3390. ISSN: 2156-7085. <https://www.osapublishing.org/abstract.cfm?URI=boe-7-9-3390> (Sept. 2016).

176. Qin, W. *et al.* Depth-resolved 3D visualization of coronary microvasculature with optical microangiography. *Physics in Medicine and Biology* **61**, 7536–7550. ISSN: 0031-9155. <http://stacks.iop.org/0031-9155/61/i=21/a=7536?key=crossref.0731d2fb478dd8a1b0065814faec969b> (Nov. 2016).
177. Lehr, H. a., Leunig, M., Menger, M. D., Nolte, D. & Messmer, K. Dorsal skinfold chamber technique for intravital microscopy in nude mice. *The American journal of pathology* **143**, 1055–62. ISSN: 0002-9440. <http://www.ncbi.nlm.nih.gov/pubmed/7692730><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1887078> (Oct. 1993).
178. Laschke, M. W. & Menger, M. D. Prevascularization in tissue engineering: Current concepts and future directions. *Biotechnology Advances* **34**, 112–121. ISSN: 07349750. <http://dx.doi.org/10.1016/j.biotechadv.2015.12.004><http://linkinghub.elsevier.com/retrieve/pii/S0734975015300598> (Mar. 2016).
179. Mian, R. *et al.* Formation of New Tissue from an Arteriovenous Loop in the Absence of Added Extracellular Matrix. *Tissue Engineering* **6**, 595–603. ISSN: 1076-3279. <http://www.ncbi.nlm.nih.gov/pubmed/11103081><http://www.liebertonline.com/doi/abs/10.1089/10763270050199541> (Dec. 2000).
180. Tee, R. *et al.* Transplantation of Engineered Cardiac Muscle Flaps in Syngeneic Rats. *Tissue Engineering Part A* **18**, 1992–1999. ISSN: 1937-3341. <http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2012.0151> (Oct. 2012).
181. Morritt, A. N. *et al.* Cardiac Tissue Engineering in an In Vivo Vascularized Chamber. *Circulation* **115**, 353–360. ISSN: 0009-7322. <http://circ.ahajournals.org/cgi/doi/10.1161/CIRCULATIONAHA.106.657379> (Jan. 2007).

182. Meecham, L. *et al.* Simultaneous Iliac Vein Bovine Pericardial Patch Venoplasty and Creation of PTFE Lower Limb Arteriovenous Fistula Graft for Rescue Vascular Access. *Annals of Vascular Surgery* **36**, 9–292. ISSN: 08905096. <http://dx.doi.org/10.1016/j.avsg.2016.03.018><http://linkinghub.elsevier.com/retrieve/pii/S0890509616304885> (Oct. 2016).
183. Utzinger, U., Baggett, B., Weiss, J. A., Hoying, J. B. & Edgar, L. T. Large-scale time series microscopy of neovessel growth during angiogenesis. *Angiogenesis* **18**, 219–232. ISSN: 0969-6970. <http://link.springer.com/10.1007/s10456-015-9461-x> (July 2015).
184. White, S. M. *et al.* Implanted Cell-Dense Prevascularized Tissues Develop Functional Vasculature That Supports Reoxygenation After Thrombosis. *Tissue Engineering Part A* **20**, 2316–2328. ISSN: 1937-3341. <http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2013.0311><https://www.liebertpub.com/doi/10.1089/ten.tea.2013.0311> (Sept. 2014).
185. Chen, X. *et al.* Prevascularization of a Fibrin-Based Tissue Construct Accelerates the Formation of Functional Anastomosis with Host Vasculature. *Tissue Engineering Part A* **15**, 1363–1371. ISSN: 1937-3341. <http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2008.0314> (June 2009).
186. Herwig, L. *et al.* Distinct Cellular Mechanisms of Blood Vessel Fusion in the Zebrafish Embryo. *Current Biology* **21**, 1942–1948. ISSN: 09609822. <http://dx.doi.org/10.1016/j.cub.2011.10.016><http://linkinghub.elsevier.com/retrieve/pii/S0960982211011407> (Nov. 2011).
187. Eppler, S. M. *et al.* A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. *Clinical Pharmacology and Therapeutics* **72**, 20–32. ISSN: 00099236 (2002).

Study	Model	Biomaterial(s)	In Vitro Study Cell Type(s)	In Vivo Study Cell Type(s)	Angiogenesis-Inducing Molecule(s)	Technique(s)	Results
Chiu et al. (2010)	CAM (Chicken)	Scaffold: Collagen	H5V Cells (Murine heart endothelium)	N/A	VEGF-165, Ang-1	Incorporation - covalent EDC	Increased tubule formation and vessel penetration into scaffold compared to independently immobilized growth factors.
Dashnyam et al. (2016)	CAM (Chicken)	Carrier: Silica	HUVECs	N/A	Si, VEGF-165	Encapsulation	Si release caused HIF1 α upregulation, resulting in downstream expression of angiogenic molecules including bFGF, eNOS, and VEGF. Stimulated EC migration, sprouting, and tubule networking.
Wu et al. (2016)	CAM (Chicken)	Scaffold: Decellularized liver	HUVECs	N/A	VEGF-165	Incorporation - heparin-binding	Higher vessel density, as determined by quantifying number of branch points.
Izadifar et al. (2016)	Aortic ring (Rat)	Scaffold: Fibrin; Carrier: PLGA core, PLLA shell	None	N/A	VEGF, bFGF, PDGF	Encapsulation	Bilayer sequential release of VEGF and bFGF followed by PDGF from core. Increased microvessel sprout density by day 8, pericyte recruitment by day 5.
Lai et al. (2014)*	Diabetic skin wound (Rat)	Scaffold: Collagen-hyaluronic acid; Carrier: Felatin	HUVECs	HUVECs	bFGF, EGF; VEGF; PDGF-BB	Incorporation - electrospinning; encapsulation	Complete wound closure by week 6, greater density of matured vessels and collagen deposition.
Brudno et al. (2013)*	Subcutaneous implant (Mouse)	Scaffold: Fibrin (<i>in vitro</i>), PLG (<i>in vivo</i>); Carrier: PLG (<i>in vivo</i>)	HUVECs, Placental pericytes	None	VEGF, Ang-2, Ang-1, PDGF-BB	Incorporation; encapsulation	Simultaneous release of all four factors resulted in lower vessel density than VEGF+Ang-2 alone, or VEGF+Ang-1 followed by delayed release of PDGF and Ang1. Delayed release of PDGF and/or Ang-1 resulted in more mature vessels measured by SMA staining.
Güç et al. (2017)*	Subcutaneous implant (Rat)	Scaffold: Collagen-fibrin	Human dermal LECs	None	FB-VEGF-C	Incorporation	Increased lymphangiogenesis without interfering with angiogenic sprouts. Lymphangiogenic effects were localized with no downstream remodeling to collecting vessels, and unaltered fluid clearance.
Poldervaart et al. (2014)*	Subcutaneous implant (Mouse)	Scaffold: Matrigel-alginate; Carrier: Gelatin	Human cord blood EPCs	Human cord blood EPCs	VEGF	3D bioprinting - pressure-assisted; Encapsulation	Increased neovessel formation in sustained release group, but immature vessels due to negative SMA staining. Slower VEGF release encouraged vessel formation.
Jiang et al. (2013)	Subcutaneous implant (Rat)	Scaffold: PEG-PLLA-DA & PEG-DA, Fibrin; Carrier: PLGA	HUVECs	HUVECs	FGF-1; PDGF-BB	Incorporation; Encapsulation	Sequential release of FGF-1 followed by PDGF-BB to promote mature vascular network formation and scaffold ingrowth.
Akar et al. (2015)*	Subcutaneous implant (Rat)	Scaffold: PEG-DA-fibrin, PEG-PLLA-DA; Carrier: PLGA	None	None	PDGF-BB	Encapsulation	Formation of a chemotactic gradient that resulted in increased tissue invasion and blood vessel density at higher magnitudes.
Park et al. (2015)**	Subcutaneous implant (Mouse)	Scaffold: PCL, collagen, gelatin-alginate	hDPSCs	hDPSCs	BMP-2, VEGF	3D printing - pressure-assisted	Temporal and spatially-distinct release of BMP-2 and VEGF allowing vascularization towards the center of the scaffold. Vasculogenesis from DPSCs in hypoxic areas and angiogenesis from host tissue. Accelerated bone regeneration.
Kang et al. (2016)	Mandible, calvarial bone, ear cartilage, skeletal muscle (Rat)	Scaffold: PCL, Pluronic	hAFSCs, rabbit ear chondrocytes, mouse	hAFSCs, rabbit ear chondrocytes, mouse	N/A	3D bioprinting - pressure-assisted, topographical engineering	Multi-cartridge extrusion for patterned scaffolds with integrated porous lattice channels to facilitate nutrient exchange. Tissue formation demonstrated with vessel formation and no necrosis.
Jang et al. (2017)	Subcutaneous implant (Mouse)	Scaffold: Decellularized porcine ECM, PCL mesh	hCPCs, hMSCs	hCPCs, hMSCs	VEGF	3D Bioprinting - pressure-assisted	hCPCs and hMSCs differentiated into cardiac and endothelial cells. Increased vascularization penetrating the scaffold. Increased ejection fraction, reduced fibrosis.
Fleischer et al. (2017)	Subcutaneous implant (Rat)	Scaffold: Albumin; Carrier: PLGA	NRVMs, HUVECs	NRVMs, HUVECs	VEGF	Encapsulation	Generation of 5mm thick scaffolds with electrical coupling in z direction. Increased capillary density, increased area occupied by vessels, anastomosis of predefined channels
Zhang et al. (2016)	Femoral vessel ligation (Rat)	Scaffold: POMaC	HUVECs, hMSCs, hESCs	RVECs, NRVMs	N/A	Topographical engineering - soft lithography, photolithography	Enabled artery bypass and artery-to-vein anastomosis, instant and pulsatile perfusion. Angiogenesis and minimal clotting one week after surgery.
Yuen et al. (2010)*	Ischemic hind limb (Rat)	Scaffold: PLG	hDVECs	hDVECs	VEGF-165, anti-VEGF	Incorporation	Temporally stable release profile, spatially sharp angiogenic sections, and moderate necrosis prevention.

Table 2.1: Spatiotemporal control of physical and chemical cues. Full caption below.

Wu et al. (2016)	Greater omentum (Rat)	Scaffold: Decellularized liver	HUVECs	HUVECs	VEGF-165	Incorporation - heparin-binding	Higher number, distribution, and diameter of blood vessels in and around implant.
Dvir et al. (2009)	Greater omentum, then MI - LAD permanent occlusion (Rat)	Scaffold: Alginate-sulfate	None	NRVMs	IGF-1, SDF-1, VEGF	Incorporation	Cardiac patches populated with dense vasculature when on the omentum. After transplantation, patches improved cardiac function and electrically coupled with the host.
Sonnenberg et al. (2015)*	MI - LAD IR (Rat)	Scaffold: Porcine pericardium-derived ECM hydrogel	RASMCs, NRVMs	None	HGF fragment	Incorporation	Increase in fractional area change, arteriole density, and neovascularization while mitigating changes in LV dimensions.
Boopathy et al. (2015)*	MI - LAD IR (Rat)	Scaffold: Self-assembling peptide hydrogel	None	None	Jagged1 peptide mimic	Incorporation	Improved LV morphology, angiogenesis in implant area, EC proliferation, and stem cell recruitment. Reduced fibrosis.
Gao et al. (2017)	MI - LAD permanent ligation (Mouse)	Scaffold: Methacrylated gelatin	hiPSC-CMs, hiPSC-ECs, hiPSC-SMCs	hiPSC-CMs, hiPSC-ECs, hiPSC-SMCs	N/A	3d bioprinting - multiphoton excitation	Increased vascular area density, reduced apoptosis, improved ejection fraction, fractional shortening, and reduced infarct size.
Song et al. (2014)	MI - LAD permanent ligation (Rat)	Scaffold: Acrylated hyaluronic acid	hMSCs	None	SDF-1, Ac-SDKP	Incorporation	Reduced infarct size and improved stroke volume, ejection fraction, cardiac output. Higher number of mature arterioles and capillaries.
Lakshmana n et al. (2016)	MI - LAD, DCx, PDA permanent ligation (Rabbit)	Scaffold: PLCL/PEOz	HUVECs	None	VEGF, bFGF	Incorporation - electrospinning	Improved cardiac function based on M-mode echocardiographic measurements of LV dimensions and performance. Increased viable myocardium, vessel penetration and sprouting capillary formation.
Riemenschneider et al. (2016)	MI - LAD permanent ligation (rat)	Scaffold: Fibrin	human blood outgrowth EPCs, human brain vascular pericytes	human blood outgrowth EPCs, human brain vascular pericytes	SCF, IL-3, SDF-1 α	Incorporation	High capillary density within 6 days in aligned scaffold implants with 40% perfused, slight increase in capillary diameter, insignificant increase in ejection fraction and fractional shortening.
Cheng et al. (2016)**	MI - LAD permanent ligation (Rat)	Carriers: PLGA-PNIPAM	HUVECs, NRVMs	None	NaB	Encapsulation	Stimulated EC proliferation by promoting mRNA expression of VEGF-A, HGF, bFGF, and PFGF. Reduced generation of reactive oxygen species and cellular autophagy. Increased LVEF, reduced infarct size.
Rodness et al. (2016)**	MI - LAD permanent ligation (Rat)	Scaffold: Chitosan sheet; Carrier: Alginate	HSVECs	None	VEGF-164 (recombinant rat)	Encapsulation - fused microparticles	Higher capillary density and mature arteriolar vessels in patch. Scars were thicker but smaller in area and length. Improved fractional shortening.
Awada et al. (2017)**	MI - LAD permanent ligation (Rat)	Scaffold: Fibrin; Carrier: PEAD, heparin	None	None	TIMP-3, bFGF, SDF-1 α	Incorporation, encapsulation	Reduced fibrosis due to TIMP-3 release, reduced inflammation, improved cardiomyocyte survival, enhanced angiogenesis and SMA+ vessels by 8 weeks.

Table 2.1: Spatiotemporal control of physical and chemical cues. Full caption below.

Table 2.1: Studies applying spatiotemporal control of physical and chemical cues in an *in vivo* environment. Articles are arranged primarily by model, then appearance in section 5. Articles marked with * are referenced only outside of section 5, and those marked with ** are not discussed in this review but are provided for additional examples and further readings. Ang-1: angiopoietin 1; bFGF: basic fibroblast growth factor; BMP2: bone morphogenetic protein 2; CM: cardiomyocyte; CAM: chorioallantoic membrane assay; DCx: distal circumflex artery; DPSC: dental pulp stem cell; EC: endothelial cell; ECM: extra-cellular matrix; EDC: 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride chemistry; EGF: endothelial growth factor; eNOS: endothelial nitric oxide synthase; EPC: endothelial progenitor cell; FB-VEGF-C: fibrin-binding vascular endothelial growth factor C variant; FGF-1: fibroblast growth factor 1; hAFSCs: human amniotic fluid-derived stem cells; hCPCs: human cardiac progenitor cells; hDPSCs: human dental pulp stem cells; hDVECs: human dermal vascular endothelial cells; HGF: hepatocyte growth factor; hiPSC: human induced pluripotent stem cell; hMSCs: mesenchymal stem cells; HUVECs: human umbilical vein endothelial cells; HIF1 α : hypoxia-inducible factor 1 α ; HSVECs: human saphenous vein endothelial cells; IGF-1: insulin-like growth factor 1; IR: ischemia-reperfusion; LAD: left anterior descending artery; LECs: lymphatic endothelial cells; LV: left ventricle; LVEF: left ventricle ejection fraction; NaB: sodium butyrate; NRVMs: neonatal rat ventricular myocytes; PCL: polycaprolactone; PDA: distal posterior descending artery; PDGF: platelet-derived growth factor; PEAD: poly(ethylene argininy laspartate diglyceride); PEG: polyethylene glycol; PEG-DA: polyethylene glycol diacrylate; PEOz: poly(2-ethyl-2-oxazoline) ; PLCL: poly(L-lactide-co-caprolactone); PLG: copolymer of D,L-lactide and glycolide; PLGA: poly(lactic-co-glycolic acid); PLLA: poly(lactic acid); PNIPAM: poly(N-isopropylacrylamide); POMaC: poly(octamethylene maleate (anhydride) citrate); RASMCs: rat aortic smooth muscle cells; RVECs: rat vein endothelial cells; SDF-1: stromal cell-derived factor 1; α -SMA: α -smooth muscle actin; SMC: smooth muscle cell; TIMP-3: tissue inhibitors of metalloproteinases-3; VEGF: vascular endothelial growth factor; Si: silicon.

Chapter 3

Tissues with patterned vessels or protein release induce vascular chemotaxis in an *in vitro* platform

Reproduced with permission from R. J. Kant, C. F. Bare, and K. L. K Coulombe, “Tissues with patterned vessels or protein release induce vascular chemotaxis in an *in vitro* platform”, *Tissue Eng. Part A*, 27 (19-20), 1290-1304, Oct 2021.

3.1 Abstract

Engineered tissues designed for translational applications in regenerative medicine require vascular networks to deliver oxygen and nutrients rapidly to the implanted cells. A limiting factor of *in vivo* translation is the rapid and successful inosculation, or connection, of host and implanted vascular networks and subsequent perfusion of the implant. An approach gaining favor in vascular tissue engineering is to provide instructive cues from the engineered tissue to enhance host vascular penetration and connection with the implant.

Here, we use a novel *in vitro* platform based on the aortic ring assay to evaluate the impact of patterned, endothelialized vessels or growth factor release from engineered constructs on preinoscultative vascular cell outgrowth from surrogate host tissue in a controlled, defined environment, and introduce robust tools for evaluating vascular morphogenesis and chemotaxis. We demonstrate the creation of engineered vessels at the arteriole scale, which develop basement membrane, exhibit tight junctions, and actively sprout into the surrounding bulk hydrogel. Vessel-containing constructs are co-cultured adjacent to rodent aortic rings, and the resulting heterocellular outgrowth is quantified. Cells originating from the aortic ring migrate preferentially toward constructs containing engineered vessels with 1.5-fold faster outgrowth kinetics, 2.5-fold increased cellular density, and 1.6-fold greater network formation versus control (no endothelial cells and growth factor-reduced culture medium). Growth factor release from constructs with nonendothelialized channels and in reduced factor medium equivalently stimulates sustained vascular outgrowth distance, cellular density, and network formation, akin to engineered vessels in endothelial growth medium 2 (EGM-2) medium. In conclusion, we show that three-dimensional endothelialized patterned vessels or growth factor release stimulate a robust, host-derived vascular cell chemotactic response at early time points critical for instructive angiogenic cues. Further, we developed robust, unbiased tools to quantify metrics of vascular morphogenesis and preinoscultative heterocellular outgrowth from rat aortic rings and demonstrated the utility of our complex, controlled environment, heterocellular *in vitro* platform.

3.2 Introduction

A major focus in tissue engineering is the creation of implantable, cellularized therapeutics that replace or augment damaged tissue. In considering the scalability of engineered constructs for clinical translation, vascularization becomes a crucial design criterion due to oxygen diffusion limits of 100–200 μm [1–3] in thick, cellularized tissue. Vascularization

is particularly important in applications such as cardiac, skin, bone, liver, and pancreatic tissue engineering that necessitate dense and hierarchically organized tissues, and thus require efficient convection of oxygen and nutrients via a robust vascular transport system [4, 5]. Such tissues fabricated without vasculature and thicker than oxygen diffusion limits can suffer hypoxia and form necrotic cores [3, 6]. Providing adequate conduits for the convection of oxygen and nutrients is necessary for the survival and function of implanted tissues both before and after implantation [7–9].

Engineering vasculature within tissues *in vitro* can be achieved by using many different techniques, but it primarily involves combinatorial manipulation of physical cues (e.g., co-culture vessel stabilization [8], fabricated geometries [9–11], biomaterials [2, 12, 13], or three-dimensional [3D] bioprinting [14, 15]) and chemical cues (e.g., peptide [16] and growth factor treatment/release [17], or co-culture paracrine signaling [8, 18]) to orchestrate vessel formation [6, 19]. Prevascularizing a patch before implantation has demonstrated perfusion of the implanted networks and improved survival of the construct [9–11]. The overall mechanism by which this occurs is termed inosculation, or the connection of disparate vascular networks [20]. In this process, independent vessels recognize and sprout toward one another over tens to hundreds of microns, anastomose, and remodel to form a continuous, integrated network for blood flow [21]. Inosculation can enable rapid perfusion to implanted tissues within 1–6 days after implantation [11, 22–24], which has implications for the survival, engraftment, and function of the implant.

Despite the great need for rapid vessel perfusion on implantation, preinosculative dynamics of endothelial and vascular stromal cells are not well understood. Primary literature in this area focuses on *in vitro* monolayer-based or microfluidic models that do not sufficiently recreate the complex environment and stimuli of angiogenesis at larger scales with engineered tissues [24–27], or *in vivo* experiments that report intra-patch vessel structures or perfusion at terminal endpoints [9, 11, 23, 28]. Thus, there is a need to examine sig-

naling and migration patterns between host and engineered tissues before inosculation at intermediate scales in terms of time and complexity. Further, several cellular populations and microenvironmental cues engage in the inosculation process from both the host and implanted tissue, and understanding how these cues drive this process is necessary to engineer an adaptive and timely vascularization response upon tissue implantation.

Utilizing animal-derived tissue provides a compromise between the simplicity of *in vitro* systems and complexity of *in vivo* models [6, 27, 29–32] and is often used to study vascularization mechanisms, often by treatment with angiogenic agents or genetic alteration [33, 34]. One such assay is the heterocellular aortic ring assay, which examines microvessel sprouting in a matrix protein (historically Matrigel) from sectioned rodent aortas stimulated primarily by VEGF, bFGF, and/or fetal bovine serum (FBS) over a 7–14 day period [27, 32]. The primary outputs of this assay are microvessel counts, histological staining of endothelial and supportive stromal cells (including smooth muscle cells, fibroblasts, pericytes, etc.), and morphological assessment in response to treatment with an agent [17, 32, 35, 36]. The aortic ring assay provides a useful starting point from which to develop a defined, robust platform for bridging studies in vascularization between *in vitro* and *in vivo* research.

In the present study, we describe and evaluate our *in vitro* platform for examining preinosculative vascular outgrowth from aortic rings in response to vascularization cues from engineered constructs in a defined and controlled environment by using a novel adaptation of the aortic ring assay. We report the global and regional migratory responses of outgrowing vascular cells adjacent to constructs containing engineered vessels and evaluate responses to physical, cellular, and chemical effectors of migration and angiogenesis by using unbiased automated analyses (in MATLAB and available online). Our data demonstrate that preinosculative outgrowth from aortic rings is sensitive to vascular stimuli and co-culture with engineered constructs, and that endothelialized vessels or growth factor

release similarly prompt early vascular morphogenesis, sustained vascular outgrowth, cellular density, and cohesive network formation. We show that this platform is useful for investigation of preinosculative vascular communication and signaling between surrogate host and engineered tissues.

3.3 Materials & methods

3.3.1 Sacrificial alginate fiber fabrication and construct gel casting

Alginate fibers were fabricated by using a wet spinning process. Briefly, 1% w/w sodium alginate (MilliporeSigma) solution was extruded through a 30G needle by using a syringe pump at 50 $\mu\text{L/s}$ into a cross-linking bath of 100 mM calcium chloride (Acros Organics). Wet fibers were collected and dried on a mandrel (**Suppl. Fig. A.1**). Custom polydimethylsiloxane (PDMS) molds were fabricated as previously described [37]. Briefly, vector-based design templates were created in Adobe Illustrator (Adobe, Inc.) and etched into a 1/4" acrylic sheet by using a Universal Laser Systems 6.75 Laser Cutter (Universal Laser Systems, Brown Design Workshop). Sylgard 184 (Dow) was cast on the acrylic master and cured overnight, creating an autoclavable PDMS mold for use in sterile tissue culture (**Fig. 3.1A**). Dried sections of alginate fibers were embedded between the walls of autoclaved molds by using a 23G needle. A temporary PDMS gate was placed to prevent collagen from spilling into the ring area, and a 2 mg/mL type-1 collagen solution (Advanced BioMatrix) was then cast in the construct region. The plate was incubated at 37°C and 5% CO₂ for 30 min; then, the construct gel was immersed in phosphate-buffered saline (PBS; Gibco).

Channel patency was established by treating the construct gels with chelation media containing 1 mM sodium citrate (Fisher Scientific) in PBS for 10 min on an orbital shaker

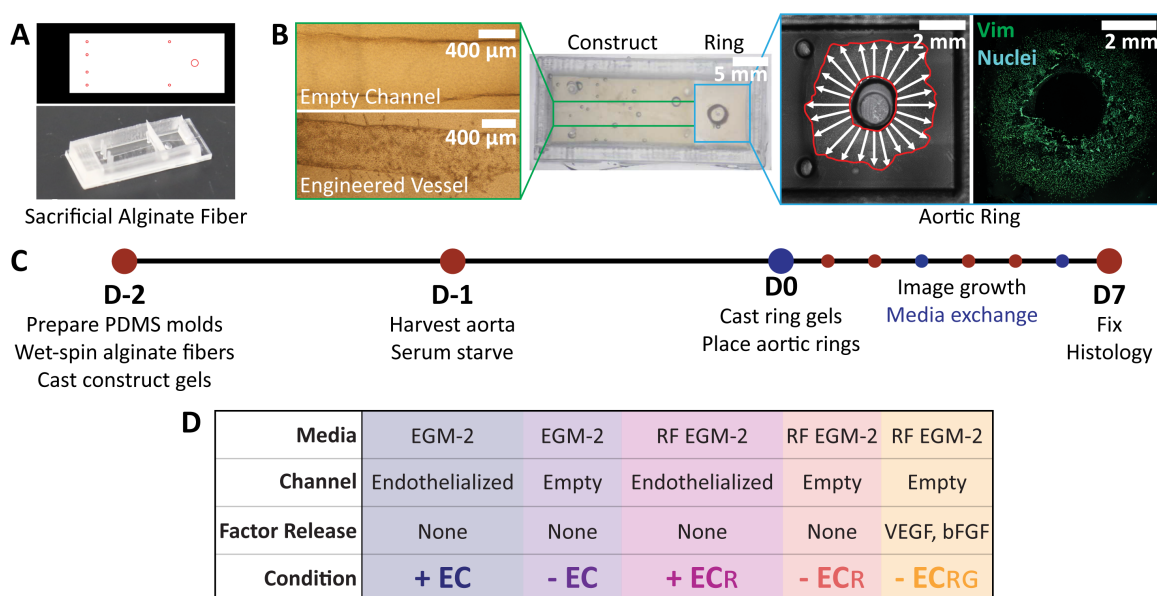


Figure 3.1: Mold and experiment design. **(A)** Adobe Illustrator design file and corresponding PDMS mold embedded with sacrificial alginate fibers to pattern **(B)** empty channels (top) and engineered vessels (bottom; 4x magnification) within construct gels and co-culture next to outgrowing aortic rings (10x magnification). **(C)** Experimental preparation and timeline indicating media exchange. **(D)** Experimental conditions tested, denoted as engineered vessels (+ EC) or empty channels (– EC) cultured in EGM-2 or RF EGM-2 media ($\pm$ EC_R), and constructs with empty channels but loaded with growth factors in RF EGM-2 (– EC_{RG}). EGM-2: Endothelial Growth Media-2; RF EGM-2: reduced factor media (complete EGM-2 without VEGF and bFGF).

to un-crosslink the calcium alginate, and patency was confirmed by perfusion of fluorescent FluoSpheres (Fisher Scientific) or PBS (**Suppl. Mov. S1, see web article for video file**). To create growth factor-loaded construct gels, 10 ng/mL VEGF and 10 ng/mL bFGF were mixed into the collagen solution before casting, and chelation media contained VEGF and bFGF as well to ameliorate early burst release before ring placement. Construct gels were typically fabricated 2 days before aortic ring placement, except for growth factor releasing constructs that were created the day of ring placement.

3.3.2 Endothelial cell culture

Human umbilical cord endothelial cells (HUVECs; Lonza) were cultured on 0.1% gelatin-coated (MilliporeSigma) 10 cm² tissue culture plates (Fisher Scientific) in endothelial growth medium 2 (EGM-2; Lonza) with media exchange every day. Cells were passaged every 4–5 days at 75% confluency by enzymatic dispersion with 0.05% trypsin (Life Technologies) in versene (0.5 mM EDTA [MilliporeSigma] and 1.1 mM d-glucose [MilliporeSigma] in PBS). Cells between passages 2 and 7 were used in experiments.

3.3.3 Engineered vessel formation

Patent channels in constructs were seeded with 3.75×10^7 cells/mL of HUVECs through the channel opening adjacent to the aortic ring and incubated upside down for 45 min and then right side up for 30 min to facilitate HUVEC attachment along the entire channel circumference. Constructs were then gently washed in PBS, changed into EGM-2 or reduced factor medium (EGM-2 without VEGF and bFGF; RF EGM-2), and gently perfused the next day to rinse out nonadherent cells.

3.3.4 Aortic ring harvest and placement

Aortas were harvested from male wild-type Sprague Dawley rats between 2 and 3 months of age and prepared by using standard protocols [38]. Briefly, rodents were anesthetized by using 4% isoflurane. The chest cavity was exposed by thoracotomy, and connective tissue was cut away to isolate the thoracic aorta. The excised aorta was cleaned of fat and connective tissue, sectioned into 1 mm tall rings, and finally serum-starved overnight in endothelial basal medium-2 (EBM-2; Lonza) + 0.1 mg/mL penicillin-streptomycin (MilliporeSigma). The next day, the medium was aspirated, a 2 mg/mL collagen gel was cast

adjacent to the construct gel in the ring area, and aortic rings were aligned on the central platform. Ring-construct setups were incubated for 30 min and then immersed in EGM-2 or RF EGM-2. All procedures were approved by the Institutional Care and Use Committee (IACUC) in Brown University (IACUC Approval No. 1702000256).

3.3.5 Imaging

Setups were imaged daily on a Nikon Eclipse Ti-E microscope (Nikon) with tiling capabilities. Histologically stained whole-mount tissues were imaged by using an Olympus FV3000 Confocal Microscope (Olympus, Brown University Leduc Bioimaging Facility). Whole-mount tissues were flattened between a glass slide and coverslip for tiling.

3.3.6 Outgrowth measurements

Outgrowth measurements were performed in ImageJ. Outgrowth area was calculated by drawing a region around outgrowing cells and subtracting it from the initial area of the ring. Outgrowth distance was measured in 15° increments around the ring, originating at the outer edge of the aortic ring for each angle. Regional measurements averaged 60° angle bins facing the construct gel (interface [IF] region) or away from the gel (remote [RM] region). Outgrowth velocity was calculated as the distance of each day's outgrowth subtracted by the previous day's outgrowth at each angle increment. A MATLAB (Math-Works) script was written to generate polar plots with error bars and extract centroid shift data.

3.3.7 Model protein release

The rate of growth factor release from collagen gels preloaded with protein was extrapolated based on bovine serum albumin (BSA) release using the bicinchoninic acid (BCA) assay. Due to the more negative charge of BSA compared with growth factors VEGF and bFGF at physiological pH and the charge interactions retaining the protein governed by negatively charged collagen, the release profile of BSA may overestimate the release kinetics. Briefly, 50 µg/mL BSA was loaded into collagen gels and cultured for 7 days in PBS +0.02% sodium azide following an identical media exchange schedule as experiments with aortic rings. Each day, 600 µL of media was removed and replaced with fresh PBS, and the aliquot was frozen. At the end of the 7 days, all media aliquots were thawed and analyzed for BSA content on a Cytation 3 Cell Imaging Multi-Mode Reader (BioTek). Values were normalized relative to cumulative release on the last day.

3.3.8 Immunohistochemistry

Setups were fixed in 4% paraformaldehyde (MilliporeSigma) for 15 min at room temperature, then washed with PBS, and finally stored at 4°C in the short term. Whole-mount histology was performed for most samples to preserve spatial information. Setups were blocked with 1% BSA (MilliporeSigma) in PBS for 2 hr followed by overnight incubation with primary antibody in fresh blocking buffer and 0.1% Triton-X (MilliporeSigma). The next day, the setups underwent two 2-hr washes in PBS, blocking for 2 hr, and finally secondary antibody treatment overnight in PBS + 1% BSA. On the third day, setups were treated with bisbenzimidazole H-33342 trihydrochloride (Hoechst; MilliporeSigma) in PBS for 15 min, three 1-hr washes, and finally stored in PBS at 4°C until they were imaged. All incubation and wash steps for whole-mount histology were performed on an orbital shaker at room temperature. Some engineered vessels were fixed, processed into frozen blocks,

and sectioned on an Avantik QS12 cryostat (Brown University Molecular Pharmacology, Physiology, and Biotechnology). Histology then proceeded as described earlier. Primary and secondary antibodies used are provided in **Table 3.1**.

Antibody/Fluorophore	Dilution	Catalog No.; Vendor
Mouse monoclonal anti-vimentin	1:200	V6630-100 UL; MilliporeSigma
Mouse monoclonal anti-collagen IV	1:100	SAB4200709-100 UL; MilliporeSigma
Rabbit polyclonal anti-human VE-cadherin	1:50	36-190-0; Fisher
Bisbenzimidide H 33342 trihydrochloride (Hoechst)	1.5 µg/mL	B2261-100MG; MilliporeSigma
Goat anti-mouse Alexa Fluor 488	1:400	A-11001; Invitrogen
Goat anti-rabbit Alexa Fluor 594	1:400	A-11005 Invitrogen
Griffonia simplicifolia isolectin B4 Dylight 594-labeled	1:400	DL-1207-.5 Vector Labs

Table 3.1: List of antibodies used for immunohistochemistry.

3.3.9 Image analyses

Histological analysis was performed in MATLAB by first defining regions of interest (ROIs) within inner and outer subdivisions of the IF and RM regions. Within ROIs, Hoechst stains were evaluated by watershed segmentation to obtain nuclear counts. Nearest neighbor analysis was performed by implementing a pixel search algorithm to find particles within 20 µm of each other. Nuclear orientation was determined by excluding uniform nuclei based on aspect ratio and normalizing each particle's major axis orientation by the angle of outgrowth from the ring. Network area measurements were taken from vimentin stains and separated into isolated cell or network masks. All MATLAB codes are hosted online and free to download and use via the Harvard Dataverse at <https://doi.org/10.7910/DVN/JE3IYD>.

3.3.10 Statistical analysis

Statistics were calculated by using one-way or two-way repeated-measures analysis of variance as appropriate, followed by the Tukey-Kramer method of post hoc analysis with p-values < 0.05 being considered statistically significant. All analysis was performed in Prism 8 (GraphPad). Sample size denoted as n refers to individual aortic rings. Each condition contains between three and eight biological replicates (aortas from distinct animals) from which between two and four aortic rings were quantified per group.

3.4 Results

3.4.1 Culture mold design enables co-culture of aortic rings with constructs containing engineered vessels

Our PDMS mold-making process as previously published for *in vitro* and *in vivo* implant fabrication [17] was adapted to enable the co-culture of rodent aortic rings next to a construct [37]. For this design, cylindrical posts were arranged in the construct area to anchor the gel in place during culture (**Fig. 3.1A**). A larger cylindrical platform centrally located in the ring region enabled precise placement of aortic rings at the same distance (2 mm) from the construct gel without contacting the ring intima. The overall mold size was optimized to accommodate imaging of vascular cell outgrowth over 7 days and fit a construct approximating the size appropriate for subcutaneous or epicardial implantation in rats (**Fig. 3.1B**).

Setups were imaged daily, fed every third day, and fixed on day 7 for immunohistochemical staining and analysis (**Fig. 3.1C**). Our experimental conditions were selected to elucidate specific contributions of endothelial cells and growth factors to vascular outgrowth,

and thus inform *in vivo* implant design choices (**Fig. 3.1D**). Construct gels contained either nonendothelialized channels ($-EC$) or engineered vessels ($+EC$) and were fed with either EGM-2 or RF EGM-2 (denoted as $\pm EC_R$). Lastly, to determine how spatiotemporal presentation of growth factors affected outgrowth response in comparison to stagnant media-solubilized presentation, we loaded 10 ng/mL VEGF and bFGF into construct gels without HUVECs and co-cultured those with aortic rings in RF EGM-2 ($-EC_{RG}$).

3.4.2 Endothelialized channels form functional engineered vasculature and exhibit sprouting

Alginate fibers were fabricated by using a wet spinning technique (**Suppl. Fig. A.1**) and utilized as sacrificial templates to pattern perfusable, arteriole-scale channels in construct gels with an average diameter of $461.5 \pm 15.6 \mu\text{m}$ (**Suppl. Mov. S1**, see web article for video file). Engineered vessels were established by seeding HUVECs into channels, which proliferated to fill the circumference as confirmed by CD31 staining (**Fig. 3.2A** and **Suppl. Fig. A.2A**). En face sections of the vascular wall showed a confluent monolayer with tight junctions marked by VE-cadherin and deposition of the basement membrane protein collagen IV, indicative of appropriate vascular structure (**Fig. 3.2B**). Engineered vessels cultured in RF EGM-2 were similarly able to form fully confluent walls with VE-cadherin expression and maintain patency, although delamination from the collagen gel wall occurred to varying degrees in 88% of engineered vessels by later time points (**Fig. 3.2C**, inset, dashed lines). In contrast, engineered vessels cultured in EGM-2 medium did not exhibit any delamination. For all experiments, HUVECs were seeded in channels 2 days before embedding aortic rings (denoted as day -2) to allow for infill of the vessel wall.

Diameter measurements over the study revealed a reduction to about 80% of initial diameter independent of culture media ($79.1\% \pm 2.37\%$ and $78.0\% \pm 2.82\%$ for $+EC$

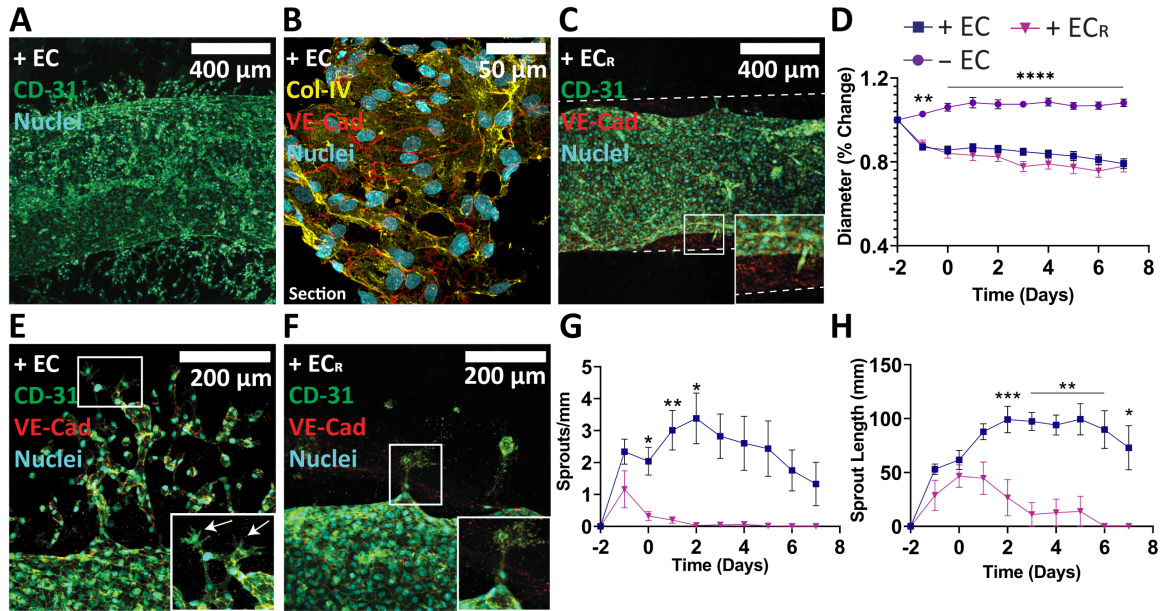


Figure 3.2: Endothelialization of empty channels. (A) Whole-mount histology of engineered vessel in complete media (10x magnification) and ((B) section view of the vessel wall (60x magnification). (C) Engineered vessel cultured in RF EGM-2 (10x magnification). Inset shows partial delamination of vessel from vessel wall (dashed lines). (D) Quantification of engineered vessel diameter. (E) Sprouting behavior in engineered vessels in EGM-2 (20x magnification). Inset shows tip-stalk phenotype of sprouts, with arrows indicating tip cells. (F) Sprout regression in RF EGM-2 (20x magnification). (G, H) Quantification of sprouting behavior from engineered vessels. + EC (n = 14), EC (n = 11), + EC_R (n = 6). *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

and + EC_R groups, respectively) due to HUVEC remodeling of the construct gel matrix (Fig. 3.2D). Nonendothelialized channels exhibited a slight increase in diameter ($108.2\% \pm 1.82\%$), likely due to sagging of the channel into an ellipsoid cross-section during the experiment.

Vascular sprouting was evident from engineered vessels in both media compositions (Fig. 3.2E-H). Engineered vessels cultured in EGM-2 demonstrated peak sprouting at 3–4 days after seeding followed by divergence into two distinct morphologies, with 57% of samples developing dense vascular plexuses with active tip-stalk sprouting throughout the experiment (Fig. 3.2A, E, inset, white arrows) and the rest exhibiting transient sprouting followed by regression and quiescence of the main vessel (Suppl. Fig. A.2B). Live/dead

staining of quiescent vessels at day 7 showed that the main vessel maintained full coverage with a few apoptotic cells, attributed to either sprout regression or death during the seeding process (**Suppl. Fig. A.2C**). In contrast, engineered vessels cultured in RF EGM-2 had significantly fewer sprouts with a peak at day -1 followed by quick regression of all sprouts (**Fig. 3.2F** inset).

Sprouting morphology varied between groups, with simple spur sprouts being the dominant morphology across + EC and + EC_R occurring either transiently or persistently in 92% and 83% of engineered vessels, respectively, during culture (**Suppl. Fig. A.2D, E**). Split sprouts (multiple spurs with a common root) occurred in 69% of + EC vessels; however, it occurred in only 17% of + EC_R vessels. Sprouts from + EC vessels additionally exhibited branches (spurs with a sprouting node above the root; 77%), loops (distinct spurs that form a linked vessel; 69%), and complex sprouts (combinations of the described morphologies including plexuses; 46%). Together, our data show that perfusable engineered vessels formed in construct gels demonstrate structural properties of native vasculature and actively sprout into the construct bulk gel in the presence of soluble pro-angiogenic growth factors.

3.4.3 Global vascular outgrowth is natively influenced by physical cues but overridden by paracrine or chemical signaling

After placement of the aortic rings adjacent to construct gels, daily phase-contrast images showed consistent outgrowth in all directions relative to the outside edge of the ring (**Fig. 3.3A**). Experiments were performed without engineered vessel perfusion to study a static, nonperfused tissue microenvironment as would occur immediately on implantation if used for translational *in vivo* studies where surgical vascular anastomosis is not used. Outgrowth measurements were taken in 15° increments around the rings each day, from

which we found significant differences between + EC and – EC groups at day 3 (**Suppl. Fig. A.3A**). Area quantification revealed improved outgrowth for the – EC_{RG} and + EC_R groups compared with the – EC group by day 5 and a significant increase in area between the + EC and – EC groups by day 6 (**Fig. A.3B**).

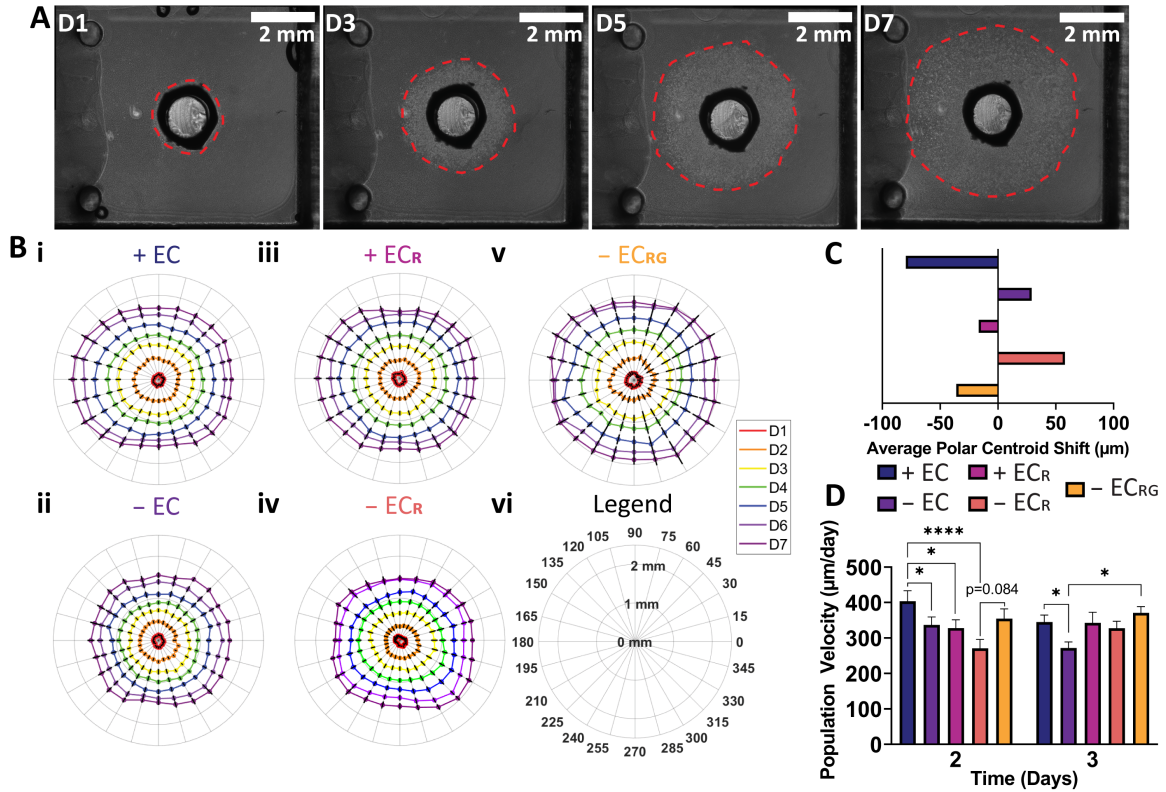


Figure 3.3: Population outgrowth from aorta-derived cells. **(A)** Aortic outgrowth progression (4x magnification) and **(B)** polar plots of vascular outgrowth for each condition (i–vi). **(C)** Average left-right centroid shift in population outgrowth for each group. **(D)** Average population velocity of aortic outgrowth at days 2 and 3. + EC (n = 17), – EC (n = 15), + EC_R (n = 11), – EC_R (n = 11), – EC_{RG} (n = 8). *: $p < 0.05$, ****: $p < 0.0001$.

By visualizing the data by using polar plots, we noted nonuniform outgrowth in aortic ring setups that appeared to be dependent on the presence of engineered vessels and growth factors solubilized in media or locally released (**Fig. 3.3B**). We quantified this bias by calculating the average horizontal shift in the centroid of outgrowth over the course of the experiment (**Fig. 3.3C**) and observed an apparent rightward shift in cellular migration (away from construct gels with nonendothelialized channels) most severe in the – EC_R

group, indicating a rightward bias in outgrowth that may be due to collagen fiber orientation during gelation, mechanical differences due to imperfect coupling of the construct and ring gels, or overall mold geometry. However, this effect was ameliorated in the – EC group and entirely reversed to a left-ward bias in migration toward engineered vessels in both media compositions as well as growth-factor loaded construct gels in RF EGM-2, showing a strong outgrowth bias in response to growth factors and engineered vessels.

In examining population velocity per day, the + EC group showed a significant increase in migration velocity at day 2 in comparison to all groups except – EC_{RG}, which itself almost reached significance relative to – EC_R ($p = 0.084$; **Fig. 3.3D** and **Suppl. Fig. A.3D**). On day 3, we found increased outgrowth velocity for both + EC and – EC_{RG} relative to – EC. At later time points, the average velocity values per day slowly decreased with no significant differences between groups. Lastly, we used the BCA assay to quantify release from collagen gels, which revealed significant burst release within the first day followed by lower, sustained release over the next 3 days (**Suppl. Fig. A.3E**). These data suggest that a combination of physical, cellular, and chemical signals bias vascular cell outgrowth distance and velocity. The culture design appears to impart a physical cue that influences migration away from the engineered construct, but this directional growth is overridden by endothelial cell paracrine signaling from engineered vessels. Further, angiogenic growth factor release from gels provides a similarly strong and sustained stimulus as compared with endothelial cell paracrine signaling, despite early burst release and in otherwise reduced factor conditions, for improving and biasing overall vascular cell outgrowth.

3.4.4 Vascular outgrowth distance is increased toward constructs by paracrine signaling from endothelial cells and growth factors

To further investigate specific biases in outgrowth direction, we binned the outgrowth measurements into 60° intervals toward or away from the construct gel, referred to as the IF and RM regions, respectively (**Fig. 3.4A**). Using this methodology, we were able to see significant increases in outgrowth distance in the IF region as early as day 2 between both + EC and – EC_{RG} relative to – EC_R that were sustained throughout the rest of the experiment. (**Fig. 3.4B, C**). At day 3, we found additional significant increases for both + EC and – EC_{RG} relative to – EC that were similarly sustained. By day 7, the + EC and – EC_{RG} groups reached an average IF outgrowth distance of $2076.32 \pm 88.04 \mu\text{m}$ and $1966.57 \pm 62.67 \mu\text{m}$, respectively, whereas the – EC and – EC_R reached IF outgrowth values of only $1607.67 \pm 83.35 \mu\text{m}$ and $1632.48 \pm 50.67 \mu\text{m}$. We were unable to find significant differences for the + EC_R group ($1935.45 \pm 102.12 \mu\text{m}$) relative to – EC ($p = 0.13$) or – EC_R ($p = 0.11$). In comparing IF and RM outgrowth at day 7, we found a significant difference only within the + EC group (**Fig. 3.4F**, + signs). Further, we examined regional velocity at early (days 1–4) and late (days 4–7) time points and found significantly increased early migration velocity in the IF region for + EC and – EC_{RG} groups relative to – EC, although neither reached significance when compared with – EC_R ($p = 0.12$ and $p = 0.20$, respectively) (**Fig. 3.4D**). In stark contrast, we did not find any significant differences between groups in the RM region in either outgrowth distance or velocity (**Fig. 3.4E-G**).

We further examined chemical effectors of outgrowth by supplementing some + EC setups with additional FBS up to 10% (+ EC_{10FBS}) and saw greater increases in population and regional outgrowth, area, and velocity in comparison to + EC, attributed to the greatest increases in the RM region (**Suppl. Fig. A.4A-E**). Setups supplemented with FBS also experienced greater compaction, which affected outgrowth measurement accuracy of some samples, and day 7 measurements were omitted from analysis due to hyper-compaction.

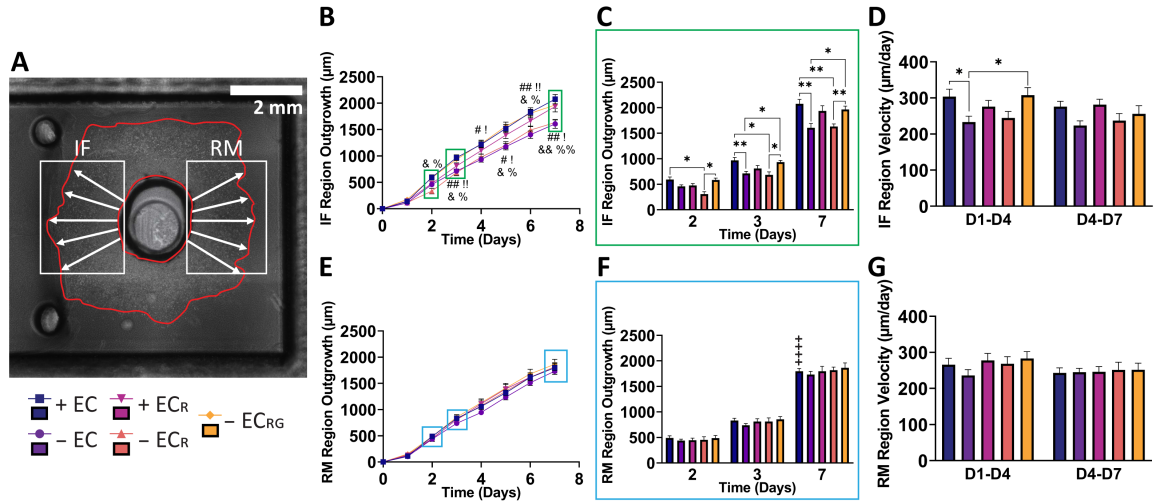


Figure 3.4: Regional outgrowth analysis. (A) Binning methodology for IF and RM regions (4x magnification). (B, E) Outgrowth over time with (C, F) differences highlighted at days 2, 3, and 7, and (D, G) regional average velocity for IF and RM regions, respectively. + EC (n = 17), - EC (n = 15), + EC_R (n = 11), - EC_R (n = 11), - EC_{RG} (n = 8). *, #, !, &, %: p < 0.05, **, ##, !!, &&, %&: p < 0.01, ***: p < 0.001, ++++: p < 0.0001. #: Significance between + EC and - EC. !: Significance between - EC_{RG} and - EC. &: Significance between + EC and - EC_R. %: Significance between - EC_R and - EC_{RG}. +: Significance between IF and RM regions for indicated group at day 7. IF: interface; RM: remote.

Together, these data show that endothelial cells and growth factors bias vascular cell outgrowth and morphogenesis. Paracrine signaling from engineered vessels in EGM-2 resulted in outgrowth matched only by growth factor release in RF EGM-2, which was sustained for the entire 7 days despite loaded factor burst release and media exchange. Engineered vessels in RF EGM-2 may provide an intermediate level of benefit in outgrowth but they suggest that implanted engineered vessels without growth factors do not provide as favorable a microenvironment as media tailored to endothelial cell growth. These cellular and chemical stimuli caused microenvironmental changes to influence outgrowth distance and velocity in the local IF region.

3.4.5 Paracrine endothelial cell signaling and growth factor release improve IF region outgrowth density and cell clustering

Imaging revealed multiple layers of outgrowth that formed over time, and we noted stark differences in morphology on comparing vascular outgrowth in the IF and RM regions between groups (**Suppl. Fig. A.5A**). The + EC and – EC groups reliably exhibited dense outgrowth relative to + EC_{RG} and – EC_R (as demonstrated by the greater white contrast, particularly adjacent to the ring), with the least density and networking apparent in the – EC_{RG} group. Growth factor release in the – EC_{RG} group appeared to ameliorate this, with average density between EGM-2 and RF EGM-2 groups. This pattern was upheld when considering network formation between neighboring cells (**Suppl. Fig. A.5A**, white arrows). We additionally observed relatively greater density and networking in the IF regions of + EC, + EC_R, and – EC_{RG} groups when compared with their respective RM regions.

We thus sought to quantify the differences in density and networking observed between treatment groups through whole-mount histology at day 7 to best preserve gross morphology and orientation. Histological analysis in MATLAB was performed by defining inner and outer subregions for both IF and RM regions from which to specify ROIs (**Fig. 3.5A**). This subdivision allowed us to capture heterogeneity in cellular density at different areas within the outgrowth regions that were otherwise not as clear in whole-region analysis (**Suppl. Fig. A.6A-C**). Nuclear density was quantified on Hoechst stains followed by nearest-neighbors analysis in a 20 μm search radius to characterize local clustering as a microenvironmental output of cell–cell interactivity relative to the macroscale-focused nuclear density metric (**Fig. 3.5B, C**). In the inner IF subregion, the + EC group had significantly higher nuclear density at 2749.65 ± 271.29 nuclei/ mm^2 relative to both RF EGM-2 groups (1507.91 ± 154.90 nuclei/ mm^2 and 1116.00 ± 184.23 nuclei/ mm^2 for + EC–R and – EC_R, respectively). The – EC (2230.39 ± 138.80 nuclei/ mm^2) and – EC_{RG} (2288.50 ± 305.71 nuclei/ mm^2) groups were significantly higher compared with – EC_R, but not rela-

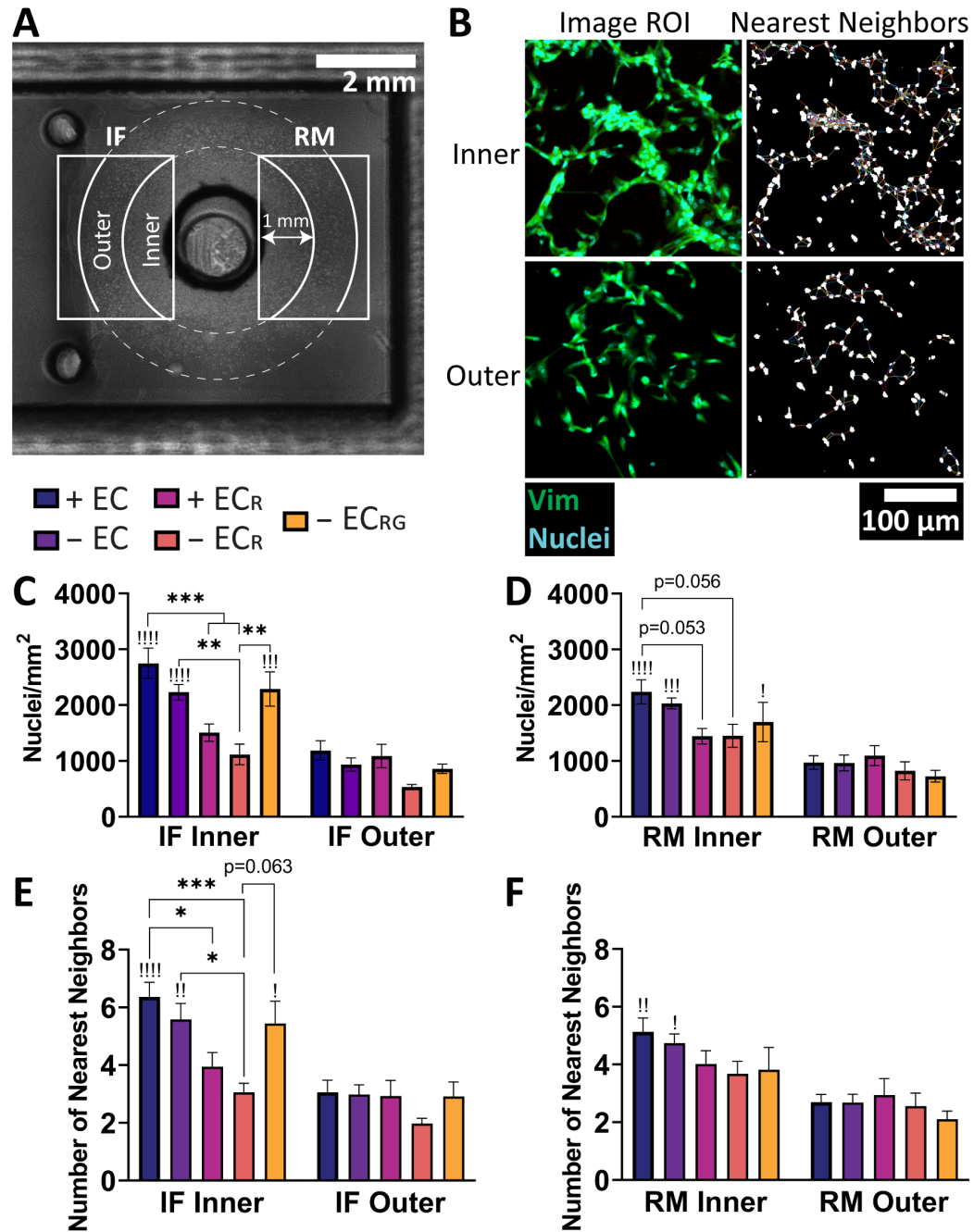


Figure 3.5: Histological evaluation of outgrowth regions. (A) Subregion specification within interface and remote regions used for quantification (4x magnification) due to (B) observed differences in density and morphology in outgrowing cells (10x magnification). Quantification of (C, D) nuclear density and (E, F) nearest neighbors within 20 μ m in the interface and remote regions. !: Significance between inner and outer subregion for that condition. + EC (n = 8), - EC (n = 8), + EC_R (n = 6), - EC_R (n = 6), - EC_{RG} (n = 5). *: p < 0.05, **: p < 0.01, ***: p < 0.001. !: Significance between inner and outer subregions within that group.

tive to + EC_R ($p = 0.164$ and $p = 0.202$, respectively). We also found significant decreases in nuclear count in the outer subregions for + EC (1186.03 ± 174.57 nuclei/mm²), – EC (935.62 ± 120.09 nuclei/mm²), and – EC_{RG} (859.52 ± 85.85 nuclei/mm²) groups when compared with their inner region counterparts (indicated with exclamation points). In contrast, we found shifts in the inner RM region that did not quite reach significance between + EC (2239.85 ± 213.96 nuclei/mm²) and both + EC–R (1444.40 ± 138.90 nuclei/mm², $p = 0.053$) and – EC_R (1451.17 ± 205.70 nuclei/mm², $p = 0.056$) groups, and significant differences in inner-outer subregion density for the + EC, – EC, and – EC_{RG} groups (**Fig. 3.5D**).

Nearest-neighbors analysis revealed greater clustering in the + EC group (6.36 ± 0.51 neighbors) relative to + EC_R (3.95 ± 0.49 neighbors) and – EC_R (3.06 ± 0.31 neighbors) groups (**Fig. 3.5E**). The – EC group (5.59 ± 0.55 neighbors) was significant only compared with – EC_R ($p = 0.306$ relative to + EC_R). – EC_{RG} (5.44 ± 0.78 neighbors) did not reach significance relative to + EC_R ($p = 0.600$) but was almost significant when compared with – EC_R ($p = 0.063$), suggesting a possible contribution of short-term factor release to clustering in depleted factor conditions. Clustering in the IF outer regions dropped significantly for + EC (3.06 ± 0.42 neighbors), – EC (2.99 ± 0.33 neighbors), and – EC_{RG} (2.92 ± 0.50 neighbors) groups, and by comparison, only decreased appreciably between the RM subregions for the + EC (5.13 ± 2.69 neighbors to 2.69 ± 0.27 neighbors) and – EC (4.74 ± 2.68 neighbors to 2.68 ± 0.29 neighbors) groups (**Fig. 3.5F**). In quantifying numbers of cells without neighbors in the 20 μ m search radius, we found significant increases in the IF region between – EC_R and both + EC and – EC, but not + EC_R ($p = 0.183$) or – EC_{RG} ($p = 0.205$) groups (**Suppl. Fig. A.6D**). We additionally examined nuclear orientation of segmented particles within the inner and outer regions of each group (**Suppl. Fig. A.6E,i-v**). Histogram plots showed a relatively flat distribution of cell angles, although the RF EGM-2 groups appeared to have greater variability in distribution at extreme angles.

These results show that engineered vessels and growth factor conditions drive differences in regional and sub-regional nuclear density and clustering. Growth factors had a dominant effect on these parameters when presented in media with engineered vessels or if factor release was controlled, whereas engineered vessels in RF EGM-2 were unable to significantly improve outgrowth. Release of growth factors improved outgrowth density and clustering in a manner comparable to groups in EGM-2 media without endothelial cells and was most pronounced in the IF region, indicating regional differences in the microenvironment closer to the construct.

3.4.6 Network morphology shows cohesive structures with locally released growth factors or endothelial cells in a growth factor-rich environment

Lastly, we sought to quantify population-level network formation in the heterocellular outgrowth as it is a vital component of preinosculative migration. Histological staining with isolectin B4 (IB4) confirmed the presence of outgrowing endothelial cells but did not quite capture all outgrowing cells (**Suppl. Fig. A.7A**). In contrast, staining for the intermediate filament protein vimentin (present in a variety of cell types including endothelial cells [39], pericytes [40], and smooth muscle cells [41, 42]) showed wide expression in outgrowing cells and was thus chosen for population-level quantification. Vimentin stains in each defined subregion were analyzed by applying threshold masks to distinguish networks and isolated cells based on size (**Fig. 3.6A**). In comparing our original ROIs and network masks, we noted differing morphologies that corresponded to either true network-like structures associated with multiple nuclei, cell–cell adhesions, and tube-like structures (**Fig. 3.6A**, white arrows), or elongated cells that did not appear to robustly interact with neighboring cells or were overlapping from a different plane (**Fig. 3.6A**, yellow arrows). These true networks were most often seen in both EGM-2 groups and comparably in the –

EC_{RG} group. The mask of isolated cells additionally captured two distinct cell morphologies consisting of cells with elongated projections characteristic of mesenchymal cells and more compact, amoeboid-like morphologies. Isolated cells appeared most frequently in the + EC_{RG} and – EC_R groups and to a relatively lesser extent in the – EC_{RG} group, although the masking algorithm erroneously labeled some clusters or overlapping cells as networks.

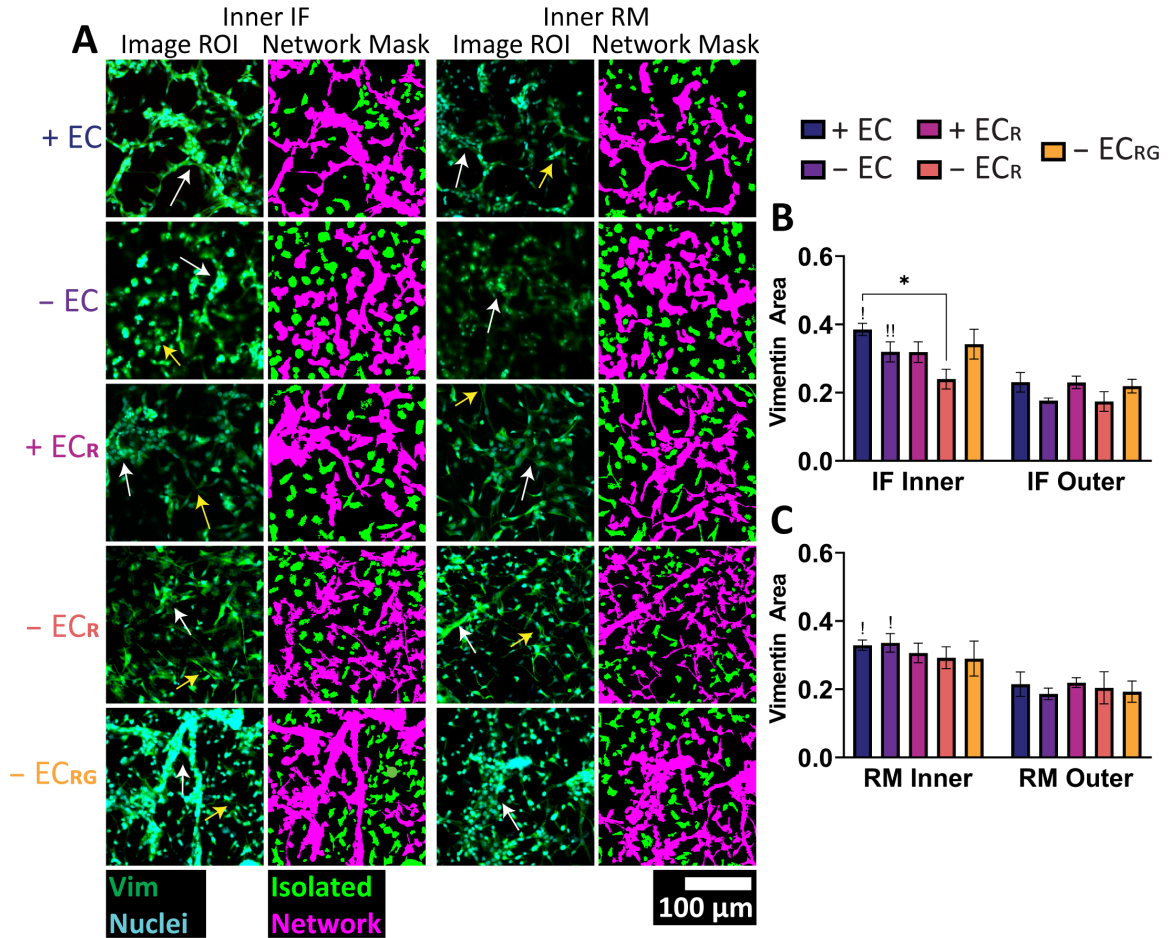


Figure 3.6: Vascular histomorphological evaluation in outgrowth regions. (A) Representative histological stains in the inner interface and remote regions for each group and corresponding isolated cell and network masks (10x magnification). White arrows indicate true networks and yellow arrows indicate overlapping cells. Cell area as quantified by vimentin stains in the (B) interface and (C) remote regions. + EC (n = 5), – EC (n = 7), + EC_R (n = 6), – EC_R (n = 6), – EC_{RG} (n = 5). *: p < 0.05, **: p < 0.01, !: Significance between inner and outer subregions within that group.

Consequently, in quantifying average vimentin stain area, we found significant increases in vimentin area in the + EC group at $38.5\% \pm 1.8\%$ compared with $24.0\% \pm 2.9\%$ for – EC_R

in the inner IF region and significantly decreased area between inner and outer subregions for + EC and – EC groups in both IF and RM regions (**Fig. 3.6B, C**). Vimentin area for the – EC_{RG} group ($34.2\% \pm 4.4\%$ in IF inner, $21.9\% \pm 2\%$ in IF outer, IF inner-outer $p = 0.095$) was not quite significant relative to – EC_R ($p = 0.21$) and not different compared with + EC ($p = 0.98$). We were also unable to find any significant differences across subregions nor in our regional analysis of single-cell and network masks (**Suppl. Fig. A.8A-D**) due to the multi-layered outgrowth observed and confirmed with high-magnification Z-stacks showing a distinct and dense primary layer of extended and networked cells, and additional upper layers of sparsely populated and increasingly compact and noninteractive cells (**Suppl. Fig. A.8E**).

Together, these results show that vascular outgrowth is multilayered and heterocellular in nature. Network formation is most significantly impacted by paracrine signaling from engineered vessels in medium containing angiogenic factors, and removal of these cues impacts robust network interactions. Growth factors alone, whether solubilized in media or presented via localized release from the hydrogel, are similarly able to initiate early endothelial cell morphogenesis and develop a comparable amount of cohesive network formation despite the lack of endothelialized channels in the latter case. This suggests the importance of timely presentation of chemical factors and paracrine signaling from engineered vessels for preinosculative network formation, stromal cell recruitment, and chemotaxis toward engineered constructs.

3.5 Discussion

Inosculation is a crucial process in establishing blood flow to implanted, engineered tissues containing prepatterned vasculature, and thus it is imperative to understand how engineered tissues influence host-derived pro-inosculative migration and cell recruitment in a

complex but controlled, heterocellular environment. In this study, we utilized our novel co-culture platform between heterocellular aortic rings and engineered constructs (**Fig. 3.1**) containing patterned, arteriole-scale engineered vessels (**Fig. 3.2**) to investigate factors beneficial for preinosculative outgrowth. Migration distance was regionally biased by engineered vessels and growth factors whether in the culture medium or locally released (**Figs. 3.3 and 3.4**), and both tissue-engineered approaches also improved cellular density and clustering (**Fig. 3.5**). Combined endothelial cell signaling and culture medium containing angiogenic factors best improved network formation relative to control, with growth factor release inducing comparable network formation (**Fig. 3.6**). Our novel *in vitro* co-culture platform demonstrates that preinosculative outgrowth can be spatiotemporally directed by appropriate and timely presentation of cellular or chemical cues to facilitate equivalently effective early vascular morphogenesis and chemotactic sprouting toward engineered vasculature.

Selection of an assay or system for evaluating angiogenesis and vascular morphogenesis is an important design choice based on the desired outcome and downstream goals. *In vitro* assays [27] such as the two-dimensional (2D) network formation assay or Boyden chamber can provide a highly simplified approach to characterizing basic angiogenic phenomena, and organ-on-chip systems can provide more sophisticated methods of fabricating tightly controlled systems to study specific mechanisms such as capillary perfusion [43] and interstitial flow [19]. However, these are not inherently translational systems designed to accommodate large engineered tissues, can be limited to shorter duration studies, and typically do not involve complex, dynamic, heterocellular environments. *In vivo* assays such as the arterio-venous (AV) loop [44] and dorsal skinfold chamber [45, 46] provide opportunities for studying vascularization at the arteriole and capillary scales, respectively, and can be relatively controlled in comparison to subcutaneous or organ-specific implantation sites due to the isolated chambers in which an implanted tissue is cultured. However, size and throughput are limiting factors for assessing preinosculative vascular dynamics, and they

do not provide the same design flexibility of the aortic ring assay. Other assays using animal derived tissues such as the chicken chorioallantoic membrane [29] or murine/leporine retina [30] assays can also provide a complex, heterocellular pro-angiogenic site with which to evaluate a therapeutic, with the latter being similar in nature to the aortic ring assay. However, the former assay utilizes an actively developing, multi-organ, and nonmammalian tissue.

Thus, we selected the aortic ring assay as an appropriately complex, mammalian, medium throughput for the goals of the study. Previous literature has utilized the aortic ring in a co-culture environment, with the inferior vena cava of rodents to study AV anastomosis [47] and co-culture with human plaques to examine atherosclerotic-induced angiogenesis [48]. To the best of our knowledge, this study is the first to examine aortic ring co-culture with vascularized engineered constructs in a translationally relevant platform to investigate preinosculation host-implant vascular growth. In our study, we focused primarily on pertinent chemical and cellular cues driven by engineered construct composition, and their effect on surrogate host vascular morphogenesis and chemotaxis using robust quantitative tools.

The diameter of engineered vessels in our constructs was informed by published literature showing an inability of patterned microvessels $< 200 \mu\text{m}$ diameter to maintain geometry and perfusion after implantation, whereas larger vessel diameters promoted greater perfusion. Redd et al. demonstrated rapid vascularization and perfusion of cardiac patches with microfabricated $150 \mu\text{m}$ diameter vessels implanted on the relatively avascular postinfarct rat heart but were unable to confirm the maintenance of original channel geometry [9], whereas Baranski et al. successfully maintained the geometry of patterned, $75 \mu\text{m}$ diameter endothelial cords for 14 days and were perfused in as little as 3 days after implantation on the parametrial fat pad of athymic mice [11]. Debbi et al. recently reported improved inosculation, perfusion, and vessel coverage of subcutaneously implanted patches with self-

assembled capillaries and 350 μm patterned vessels when compared with those with only microvasculature, but reported finding perfused vessels no larger than 50 μm at explant [28]. These findings indicate that implant location, host tissue state, and remodeling play important roles in the maintenance of patterned vessel geometries. Further, Mirabella et al. showed that patterned 400 μm vessels were better able to direct collateralization and rescue perfusion in an ischemic hind limb model compared with 200 μm channels [15], suggesting that patterned vessel diameter is a key consideration for developing vessel-patterning therapeutics and enabling oxygen and nutrient transfer to ischemic regions. Our wet spinning parameters were consequently optimized to create alginate fibers and thus channels with an initial diameter of 450 μm before compaction by resident HUVECs. The vessels were patterned in collagen gels with dimensions suitable for future *in vivo* subcutaneous or epicardial implantation studies in rodents [17], as well as appropriately sized for co-culture with aortic rings.

The engineered vessels exhibited sprouting into the construct gel in EGM-2 medium, often in localized regions and forming complex vascular plexuses. We hypothesize that nonuniform concave pockets in alginate fibers due to rapid cross-linking kinetics [49] and swelling could result in channel wall concavities, causing heterogeneous HUVEC seeding and thus plexus formation. Conversely, uniform fibers would allow homogenous seeding and correspondingly less sprouting into the bulk. Sprout regression followed to varying degrees, as was expected in vessel sprouts with no pericytes or other supportive stromal cells [50]. The contrasting engineered vessel phenotypes observed could differentially bridge a host-implant vascular gap via a bottom-up approach of angiogenic sprout inosculation or a hierarchical top-down style of inosculation with a quiescent vessel. Studies, including Redd et al. [9] and Debbi et al. [28], suggest there are benefits to this multi-scaled approach to implant vascularization, and our platform provides an adequately complex foundation from which host-implant vascular interactions and preinosculative migration can be pursued without many of the complications or considerations of *in vivo* experiments [27,

32, 51].

Two growth factors, VEGF and bFGF, are secreted by endothelial cells and vascular stromal cells and are primary drivers of angiogenic outgrowth, necessitating careful consideration of their presence (or absence) in our study. Angiogenesis is mediated by Notch signaling upstream of VEGF activation via Dll-4 and molecularly linked Wnt signaling to enable tip cell specification and consequent stalk cell specification of surrounding endothelial cells [52, 53]. This enables new sprouts to form from activated endothelial cells and undergo chemotaxis along pro-angiogenic growth factor gradients and has been demonstrated in aortic rings [32, 48]. bFGF is shown to participate in angiogenesis via multiple downstream signaling cascades, including PI3K, Ras, and Src pathways [54]. Other angiogenic signaling molecules may also initiate and modulate vascular responses, such as angiopoietins, which can induce inflammatory-mediated angiogenic remodeling via the TEK pathway to generate new sprouts and recruit mural cells for stabilization [20, 55]. These angiogenesis pathways are highly relevant during inosculation wherein separate vascular sprouts migrate toward each other and anastomose to form a cohesive network. However, it is not clear what pathways facilitate the recognition process preceding migration, or whether paracrine and autocrine regulatory mechanisms differentiate between independent sprouting and inosculation processes in endothelial cells, which are questions that further studies could address.

The vascular outgrowth we observed was modulated by engineered vessels and presentation of growth factors, suggesting that these cues heightened growth factor gradients to drive preinosculative directional migration. In contrast, the reduced factor culture medium composition (no exogenous VEGF and bFGF) may be more akin to an *in vivo* microenvironment with regards to readily available growth factors, likely compromising the proliferative, migrative, and tubulogenic capacity of outgrowing cells as well as the ability of engineered vessels to engage in paracrine signaling and appreciably affect outgrowth

[56, 57]. Nuclear analysis in our study confirmed deficits in cellular outgrowth density and clustering with RF EGM-2 medium for the + EC_R and – EC_R groups when compared with the growth factor-containing EGM-2 counterparts. Further studies could elucidate specific mechanisms through histological staining of intracellular proteins [36] and analysis of media with protein detection kits to refine our understanding of the angiogenic and preinosculative signaling between engineered vessels and the vascular outgrowth population. In contrast, nonendothelialized channels were similarly unable to stimulate a similar level of outgrowth over the 7 days. Literature showed that channels lacking endothelial monolayers do not inosculate with the host *in vivo* [15], though they may play a role in facilitating heterocellular ingrowth after migration to the construct [58] and could be pursued in future studies using our platform.

Owing to the short half-life of soluble growth factors both *in vitro* and *in vivo*, controlled release from a biomaterial is a common method of presenting multiple angiogenic stimuli simultaneously [17] or delayed [59] to mimic chemotactic gradients and direct vascularization [6]. We explored the efficacy of growth factor signaling in the absence of endothelialized vessels by loading 10 ng/mL VEGF and bFGF into construct gels cultured in RF EGM-2 and examining the vascular outgrowth response. This concentration was chosen based on our previously published work [37], and it is low or on par with reported literature on the aortic ring assay (10–50 ng/mL) [32, 48, 60] and other tissue engineering literature (10–100+ ng/mL) [6, 12, 59, 61].

Increased – EC_{RG} outgrowth as early as day 2 relative to – EC_R kept pace with + EC outgrowth up to day 7, suggesting an equivalently strong and sustained migratory stimulus despite burst release of factor from the constructs [62] and washout from medium exchange on days 3 and 6. Nuclear density and clustering with growth factor release were comparable to the + EC group, and we found no significant differences in – EC_{RG} vimentin-based network formation relative to the negative control or + EC but observed similarly cohesive

morphologies to the latter group. These data suggest that local growth factor release is effective in inducing early and somewhat sustained network-like vascular outgrowth on par with EC-optimized medium containing VEGF and bFGF. Proper combinatorial orchestration of angiogenic factors may effectively instruct chemotactic vascular outgrowth and, in the case of *in vivo* implementation, host inosculation, thus enabling blood flow throughout the bulk of the implant. In translational implementation, there are important tradeoffs to consider in pursuing either localized growth factor release or 3D endothelialized, patterned engineered vessels. Growth factors typically benefit from facile implementation and reduced regulatory burdens but are finite in reserve, whereas patterned, cellularized channels require greater biological engineering to provide an appropriate environment for survival but can persist longer to instruct host vascularization for inosculation. Practical implementation in studies of inosculation necessitate patterned vessels, so future studies could pursue combinatorial implementation of growth factor release from tissues with patterned vessels to provide synergistic benefits in revascularization.

Heterocellular interactions are essential for matrix remodeling and vessel stabilization to vascularize tissues, and histomorphological evaluation of vimentin-positive stromal cells tagged almost all outgrowing cells (verified by nuclear co-staining) and enabled population-level analysis inclusive of the heterocellular population. In addition to the prominent role of endothelial cells (commonly stained using CD31, IB4) (**Suppl. Fig. A.6**), other cells that support angiogenesis and vascular bed stabilization include pericytes (often labeled by α -SMA, NG-2), smooth muscle cells (α -SMA, SM22), fibroblasts (many possible overlapping markers), and, to a lesser extent, macrophages (CD-45) and dendritic cells (CD-54) [27, 32, 34]. These cell types are present in aortic rings and engage in complex paracrine signaling pathways between endothelial, stromal, and inflammatory cells [63] that potentially provide a more physiologically relevant setting for evaluating angiogenesis, but also additional difficulty in data interpretation [6, 34]. Fibroblasts have been shown to be beneficial in supporting the development of functional vasculature [22], even if only transiently

present [64]. VEGF and bFGF additionally act on vascular stromal cell types in a variety of ways (e.g., stimulating smooth muscle migration [65], proliferation and remodeling in fibroblasts [66], and inhibiting pericyte vessel stabilization [67]). IGF and EGF are other growth factors present in media compositions and angiogenesis *in vivo* that facilitate endothelial cell growth and tubulogenesis [36].

We observed qualitatively more cohesive network morphologies in the + EC and – EC_{RG} groups that could be due to greater network formation between endothelial cells and/or recruitment of pericytes or other stromal cells to stabilize new networks and prevent regression. Additional immunohistochemical staining could better elucidate the causes of network differences and identify specific supportive cell types recruited to aortic ring-derived neovessels. Our platform presents a unique foundation to better perturb these complex paracrine signaling interactions in angiogenesis [63], as they relate to host vascular migration toward inosculation with prepatterned vessels to advance translationally focused tissue engineering.

The versatility of our PDMS mold-based platform allows controlled examination of a broad array of parameters relevant to engineered tissue design, angiogenesis, and complex paracrine and host-implant signaling interactions with aortic rings. Perturbations may include physical cues such as perfusion, distance between and relative orientation of engineered vessels and aortic rings, channel diameter, and gel composition and cellular interactions such as doping in stromal populations into engineered constructs. Further, our platform and the provided analysis tools are adaptable to specific soft tissue engineering applications without the need for significant redesign (e.g., cardiac [37], skin [28], or pancreatic [5, 68] tissue engineering) by mixing appropriate organ-specific cell types into the collagen-casting mix and seeding in molds with a desired channel geometry, or by placing a prefabricated cellularized scaffold in the construct region.

The platform is also compatible with other prevascularization systems such as self-

assembled capillaries [8, 22] and patterned cords [10, 11], although our sacrificial templating process provides an additional layer of customization by arranging alginate fibers in different orientations or casting/3D bioprinting alginate in premade templates to create more complex 3D vascular structures [2, 28]. This provides additional benefits relative to 2D angiogenesis assays, endothelial cell monolayers seeded on 3D gels that are often utilized in microfluidic/organ-on-a-chip systems, or EC-coated beads embedded in 3D gels [27]. These systems are often highly specialized to focus study on specific mechanisms of vascular biology, such as barrier function [69] and flow dynamics [19], but can result in limited flexibility with regards to cell–cell interactions and thus signaling, macro-morphology [26], appropriate substrates and stiffnesses [24], or otherwise be unsuitable for direct comparison to an implantable therapeutic at preclinical scales [70].

The platform that we designed and used in this study has limitations due to adoption of the aortic ring assay itself. As with any animal-based study, there is an inherent heterogeneity between animals, including factors controlled for in study design (e.g., animal age, location of aortic sections, intima damage during preparation [27]). The larger volume and intermittent exchange of media necessary to accommodate larger engineered tissues in our platform can dilute paracrine signaling from both rings and engineered vessels [63, 71]. A broader translational limitation of the platform is its inability to fully predict the host inflammatory response, which plays a key role in stimulating angiogenesis and remodeling during wound healing [72]. Further, longer term (> 14 days) assessment of aortic rings is typically not possible as they begin to shift into a regressive state in which sprouts begin to recede and undergo apoptosis. Importantly, there is a lack of perfusion through the aortic rings, which *in vivo* would induce mechanotransductive and mechanochemical signaling to affect endothelial cell outgrowth [73]. However, our platform allows for perfusion of engineered vessels, providing additional versatility that may elucidate flow-driven mechanisms of pro-angiogenic vascular signaling between host and implanted tissue.

3.6 Conclusion

In summary, our novel platform uses the aortic ring assay in co-culture with engineered constructs in a complex, defined environment to investigate preinoscultative vascular cell outgrowth with greater ability to control environmental signals as compared with *in vivo* studies. Using robust, open-source quantitative tools, we report for the first time that both endothelial cell-lined vessels and local growth factor release comparably promote early chemotaxis from surrogate host heterocellular host vascular populations, which is less pronounced with nonendothelialized channels, media lacking key angiogenic growth factors, or both. Our platform provides a flexible foundation for perturbing effectors of preinoscultative heterotypic vascular outgrowth and evaluating host-instructive vascularization cues from engineered therapeutics for regenerative medicine applications.

3.7 References

1. Radisic, M. *et al.* Oxygen gradients correlate with cell density and cell viability in engineered cardiac tissue. *Biotechnology and Bioengineering* **93**, 332–343. ISSN: 00063592 (2006).
2. Vollert, I. *et al.* In vitro perfusion of engineered heart tissue through endothelialized channels. *Tissue engineering. Part A* **20**, 854–63. ISSN: 1937-335X. <http://www.ncbi.nlm.nih.gov/pubmed/24156346> (2014).
3. Radisic, M. *et al.* Medium perfusion enables engineering of compact and contractile cardiac tissue. *American Journal of Physiology-Heart and Circulatory Physiology* **286**, H507–H516. ISSN: 0363-6135. <https://www.physiology.org/doi/10.1152/ajpheart.00171.2003> (Feb. 2004).

4. Laflamme, M. A. & Murry, C. E. Regenerating the heart. *Nature Biotechnology* **23**, 845–856. ISSN: 1087-0156. <http://www.nature.com/articles/nbt1117> (July 2005).
5. Salg, G. A. *et al.* The emerging field of pancreatic tissue engineering: A systematic review and evidence map of scaffold materials and scaffolding techniques for insulin-secreting cells. *Journal of Tissue Engineering* **10**, 1–15. ISSN: 2041-7314. <http://journals.sagepub.com/doi/10.1177/2041731419884708> (Jan. 2019).
6. Kant, R. J. & Coulombe, K. L. Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues. *Acta Biomaterialia* **69**, 42–62. ISSN: 17427061. <https://doi.org/10.1016/j.actbio.2018.01.017> %20<https://linkinghub.elsevier.com/retrieve/pii/S174270611830028X> (Mar. 2018).
7. Miller, J. S. *et al.* Rapid casting of patterned vascular networks for perfusable engineered three-dimensional tissues. *Nature Materials* **11**, 768–774. ISSN: 1476-1122. <http://www.nature.com/doifinder/10.1038/nmat3357> (July 2012).
8. Schaefer, J. A., Guzman, P. A., Riemenschneider, S. B., Kamp, T. J. & Tranquillo, R. T. A cardiac patch from aligned microvessel and cardiomyocyte patches. *Journal of Tissue Engineering and Regenerative Medicine* **12**, 546–556. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.2568> (Feb. 2018).
9. Redd, M. A. *et al.* Patterned human microvascular grafts enable rapid vascularization and increase perfusion in infarcted rat hearts. *Nature Communications* **10**, 1–14. ISSN: 20411723. <http://dx.doi.org/10.1038/s41467-019-08388-7> (2019).

10. Brady, E. L. *et al.* Guided vascularization in the rat heart leads to transient vessel patterning. *APL Bioengineering* **4**, 016105. ISSN: 2473-2877. <http://aip.scitation.org/doi/10.1063/1.5122804> (Mar. 2020).
11. Baranski, J. D. *et al.* Geometric control of vascular networks to enhance engineered tissue integration and function. *Proceedings of the National Academy of Sciences* **110**, 7586–7591. ISSN: 0027-8424 (2013).
12. Wu, Q. *et al.* The effect of heparinized decellularized scaffolds on angiogenic capability. *Journal of Biomedical Materials Research - Part A* **104**, 3021–3030. ISSN: 15524965 (2016).
13. Abaci, H. E. *et al.* Human Skin Constructs with Spatially Controlled Vasculature Using Primary and iPSC-Derived Endothelial Cells. *Advanced Healthcare Materials* **5**, 1800–1807. ISSN: 21922640. <https://linkinghub.elsevier.com/retrieve/pii/S0031938416312148%20http://doi.wiley.com/10.1002/adhm.201500936> (July 2016).
14. Noor, N. *et al.* 3D Printing of Personalized Thick and Perfusable Cardiac Patches and Hearts. *Advanced Science* **6**, 1900344. ISSN: 2198-3844. <https://onlinelibrary.wiley.com/doi/abs/10.1002/advs.201900344%20https://onlinelibrary.wiley.com/doi/10.1002/advs.201900344> (June 2019).
15. Mirabella, T. *et al.* 3D-printed vascular networks direct therapeutic angiogenesis in ischaemia. *Nature Biomedical Engineering* **1**, 0083. ISSN: 2157-846X. <http://www.nature.com/articles/s41551-017-0083> (June 2017).
16. Song, M. *et al.* Regeneration of chronic myocardial infarction by injectable hydrogels containing stem cell homing factor SDF-1 and angiogenic peptide Ac-SDKP. *Biomaterials* **35**, 2436–2445. ISSN: 01429612. <http://dx.doi.org/10.>

- 1016/j.biomaterials.2013.12.011%20https://linkinghub.elsevier.com/retrieve/pii/S0142961213014804 (Mar. 2014).
17. Munarin, F., Kant, R. J., Rupert, C. E., Khoo, A. & Coulombe, K. L. Engineered human myocardium with local release of angiogenic proteins improves vascularization and cardiac function in injured rat hearts. *Biomaterials* **251**, 120033. ISSN: 01429612. <https://doi.org/10.1016/j.biomaterials.2020.120033%20https://linkinghub.elsevier.com/retrieve/pii/S0142961220302799> (Aug. 2020).
18. Morrisette-McAlmon, J. *et al.* Biomimetic Model of Contractile Cardiac Tissue with Endothelial Networks Stabilized by Adipose-Derived Stromal/Stem Cells. *Scientific Reports* **10**, 8387. ISSN: 2045-2322. <http://dx.doi.org/10.1038/s41598-020-65064-3%20https://www.nature.com/articles/srep29933> (Dec. 2020).
19. Kim, S., Chung, M., Ahn, J., Lee, S. & Jeon, N. L. Interstitial flow regulates the angiogenic response and phenotype of endothelial cells in a 3D culture model. *Lab Chip* **16**, 4189–4199. ISSN: 1473-0197. <http://xlink.rsc.org/?DOI=C6LC00910G> (2016).
20. Laschke, M. W., Vollmar, B. & Menger, M. D. Inosculation: Connecting the Life-Sustaining Pipelines. *Tissue Engineering Part B: Reviews* **15**, 455–465. ISSN: 1937-3368. <https://www.liebertpub.com/doi/10.1089/ten.teb.2009.0252> (Dec. 2009).
21. Utzinger, U., Baggett, B., Weiss, J. A., Hoying, J. B. & Edgar, L. T. Large-scale time series microscopy of neovessel growth during angiogenesis. *Angiogenesis* **18**, 219–232. ISSN: 0969-6970. <http://link.springer.com/10.1007/s10456-015-9461-x> (July 2015).

22. Chen, X. *et al.* Rapid Anastomosis of Endothelial Progenitor Cell–Derived Vessels with Host Vasculature Is Promoted by a High Density of Cotransplanted Fibroblasts. *Tissue Engineering Part A* **16**, 585–594. ISSN: 1937-3341. <http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2009.0491> (2010).
23. White, S. M. *et al.* Implanted Cell-Dense Prevascularized Tissues Develop Functional Vasculature That Supports Reoxygenation After Thrombosis. *Tissue Engineering Part A* **20**, 2316–2328. ISSN: 1937-3341. <http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2013.0311%20https://www.liebertpub.com/doi/10.1089/ten.tea.2013.0311> (Sept. 2014).
24. Wang, X. *et al.* Engineering anastomosis between living capillary networks and endothelial cell-lined microfluidic channels. *Lab on a Chip* **16**, 282–290. ISSN: 1473-0197. <https://linkinghub.elsevier.com/retrieve/pii/S0031938416312148%20http://xlink.rsc.org/?DOI=C5LC01050K> (July 2016).
25. Kim, J. J., Hou, L. & Huang, N. F. Vascularization of three-dimensional engineered tissues for regenerative medicine applications. *Acta Biomaterialia* **41**, 17–26. ISSN: 18787568. <http://dx.doi.org/10.1016/j.actbio.2016.06.001> (2016).
26. De Graaf, M. N. S. *et al.* Scalable microphysiological system to model three-dimensional blood vessels. *APL Bioengineering* **3**, 026105. ISSN: 2473-2877. <http://aip.scitation.org/doi/10.1063/1.5090986> (June 2019).
27. Nowak-Sliwinska, P. *et al.* Consensus guidelines for the use and interpretation of angiogenesis assays. *Angiogenesis* **21**, 425–532. ISSN: 0969-6970. <https://linkinghub.elsevier.com/retrieve/pii/S0031938416312148%20https://www.elsevier.com/locate/angiogenesis>

- 20<http://link.springer.com/10.1007/s10456-018-9613-x>
(Aug. 2018).
28. Debbi, L., Zohar, B., Shandalov, Y. & Levenberg, S. Integrating Engineered Macro Vessels with Self-assembled Capillaries in 3D Implantable Tissue for Promoting Vascular Integration In-vivo. *BioRxiv* (2020).
 29. Nowak-sliwinska, P., Segura, T., Iruela-Arispe, M. L. & Angeles, L. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. *Angiogenesis* **17**, 779–804. ISSN: 0969-6970. <http://link.springer.com/10.1007/s10456-014-9440-7> (Oct. 2014).
 30. Rezzola, S. *et al.* In vitro and ex vivo retina angiogenesis assays. *Angiogenesis* **17**, 429–442. ISSN: 0969-6970. <http://link.springer.com/10.1007/s10456-013-9398-x> (July 2014).
 31. Stiffey-Wilusz, J., Boice, J. A., Ronan, J., Fletcher, A. M. & Anderson, M. S. An ex vivo angiogenesis assay utilizing commercial porcine carotid artery: Modification of the rat aortic ring assay. *Angiogenesis* **4**, 3–9. ISSN: 09696970 (2001).
 32. Nicosia, R. F. The aortic ring model of angiogenesis: a quarter century of search and discovery. *Journal of Cellular and Molecular Medicine* **13**, 4113–4136. ISSN: 15821838. <http://doi.wiley.com/10.1111/j.1582-4934.2009.00891.x> (Oct. 2009).
 33. Masson, V. *et al.* Mouse aortic ring assay: A new approach of the molecular genetics of angiogenesis. *Biological Procedures Online* **4**, 24. ISSN: 1480-9222. <http://link.springer.com/10.1251/bpo30> (June 2002).
 34. Chiaverina, G. *et al.* Dynamic Interplay between Pericytes and Endothelial Cells during Sprouting Angiogenesis. *Cells* **8**, 1109. ISSN: 2073-4409. <https://www.mdpi.com/2073-4409/8/9/1109> (Sept. 2019).

35. Rawal, S. *et al.* Down-regulation of proangiogenic microRNA-126 and microRNA-132 are early modulators of diabetic cardiac microangiopathy. *Cardiovascular Research* **113**, 90–101. ISSN: 0008-6363. <https://academic.oup.com/cardiovascres/article-lookup/doi/10.1093/cvr/cvw235> (2017).
36. Kapoor, A., Chen, C. G. & Iozzo, R. V. A simplified aortic ring assay: A useful ex vivo method to assess biochemical and functional parameters of angiogenesis. *Matrix Biology Plus*, 100025. ISSN: 25900285. <https://doi.org/10.1016/j.mbps.2020.100025><https://linkinghub.elsevier.com/retrieve/pii/S2590028520300065> (Feb. 2020).
37. Munarin, F., Kaiser, N. J., Kim, T. Y., Choi, B.-R. & Coulombe, K. L. K. Laser Etched Designs for Molding Hydrogel-Based Engineered Tissues. *Tissue engineering. Part C, Methods* **23**, 311–321. ISSN: 1937-3392. <http://www.ncbi.nlm.nih.gov/pubmed/28457187><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5444510> (2017).
38. Go, R. S. & Owen, W. G. The rat aortic ring assay for in vitro study of angiogenesis. *Methods in molecular medicine* **85**, 59–64. ISSN: 1543-1894. <http://www.ncbi.nlm.nih.gov/pubmed/12710197> (2003).
39. Dave, J. M. & Bayless, K. J. Vimentin as an Integral Regulator of Cell Adhesion and Endothelial Sprouting. *Microcirculation* **21**, 333–344. ISSN: 10739688. <https://doi.wiley.com/10.1111/micc.12111> (May 2014).
40. Stapor, P. C., Sweat, R. S., Dashti, D. C., Betancourt, A. M. & Murfee, W. L. Pericyte Dynamics during Angiogenesis: New Insights from New Identities. *Journal of Vascular Research* **51**, 163–174. ISSN: 1018-1172. <https://linkinghub.elsevier.com/retrieve/pii/S0031938416312148><https://www.karger.com/Article/FullText/362276> (July 2014).

41. Gabbiani, G. *et al.* Vascular smooth muscle cells differ from other smooth muscle cells: Predominance of vimentin filaments and a specific α -type actin. *Proc Natl Acad Sci* **78**, 298–302 (1981).
42. Avolio, E., Alvino, V. V., Ghorbel, M. T. & Campagnolo, P. Perivascular cells and tissue engineering: Current applications and untapped potential. *Pharmacology & Therapeutics* **171**, 83–92. ISSN: 01637258. <http://linkinghub.elsevier.com/retrieve/pii/S016372581630211X> (2017).
43. Moya, M. L., Hsu, Y.-H., Lee, A. P., Hughes, C. C. & George, S. C. In Vitro Perfused Human Capillary Networks. *Tissue Engineering Part C: Methods* **19**, 730–737. ISSN: 1937-3384. <https://www.liebertpub.com/doi/10.1089/ten.tec.2012.0430> (Sept. 2013).
44. Mian, R. *et al.* Formation of New Tissue from an Arteriovenous Loop in the Absence of Added Extracellular Matrix. *Tissue Engineering* **6**, 595–603. ISSN: 1076-3279. <http://www.ncbi.nlm.nih.gov/pubmed/11103081> <http://www.liebertonline.com/doi/abs/10.1089/10763270050199541> (Dec. 2000).
45. Lehr, H. a., Leunig, M., Menger, M. D., Nolte, D. & Messmer, K. Dorsal skinfold chamber technique for intravital microscopy in nude mice. *The American journal of pathology* **143**, 1055–62. ISSN: 0002-9440. <http://www.ncbi.nlm.nih.gov/pubmed/7692730> <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1887078> (Oct. 1993).
46. White, S. M. *et al.* Longitudinal In Vivo Imaging to Assess Blood Flow and Oxygenation in Implantable Engineered Tissues. *Tissue Engineering Part C: Methods* **18**, 697–709. ISSN: 1937-3384. <https://www.liebertpub.com/doi/10.1089/ten.tec.2011.0744> (Sept. 2012).

47. Nicosia, R. F., Zhu, W.-H., Fogel, E., Howson, K. M. & Aplin, A. C. A New ex vivo Model to Study Venous Angiogenesis and Arterio-Venous Anastomosis Formation. *Journal of Vascular Research* **42**, 111–119. ISSN: 1018-1172. <https://www.karger.com/Article/FullText/83457> (2005).
48. Aplin, A. C. & Nicosia, R. F. The plaque-aortic ring assay: a new method to study human atherosclerosis-induced angiogenesis. *Angiogenesis* **22**, 421–431. ISSN: 0969-6970. <https://doi.org/10.1007/s10456-019-09667-z> <http://link.springer.com/10.1007/s10456-019-09667-z> (Aug. 2019).
49. Lee, K. Y. & Mooney, D. J. Alginate: Properties and biomedical applications. *Progress in Polymer Science (Oxford)* **37**, 106–126. ISSN: 00796700. <http://dx.doi.org/10.1016/j.progpolymsci.2011.06.003> (2012).
50. Watson, E. C., Grant, Z. L. & Coultas, L. Endothelial cell apoptosis in angiogenesis and vessel regression. *Cellular and Molecular Life Sciences*, 1–17. ISSN: 14209071 (2017).
51. Goodwin, A. M. *In vitro assays of angiogenesis for assessment of angiogenic and anti-angiogenic agents* 2007.
52. Tung, J. J., Tattersall, I. W. & Kitajewski, J. Tips, Stalks, Tubes: Notch-Mediated Cell Fate Determination and Mechanisms of Tubulogenesis during Angiogenesis. *Cold Spring Harbor Perspectives in Medicine* **2**, a006601. ISSN: 2157-1422. <http://perspectivesinmedicine.cshlp.org/lookup/doi/10.1101/cshperspect.a006601> (Feb. 2012).
53. Phng, L. K. *et al.* Nrarp Coordinates Endothelial Notch and Wnt Signaling to Control Vessel Density in Angiogenesis. *Developmental Cell* **16**, 70–82. ISSN: 15345807. <http://dx.doi.org/10.1016/j.devcel.2008.12.009> (2009).

54. Cross, M. J. & Claesson-Welsh, L. FGF and VEGF function in angiogenesis: Signalling pathways, biological responses and therapeutic inhibition. *Trends in Pharmacological Sciences* **22**, 201–207. ISSN: 01656147 (2001).
55. Iularo, M. *et al.* Rat aorta-derived mural precursor cell express the Tie2 receptor and respond directly to stimulation by angiopoietins. *Journal of Cell Science* **116**, 3635–3643. ISSN: 00219533 (2003).
56. Leopold, B. *et al.* Outgrowth, proliferation, viability, angiogenesis and phenotype of primary human endothelial cells in different purchasable endothelial culture media: feed wisely. *Histochemistry and Cell Biology* **152**, 377–390. ISSN: 0948-6143. <https://doi.org/10.1007/s00418-019-01815-2> <http://link.springer.com/10.1007/s00418-019-01815-2> (Nov. 2019).
57. Lafleur, M. A., Handsley, M. M., Knäuper, V., Murphy, G. & Edwards, D. R. Endothelial Tubulogenesis Within Fibrin Gels Specifically Requires the Activity of Membrane-Type-Matrix Metalloproteinases (MT-MMPs). *J Cell Sci* **1**, 3427–38 (2002).
58. Tang, F. *et al.* Microchannels are an architectural cue that promotes integration and vascularization of silk biomaterials in vivo. *ACS Biomaterials Science and Engineering* **6**, 1476–1486. ISSN: 23739878 (2020).
59. Brudno, Y., Ennett-Shepard, A. B., Chen, R. R., Aizenberg, M. & Mooney, D. J. Enhancing microvascular formation and vessel maturation through temporal control over multiple pro-angiogenic and pro-maturation factors. *Biomaterials* **34**, 9201–9209. ISSN: 01429612. <http://linkinghub.elsevier.com/retrieve/pii/S0142961213009381> (Dec. 2013).
60. Baker, M. *et al.* Use of the mouse aortic ring assay to study angiogenesis. *Nature Protocols* **7**, 89–104. ISSN: 1754-2189. [10.1038/nprot.2011.435](http://www.nature.com/articles/nprot.2011.435) <http://www.nature.com/articles/nprot.2011.435> (Jan. 2012).

61. Izadifar, M., Kelly, M. E. & Chen, X. Regulation of sequential release of growth factors using bilayer polymeric nanoparticles for cardiac tissue engineering. *Nanomedicine* **11**, 3237–3259. ISSN: 1743-5889 (2016).
62. Tabata, Y., Miyao, M., Ozeki, M. & Ikada, Y. Controlled release of vascular endothelial growth factor by use of collagen hydrogels. *Journal of Biomaterials Science, Polymer Edition* **11**, 915–930. ISSN: 0920-5063. <https://www.tandfonline.com/doi/full/10.1163/156856200744101> (Jan. 2000).
63. Nicosia, R. F., Zorzi, P., Ligresti, G., Morishita, A. & Aplin, A. C. Paracrine regulation of angiogenesis by different cell types in the aorta ring model. *International Journal of Developmental Biology* **55**, 447–453. ISSN: 02146282 (2011).
64. Song, H.-h. G. *et al.* Transient Support from Fibroblasts is Sufficient to Drive Functional Vascularization in Engineered Tissues. *Advanced Functional Materials* **2003777**, 2003777. ISSN: 1616-301X. <https://onlinelibrary.wiley.com/doi/abs/10.1002/adfm.202003777> (June 2020).
65. Grosskreutz, C. L. *et al.* Vascular Endothelial Growth Factor-Induced Migration of Vascular Smooth Muscle Cells in Vitro. *Microvascular Research* **58**, 128–136. ISSN: 00262862. <https://linkinghub.elsevier.com/retrieve/pii/S0026286299921714> (Sept. 1999).
66. Makino, T. *et al.* Basic fibroblast growth factor stimulates the proliferation of human dermal fibroblasts via the ERK1/2 and JNK pathways. *British Journal of Dermatology* **162**, 717–723. ISSN: 00070963. <http://doi.wiley.com/10.1111/j.1365-2133.2009.09581.x> (Nov. 2009).
67. Greenberg, J. I. *et al.* A role for VEGF as a negative regulator of pericyte function and vessel maturation. *Nature* **456**, 809–813. ISSN: 0028-0836. <https://linkinghub.elsevier.com/retrieve/pii/S0031938416312148%20http://www.nature.com/articles/nature07424> (Dec. 2008).

68. Kim, J. *et al.* 3D cell printing of islet-laden pancreatic tissue-derived extracellular matrix bioink constructs for enhancing pancreatic functions. *Journal of Materials Chemistry B* **7**, 1773–1781. ISSN: 2050-750X. <http://xlink.rsc.org/?DOI=C8TB02787K> (2019).
69. Polacheck, W. J. *et al.* A non-canonical Notch complex regulates adherens junctions and vascular barrier function. *Nature* **552**, 258–262. ISSN: 0028-0836. <http://www.nature.com/articles/nature24998> (Dec. 2017).
70. Shin, Y. *et al.* In vitro 3D collective sprouting angiogenesis under orchestrated ANG-1 and VEGF gradients. *Lab on a Chip* **11**, 2175. ISSN: 1473-0197. <http://xlink.rsc.org/?DOI=c11c20039a> (2011).
71. Nicosia, R. F., Lin, Y. J., Hazelton, D. & Qian, X. H. Endogenous regulation of angiogenesis in the rat aorta model: Role of vascular endothelial growth factor. *American Journal of Pathology* **151**, 1379–1386. ISSN: 00029440 (1997).
72. Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H. & Tomic-Canic, M. Growth factors and cytokines in wound healing. *Wound Repair and Regeneration* **16**, 585–601. ISSN: 10671927. <http://doi.wiley.com/10.1111/j.1524-475X.2008.00410.x> (Sept. 2008).
73. Lamalice, L., Le Boeuf, F. & Huot, J. Endothelial Cell Migration During Angiogenesis. *Circulation Research* **100**, 782–794. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/01.RES.0000259593.07661.1e> (Mar. 2007).

Chapter 4

Patterned arteriole-scale vessels enhance engraftment, perfusion, and maturation of engineered human myocardium for heart regeneration

4.1 Abstract

Despite therapeutic advances in cardiovascular health, there are patients who, after surviving a myocardial infarction (MI), are refractory to treatment options and progress into chronic heart failure (HF). Current research in heart regeneration using stem cell-derived cardiomyocytes (CMs) is accelerating as seen in large animal and human clinical trials; however, delivery of CMs by intramuscular injection or epicardially-implanted engineered cardiac tissues often fail to consider the need for a robust oxygen supply to ensure the engraftment and survival of cells in the harsh environment of the infarct. To this end, we have developed a remuscularization-revascularization therapy to regenerate the heart post-

MI that is evaluated in a rodent model of ischemia-reperfusion myocardial infarction. Our work details the differentiation, expansion, and maturation of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) using a multi-stage biphasic Wnt modulation protocol. Vascularized engineered human myocardial tissues (vEHMs) are then formed by mixing hiPSC-CMs and 5% human primary cardiac fibroblasts into a collagen-1 hydrogel. Sacrificial gelatin-alginate fibers coated with endothelial cells are embedded in cardiac tissues to pattern patent, arteriole-scale channels 400 μm in diameter. vEHMs are cultured under dynamic perfusion conditions prior to implantation for two weeks to study inosculature, revascularization, and remuscularization of the heart. *Ex vivo* optical coherence tomography reveals less tortuous vascular organization in perfused vEHMs relative to non-perfused vEHMs, and microCT-based 3D vascular reconstructions show improved presence of large vessels ($>50 \mu\text{m}$) in vEHMs perfused *in vitro*. Immunohistochemical staining shows improved engraftment of hiPSC-CMs in the presence of patterned, perfused vasculature and inosculature with host vasculature. RNA sequencing further reveals upregulation of genes related to cardiac contractility and electrophysiology in perfused vEHMs relative to control. Our remuscularization-revascularization therapy, featuring innovative approaches to study host-implant vascular inosculature and vascular remodeling, provides building blocks to enhance vEHM engraftment, perfusion, and thus maturation for future applications as a regenerative therapy.

4.2 Introduction

Myocardial infarction (MI) causes an interruption of vascular flow to downstream muscle, resulting in ischemia and necrosis of the tissue. Approaches in regenerative medicine to implant human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) seek to repopulate the infarct zone with functional tissue that can reinforce the scar-laden ventricle, electrically couple with host myocardium, augment contractile function, and ultimately

prevent further muscle deterioration and end-stage heart failure [1]. However, a robust vascular supply is needed to ensure proper engraftment and survival of implanted cardiomyocytes, particularly in the case of epicardial patch-based approaches where highly dense tissues are initially sequestered from the host vasculature [2]. Therefore, there is a great need to develop strategies to encourage rapid perfusion of implanted engineered cardiac tissues after implantation.

Strategies to prevascularize cardiac tissues *in vitro* aim to provide conduits for rapid perfusion after implantation [3]. As opposed to *in situ* methods which necessitate host vasculature to grow into and perfuse an implant [2, 4], *in vitro* prevascularization provides a high degree of control over engineered cardiac tissue design, intentional vascular development [3], and may function to improve *in vivo* engraftment of muscle [5]. Common methods include co-culture of vascular units (e.g., endothelial cells [6] or isolated microvessels [7] to establish homogeneously distributed microvasculature, or patterning vessels across larger scales for top-down vascular integration by way of microfabrication techniques such as 3D printing [8–10] and pattern templating [11–13]. This host-driven vascular integration is termed inosculation, or the natural connection and perfusion of disparate vascular beds *in vivo* [14].

Evaluating the effectiveness of such vascularization techniques has historically relied on the gold standard of static 2D histological analysis, but newer methodologies show promise in providing greater insight into vascular remodeling in the infarcted heart [3]. Techniques including microtomography (microCT)-assisted reconstructions [15, 16], Optical Coherence Tomography (OCT) imaging [11, 12, 17], and fluorescence imaging [18, 19] can enable vascular visualization down to the capillary scale and flow mapping. Continued development of such techniques are invaluable in determining appropriate designs for prevascularizing engineered cardiac tissues *in vitro*.

Another key point of concern of hiPSC-based cardiac therapies is the relative immatu-

rity of the CMs directly after differentiation [20–22]. Many groups seek to use mechanical [23, 24], electrical [25], or media-based/molecular [26–28] methods to mature the fetal-like contractile apparatus, gene expression, electrophysiology, and metabolism of differentiated hiPSC-CMs. Recent work suggests that cardiomyocytes can also mature *in vivo* [29–31] hypothesized to be induced by paracrine signaling from adjacent healthy myocardium. Examination of the relationship between cardiomyocyte maturation and engraftment is ongoing, with studies suggesting that 20–40 days of *in vitro* maturation is beneficial for improving engraftment, though still morphologically immature compared to true adult CMs [22, 29].

To date, no group has examined the effect of large-scale vascular patterning and perfusion on cardiomyocyte engraftment and maturation *in vivo*. We hypothesized that establishment of engineered cardiac tissues consisting of robustly perfused implanted hiPSC-CM tissue by way of prevascularization using arteriole-scale vessels would facilitate greater patch engraftment and functional maturation. Here, we report the formation of engineered human myocardium (EHM) containing perfused arteriole-scale vessels and demonstrate their robust engraft onto infarcted myocardium, improved perfusion, and maturation of engrafted hiPSC-CMs.

4.3 Materials & methods

4.3.1 hiPSC maintenance

The WTC-11 GCaMP hiPSC line used in all experiments was gifted from Dr. Bruce Conklin (Gladstone Institute, UCSF). WTC-11s were maintained on 10 cm² dishes coated with 5 µg/mL vitronectin (Thermo Fisher) in a cell culture incubator (37 °C, 5% CO₂). WTC-11s were maintained in Essential 8 (E8) medium (Gibco) and passaged every 4–

5 days at 80% confluency with versene (0.5 M EDTA (Fisher) and 1.1 mM D-glucose (MilliporeSigma) in DPBS without calcium and magnesium (Gibco)) onto new vitronectin-coated plates.

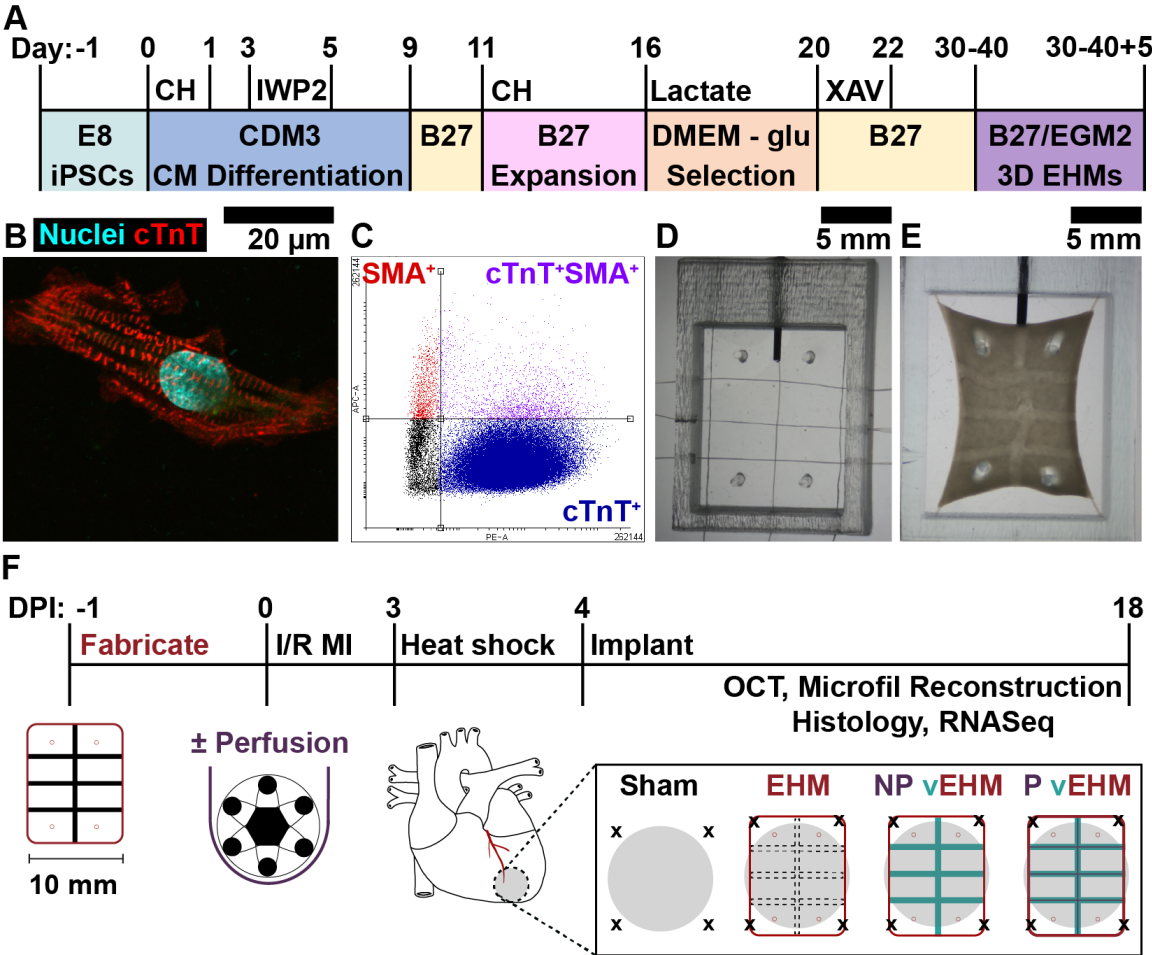


Figure 4.1: Overview of hiPSC-CM differentiation, vEHM formation, and *in vivo* implantation. (A) Differentiation timeline of hiPSCs into (B) ventricular CMs using multi-stage biphasic Wnt modulation. (C) Differentiation purity is evaluated using flow cytometry staining for cardiac troponin T (cTnT). (D, E) PDMS-based replica molding system for embedding biomaterials and forming vEHMs. (F) Overview of timeline for *in vivo* implantation of EHMs in a rodent model of ischemia-reperfusion myocardial infarction.

4.3.2 hiPSC-CM differentiation, expansion, and lactate selection

Directed differentiation of hiPSCs into cardiomyocytes (Fig. 4.1A). was achieved via multi-stage biphasic modulation of the Wnt signaling pathway in defined conditions [32–

35]. Briefly, hiPSCs were replated on Matrigel-coated (Corning) 24 well plates in E8 with 10 μ M Y-27632 (Rock Inhibitor, RI; Tocris) at a density of 40,000-70,000 cells/cm². The next day (D0), hiPSCs were treated with 4-5 μ M CHIR 99021 (Chiron; Tocris) in CDM3 media [33] for 24 hours. On day 3 (72 hours after Chiron treatment) cells were treated with 5 μ M IWP2 (Tocris) for 2 days, then fed CDM3 every other day until beating commenced (day 8-12).

On day 9, hiPSC-CMs were fed RPMI 1640 with B27 supplement (RPMI B27; Gibco) for two days, then on day 11 harvested with TrypLE Select 10x (Gibco) and re-plated on Matrigel-coated 15 cm² plates at 25,000 cells/cm² in RPMI B27 containing 2 μ M Chiron and 10 μ M RI to encourage cardiomyocyte expansion [35]. hiPSC-CMs were fed fresh RPMI B27 with 2 μ M Chiron every 2 days until 90% confluent (4-6 days). Cells were then washed with DPBS and fed DMEM – glucose (Gibco) containing 4 mM sodium lactate (MilliporeSigma) every other day for 4 days to remove non-cardiomyocytes from culture [26], followed by two days of treatment with 4 μ M XAV939 (Tocris) in RPMI B27 to encourage cell cycle exit of hiPSC-CMs [29]. Expanded, purified hiPSC-CMs (**Fig. 4.1B**) were then maintained in RPMI B27 containing 100 μ g/mL penicillin-streptomycin (pen-strep; MilliporeSigma) and used between days 30-40 of differentiation for all downstream experiments. The average purity of hiPSC-CMs used for *in vitro* and *in vivo* experiments was 94.0% and 85.05%, respectively, by flow cytometry staining for cardiac troponin T (cTnT; **Fig. 4.1C**).

4.3.3 Cardiac fibroblast maintenance

Human primary cardiac fibroblasts (hCFs; MilliporeSigma) were maintained on 15 cm² culture dishes in hCF media consisting of DMEM\F-12, 10% fetal bovine serum (FBS; Gibco), 4 ng/mL bFGF (Stemgent), and 100 μ g/mL pen-strep. hCFs were passaged every 4-5 days at 80% confluency using 0.05% trypsin (Gibco) in versene and frozen back in hCF

media with 10% dimethylsiloxane (DMSO; Fisher). All experiments used hCFs directly from thaw between passages 3-6.

4.3.4 Endothelial cell maintenance

Human umbilical vein endothelial cells (ECs; Lonza) were maintained on 0.1% gelatin-coated (MilliporeSigma) 15 cm² culture dishes in EGM-2 Media (Lonza). HUVECs were passaged every 4-5 days at 80% confluency using 0.05% trypsin diluted in versene, and frozen back in EGM-2 with 10% DMSO. All experiments used HUVECs between passages 3-7.

4.3.5 Cellular viability, proliferation, and morphological assays

EC and hiPSC-CM viability was assessed by plating cells on 0.1% gelatin-coated or MG-coated 6 well plates, respectively, and feeding with either control media (EGM-2 for ECs; 0/100, or RPMI B27 for hiPSC-CMs; 100/0), or mixed media consisting of either 70% RPMI B27 and 30% EGM-2 (70/30) or 50% RPMI B27 and 50% EGM-2 (50/50). 100 µg/mL pen-strep was additionally added to all medias. Daily images were taken to assess the number of live and dead cells in 5 separate microscope fields per condition. The morphology and structure of hiPSC-CMs was assessed with immunohistochemical staining.

4.3.6 Mechanical characterization of EHMs

Culture molds were fabricated using a replica molding technique [36, 37]. Briefly, vector-based designs were created in Adobe Illustrator (Adobe Inc., San Jose, CA) with a 9 mm x 3 mm culture area, and laser-cut into a 1/4-inch acrylic sheet using a Universal Laser

Systems 6.75 Laser Cutter (ULS) at the Brown Design Workshop (Brown University). Sylgard 184 (Dow) was cast into the acrylic templates and cured overnight at 60 °C. The cured PDMS molds were then removed from the acrylic negative and sterilized in an autoclave at 121 °C for 30 minutes. hiPSC-CMs were harvested between 30-40 days of differentiation and resuspended at a density of 1.5×10^7 cells/mL and mixed with 5% hCFs (from thaw) by number and rat tail-derived collagen-1 (final concentration 1 mg/mL; Advanced BioMatrix). The suspension was cast into each trough of the PDMS mold and allowed to set for 30 minutes at 37 °C before feeding with either 100/0, 70/30, or 50/50 media. EHMs were cultured for 1 week and paced at 1 Hz using a C-Pace EM Stimulator (IonOptix, Westwood, MA). Mechanical testing was performed as previously described [38]. Briefly, EHMs were mounted between a 5 mN load cell (Aurora Scientific) and a high-speed length controller in a temperature-controlled bath of Tyrodes solution. Tissues were stretched in 5% length step increments to 130% of L_0 and resulting force generation at each step was measured under a 1 Hz field stimulus. Tissues were then electrically paced from 1 Hz to 4 Hz in 0.5 Hz increments to determine maximum capture rate before returning length to L_0 . Tissues were then immediately fixed for downstream immunohistochemistry. Passive stiffness and active force kinetics were measured using a custom MATLAB (Mathworks Inc.) code.

4.3.7 Gelatin-alginate fiber wetspinning

Wetspinning of sacrificial fibers was adapted from a previous publication [39]. Briefly, a 1% w/w sodium alginate (Millipore Sigma) was prepared in DI water and loaded into a 5 mL syringe fitted with a 30G hypodermic needle. Crosslinking solution was separately made by dissolving 100 mM calcium chloride (Acros) and 2.5% w/w gelatin (Millipore Sigma) in DI water. Alginate was extruded into the crosslinking bath via a syringe pump at a rate of 50 μ L/sec, and the resulting cross-linked gelatin-alginate (GA) alginate fibers were collected on a mandrel. Wetspun fibers were sprayed briefly with 70% ethanol to disinfect

and stored in a biosafety cabinet. GA fiber diameter was measured using ImageJ. Node measurements were determined by counting the number of nodes that appeared normalized over the length of the coated fiber.

4.3.8 Collagen microfiber wetspinning

Collagen microfiber wetspinning was adapted as previously described [40] to create bundled collagen microfibers. Briefly, 13 mg/mL collagen-1 isolated from rat tails was extruded using a syringe pump through a spinneret and ensheathed in a coaxial flow of a polyethylene glycol-based neutralization buffer to form collagen microfibers. The formed microfibers were then washed in a bath of 70% ethanol and collected on a mandrel to dry. Microfiber bundles were formed by wrapping collagen microfibers around the same location on the mandrel 4 times and briefly rewetting the fibers with 70% ethanol to form robust bundles. Microfiber bundle diameter was measured using ImageJ.

4.3.9 EHM fabrication for *in vivo* studies

PDMS molds were fabricated to create larger 10 mm x 12 mm EHM for implantation studies and prepared as described above. In the biosafety cabinet, GA fibers and collagen microfibers were aseptically embedded in autoclaved PDMS molds [39] using a 25G needle to form an array pattern of GA fibers through the center of the tissue with a set of collagen microfibers along the longitudinal edges of the mold (**Fig. 4.1D**). The molds were then adhered to the bottom of untreated 6 well plates with silicone adhesive (Dow). A casting mix of hiPSC-CMs (1.5×10^7 cells/mL), 5% hCFs and collagen-1 was prepared as described above with volumes scaled to accommodate the larger culture molds and kept on ice. Concomitantly, HUVECs were thawed, resuspended at a density of 2.5×10^7 cells/mL in a coating solution of EGM-2, 1% w/w gelatin, 0.25% w/w alginate, and 1 mg/mL collagen-1, and

carefully pipetted onto the GA fibers to coat the surface. The casting mix of hiPSC-CMs, hCFs, and collagen-1 was then seeded into the PDMS mold around the fibers and allowed to gel for 30 minutes at 37 °C to form vascularized EHMS (vEHMS; **Fig. 4.1E**). vEHMS were maintained in 50/50 media and paced at 1 Hz under field stimulation. Control EHMS were fabricated following the same procedure except the coating solution was devoid of HUVECs.

4.3.10 GA fiber un-crosslinking and perfusion culture

After tissue formation overnight, GA fibers embedded in EHMs were un-crosslinked by a 3 hour incubation in un-crosslinking media, consisting of calcium-free DMEM (Gibco), 2 mM GlutaMAX (Gibco), 1.5 mM sodium citrate (Fisher), and 100 µg/mL pen-strep. Un-crosslinking media was manually perfused through the channels to wash out any leftover GA and confirm patency, followed by a media change back into EHM media.

Dynamic perfusion culture of patterned vessels in vEHMs was achieved with the use of a custom-built Arduino-controlled peristaltic pump, enabling independent recirculating perfusion of up to 3 tissues in vitro at once with. EHM media was directed through a media reservoir prior to infusion to prevent embolisms and dampen the flow profile to an average of 7.28 mL/hr. Media was partially exchanged every other day by replacing media buildup in the well of the plate. Control and non-perfused tissues were not connected to perfusion devices and fed every other day with EHM media. Design files are free to access and download at the Harvard Dataverse at <https://doi.org/10.7910/DVN/KARO3F>. vEHM compaction and channel diameter were evaluated using ImageJ.

4.3.11 Implantation of EHMs on infarcted hearts and explant

All animal procedures were approved by the Institutional Care and Use Committee (IACUC) at Brown University (20-02-0002). Nude athymic rats (8 weeks, male, 200 g) underwent ischemia-reperfusion MI (**Fig. 4.1F**) using protocols published previously [2, 36]. Briefly, rats were anesthetized with 2-3% isoflurane, then induced with 100 mg/kg ketamine and 90 mg/kg xylazine and placed on a ventilator. A thoracotomy was performed to expose the heart and the left anterior descending artery was ligated with 4-0 polypropylene suture for 60 minutes to induce ischemic MI, followed by reperfusion by removal of the suture and closure of the chest.

Three days after MI, EHMs were prepared for implantation by performing a heat shock protocol [41], consisting of incubation at 42 °C for 1 hour. Animals were additionally given daily subcutaneous injections of 30 mg/kg cyclosporine-A to mitigate mitochondrial permeability transition pore opening starting the day before implantation. The next morning (four days after MI), EHMs were treated with 100 ng/mL IGF-1 (RD Systems) and 200 nM cyclosporine-A (MilliporeSigma) one hour prior to the implant procedure. Animals underwent a second thoracotomy to implant EHMs over the infarcted region of the left ventricle. Sham animals received sutures but no implants. Echocardiography measurements were performed under mild sedation using a Vivid 7 Dimension Ultrasound System (GE) with a pediatric 10 S 4-10 MHz transducer. Longitudinal and short-axis views of the left ventricle at the mid-papillary level were taken to assess cardiac function before MI (baseline), 3-4 days after MI, and before sacrifice at 14 days. Echocardiography data was additionally used as exclusion criteria (fractional shortening (FS) < 45%) to ensure consistent MIs with reduced FS.

At 14 days, hearts were prepared for vascular morphological and reconstructive analysis [2, 15]. Briefly, rats were injected with 750 USP/mL Heparin (Meitheal Pharmaceuticals) via tail vein injection, then underwent a thoracotomy to expose the heart and aorta. The

superior vena cava and innominate, left common carotid, and left subclavian branches of the aortic arch were ligated, then the abdominal aorta was cannulated with an 18G catheter to provide antegrade perfusion access to the cardiac microcirculation via the aortic root. The cannula was connected to a perfusion system and the inferior vena cava cut to provide an outlet. Perfusion buffer containing 1 g/L adenosine (Millipore Sigma) in DPBS was perfused for 3 minutes to clear blood then briefly perfused with 150 mM potassium chloride (Fisher) to arrest the heart in diastole. After another 2 minutes of perfusion buffer, 4% paraformaldehyde (PF; Millipore Sigma) was perfused for 10 minutes to fix the vasculature, followed by explant of the heart, lungs, and aortic arch which were fixed overnight in 4% PF.

4.3.12 Optical coherence tomography

In preparation for *ex vivo* imaging, hearts were re-cannulated at the aortic arch using a male luer adapter prior to the innominate branch, connected to a perfusion system, and perfused with 20% Intralipid (Fresenius Kabi) solution at a pressure of 50-60 mmHg for spectral domain optical coherence tomography imaging (OCT; Thorlabs). OCT acquisition utilized a large-bandwidth near-infrared (NIR) light source with a 2048-pixel line-scan camera to achieve 147,000 A-scans/s focused on areas approximately 100-200 μm below the surface of the heart or implant. A 5x objective (3 x 3 mm FOV) or 10x objective (1.5 x 1.5 mm FOV) was used for image acquisition. For each image acquisition, two-four B-scans were taken at each y position with 20 volumetric scans each. A custom contrast-mask averaging process was utilized to process raw imaging data and remove visual artifacts due to movement. Tortuosity measurements were performed by measuring the length of a vessel initiating and terminating at branch points and normalizing by to the shortest distance between the start and end.

4.3.13 Vascular reconstructive analysis and immunohistochemistry

After OCT imaging, hearts were cleared of Intralipid then manually perfused with Microfil (Flow Tech Inc.) and scanned with a microCT machine (Providence VA Medical Center) at a voxel size of 50 μm . DICOM files were post-processed using SimVascular to segment out cavities and custom MATLAB code [42] for quantitative analysis and visualization. After scanning, hearts were processed into 2 mm slices, processed into paraffin or frozen blocks, and sectioned into 14 μm slices for immunohistochemical analysis. Infarct size was visualized and quantified using picrosirius red – fast green stains of heart sections at mid-papillary level. Sections were treated with primary antibodies overnight at 4 °C followed by secondary antibodies and nuclear counterstain for 1 hour at room temperature. Cover slips were mounted using ProLong AntiFade Glass Mountant (Invitrogen). Histological stains were imaged using an Olympus FV3000 Confocal Microscope or Olympus FV200 Slide Scanner (Olympus; Leduc BioImaging Facility, Brown University), and processed in ImageJ. Primary and secondary antibodies used are provided in **Table 4.1**. Human cell density and viable implant size were measured in ImageJ from histological stains of cTnT and Hu-Ku80 and normalized by quantified area. Engraftment was determined by normalizing density measurements to implant size. MLC2 measurements were determined in ImageJ by thresholding histological stains of MLC2a and MLC2v and quantifying area. Sarcomere length was determined in histological stains of cTnT by measuring the length of between 3-10 sarcomeres in a straight line and dividing by the number of encompassed sarcomeres. Vessel density and diameter were quantified in histological stains of isolectin B4 and Hu-Ku80 in the remote (healthy), infarct, and implant regions of each heart, and normalized by imaged area.

Antibody/Fluorophore	Dilution	Catalog No.; Vendor
Mouse monoclonal anti- α sarcomeric actinin	1:500	A7811-.2ML UL; MilliporeSigma
Mouse monoclonal anti-cTnT	1:100	MA5-12960 UL; Invitrogen
Rabbit monoclonal anti-smooth muscle actin	1:150	ab150301; Abcam
Mouse monoclonal anti-human CD31	1:100	M082301-2; Agilent
Rabbit polyclonal anti-MLC2v	1:100	10906-1-AP; ProteinTech
Mouse monoclonal anti-MLC2a	1:100	311 011; Synaptic Systems
Rabbit polyclonal anti-cTnI	1:150	ab47003; Abcam
Rabbit polyclonal anti-Cx43	1:200	C6219-100UL; MilliporeSigma
Rabbit monoclonal anti-human Ku80	1:150	2180S; Cell Signaling Technologies
Bisbenzimidazole H 33342 trihydrochloride (Hoechst)	1.5 μ g/mL	B2261-100MG; MilliporeSigma
Goat anti-mouse/rabbit Alexa Fluor 488	1:300	A-1100; A-11008; Invitrogen
Goat anti-rabbit/mouse Alexa Fluor 594	1:300	A11012; A-11005; Invitrogen
Griffonia simplicifolia isolectin B4 Dylight 594-labeled	1:200	DL-1207-.5; Vector Labs
Mouse anti-human Ki67 FITC-labeled	1:50	612472; BD Biosciences

Table 4.1: Antibodies for immunohistochemical staining.

4.3.14 Bulk RNA sequencing

A subset of implants were explanted at the 14 day timepoint for immediate RNA isolation using Tri Reagent solution (Invitrogen) followed by on-column processing and washing using a Reliaprep RNA Miniprep System (Promega). Yield and purity were analyzed on a Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher) prior to shipment for next generation sequencing (Azenta Life Sciences). RIN score of all samples was verified

to be above 7.5 before continuing with library preparation and RNA sequencing (RNASeq).

The open-source, web-based platform, Galaxy [43], was used to perform standard quality control analysis, trimming, alignment of the RNAseq raw data to appropriate reference genomes, and generation of count matrices. Sequence quality was assessed through FASTQC and trimming of the Illumina adaptor sequences was performed with Trimmomatic [44]. Sequences were mapped to the human (hg38) and rat (rn6) genome using HISAT2, with alignment scores calculated from percentage of aligned reads. Count matrices of gene counts per samples were generated using featureCounts, which was used as the input to a custom Bioconductor-based R script to filter, assess DE gene expression, and perform gene ontology (GO) enrichment. Within the R script, initial gene filtering was achieved using the function `koverA` in the `genefilter` package. Filtered genes were used as the input to the package `DESeq2` to perform size-based normalization and DGE analysis on the data set. Principal components analysis (PCA), unsupervised clustering, dispersion modeling and heat mapping of gene expression differences among samples were performed with the complete filtered gene list as well as the differentially expressed genes (DEGs). Further, gene ontology (GO) analysis was performed on sets of upregulated or downregulated genes. A false discovery rate (FDR) of < 0.05 was considered statistically significant for both the DGE and GO analysis.

4.3.15 Data and statistical analysis

RNASeq data was analyzed and graphed in RStudio, and all other data was analyzed and graphed in Prism 8 (GraphPad). Statistics were calculated using one- or two-way ANOVA as appropriate with Tukey-Kramer post-hoc analysis and p-values < 0.05 considered statistically significant.

4.4 Results

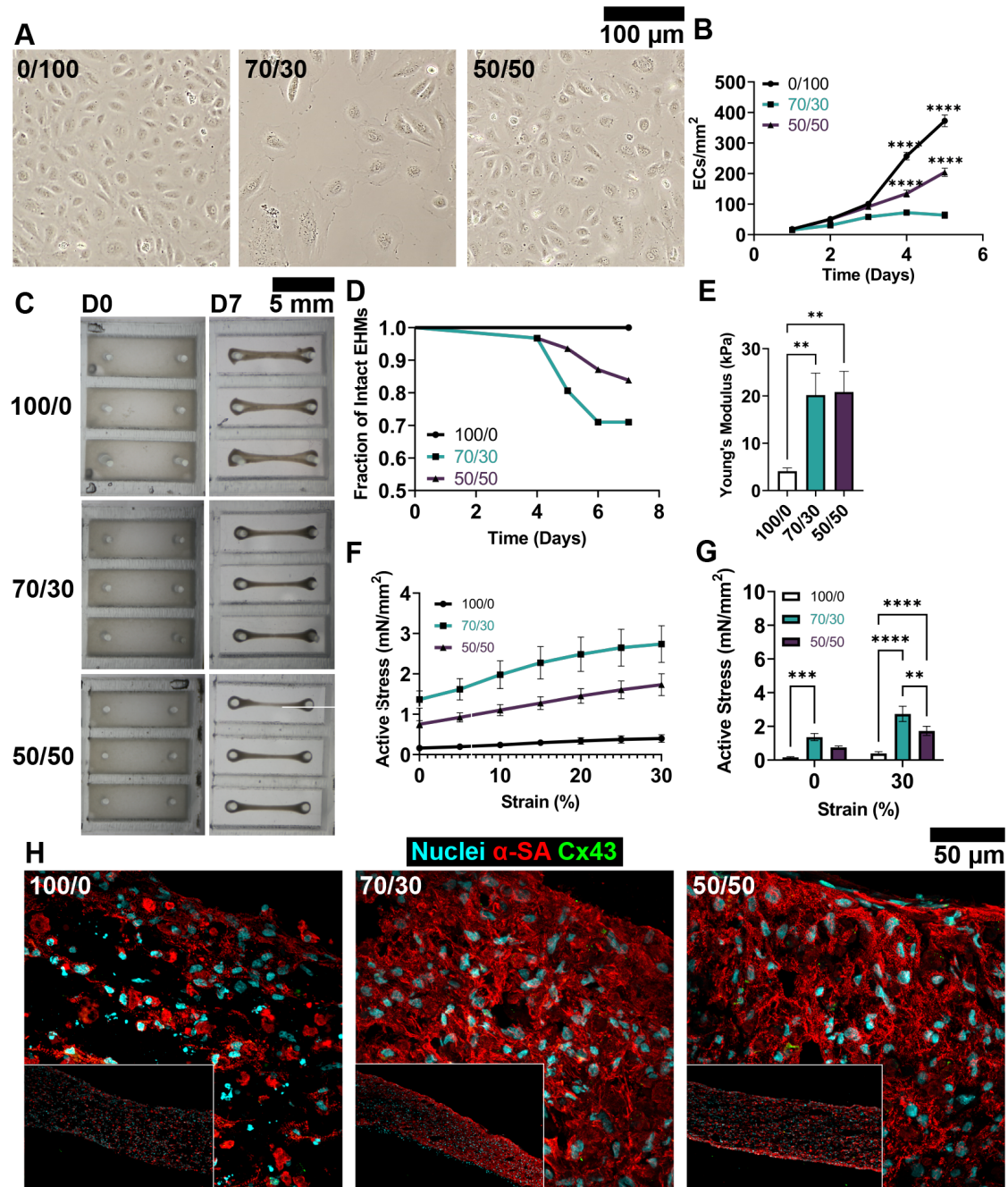


Figure 4.2: Mixed media culture differentially impacts EC and CM structure and function. (A, B) EC proliferation and morphology evaluated in mixed media monolayer culture. $n = 9$ samples per group. (C) Compaction and (D) necking of 3D linear EHMs across 7 days of culture. $n = 18-21$ samples per group. (E) Mechanical characterization of passive and (F, G) active force generation of EHMs at 5% stretch increments up to 30% stretch. $n = 18-21$ samples per group. (H) Histological staining of EHMs using α -sarcomeric actinin (α -SA) and Connexin-43 (Cx43) following mechanical characterization.

4.4.1 Media composition influences hiPSC-CM and EC structure and function

A primary consideration of fabricating complex EHMs is the impact of media on each primary cell population due to varying levels of ions, growth factors, and other supplements. ECs are primarily maintained in EGM-2, whereas iPSC-CMs are cultured in RPMI B27, so we sought to evaluate the impacts of mixed media culture on each cell type and determine an ideal composition. We first examined EC and hiPSC-CM viability in culture over 5 days in their standard media, 70/30, or 50/50 media (**Suppl. Fig. B.1**). While we found no significant differences in hiPSC-CM viability, there was a significant decrease in day 1 attachment of ECs in 70/30 media relative to control EGM-2, suggesting that media composition may play a role in EC adherence. In examining EC proliferation rate and morphology (**Fig. 4.2A, B**) we noted rapid proliferation in EGM-2 media causing confluence of the well within 5 days and a characteristic cobblestone morphology. In comparison, ECs cultured in 50/50 and 70/30 proliferated markedly slower, first reaching significance at day 3 and 4 relative to control, respectively. While ECs in 50/50 did not reach confluency within 5 days, the cells displayed primarily a cobblestone morphology in denser areas, with few cells appearing to display a more spread morphology. In contrast, ECs in the 70/30 medium displayed the poorest proliferative capacity, and the vast majority of cells displayed irregular, spread morphologies. Additionally, we noted that hiPSC-CMs in 50/50 and 70/30 media conditions demonstrated markedly increased sarcomere development and hypertrophy relative to hiPSC-CMs in control media (**Suppl. Fig. B.1C**).

To evaluate the functional consequences of mixed-media compositions on hiPSC-CMs in 3D culture, we fabricated small (9 x 3 mm) linear EHMs (**Fig. 4.2C**). We noted increased compaction of EHMs in 70/30 media relative to 50/50 media (**Suppl. Fig. B.2A**), and particularly many instances of overcompaction, necking, and failure of the EHMs (**Fig. 4.2D**). After 7 days of *in vitro* culture, we performed mechanical analysis on the tissues fol-

lowed immediately by fixation and histological analysis. Mechanical evaluation of EHMs showed significantly greater stiffness (**Fig. 4.2E**) in mixed media culture (20.18 ± 4.67 kPa in 70/30, $p = 0.0071$; and 20.10 ± 4.39 kPa in 50/50, $p = 0.0033$) relative to control tissues (4.08 ± 0.71 kPa). Active force generation was also significantly different across groups (**Fig. 4.2F, G**), with 70/30 tissues generating greater force ($1.37 \pm .21$ mN/mm², $p = 0.0006$;) relative to control tissues (0.16 ± 0.04 mN/mm²) at 0% stretch, and both mixed media groups (2.74 ± 0.46 mN/mm² in 70/30, $p < 0.0001$; and 1.73 ± 0.27 mN/mm² in 50/50, $p < 0.0001$;) outperforming control tissues (0.40 ± 0.1 mN/mm²) at 30% stretch. We consequently also saw similar significant differences in upstroke velocity (**Suppl. Fig. B.2B**), T50 (**Suppl. Fig. B.2C**), and T90 (**Suppl. Fig. B.2D**). Lastly, we performed force-frequency response analysis and noted that irrespective of condition, all tissues demonstrated an inverse relationship pacing frequency and force generation (**Suppl. Fig. B.2E** and were able to follow pacing up to 4 Hz (**Suppl. Fig. B.2F** albeit with noticeable alternans [38]. Lastly, immunohistochemical staining of tissues following mechanics analysis revealed noticeable improvements in sarcomere development and mild hypertrophy, with greatest sarcomere density in the 70/30 media group (**Fig. 4.2H**).

Together, these data suggest that a mixed media composition of EGM-2 and B27 benefits hiPSC-CMs via greater active stress output, but negatively impacted EC viability and proliferation. Ultimately, we proceeded to utilize the 50/50 media mixture for all further experiments due to the morphological and proliferative consequences of 70/30 media on ECs which could compromise proper vessel formation and function, and necking of EHMs that would cause early tissue failure.

4.4.2 Coating solution enables local delivery of ECs to lumen walls

We modified our previously published alginate fiber wet-spinning technique [3] by incorporating 2.5% w/w gelatin into the 100 mM CaCl₂ crosslinking bath, generating sacrifi-

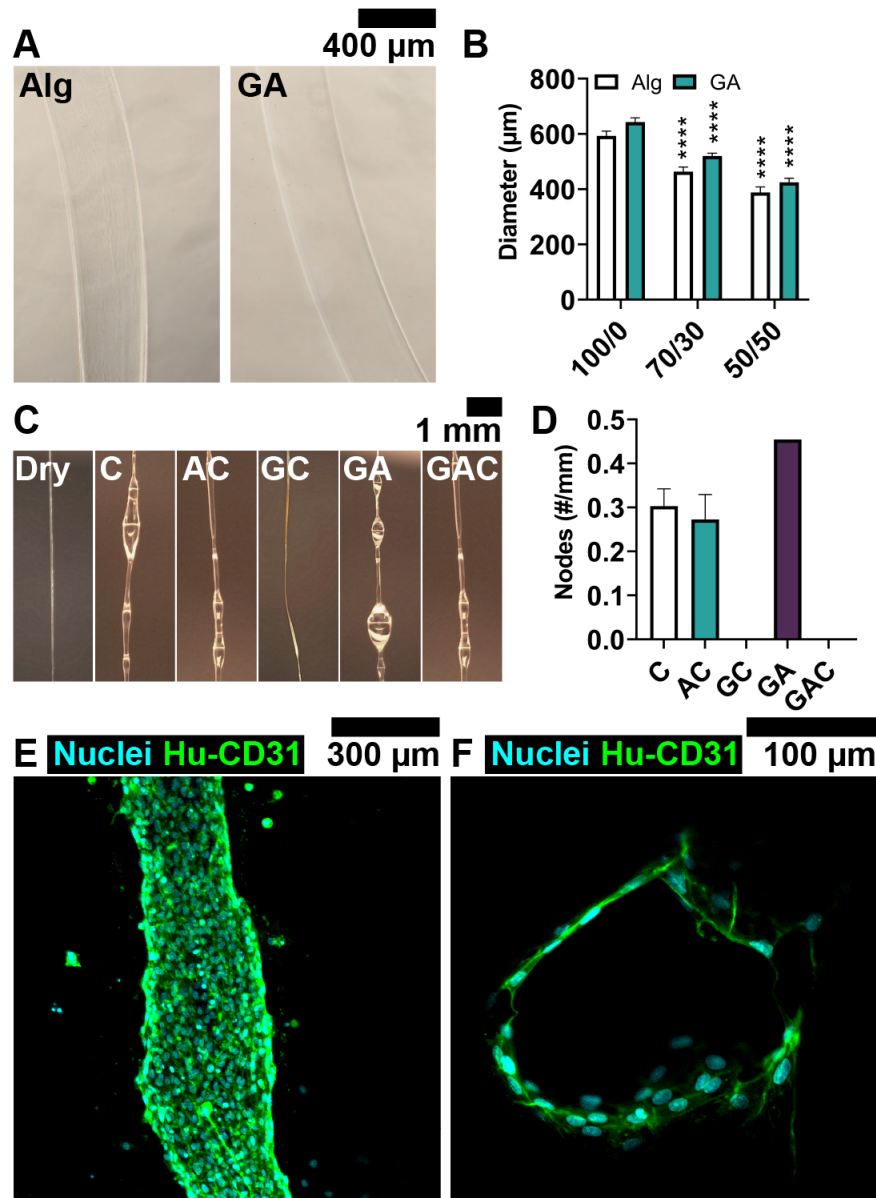


Figure 4.3: Formation of patterned vasculature using EC-coated gelatin-alginate fibers. (A, B) Equilibrium swelling and diameter assessment of gelatin-alginate fibers in mixed media culture. $n = 9$ per group. (C, D) Solutions composed of gelatin, alginate, and/or collagen used to evaluate coating smoothness for homogenous EC seeding. $n = 3$ per group. (E) EC-coated vessel using gelatin-alginate-collagen solution in whole mount and (F) transverse sections.

cial alginate fibers ensheathed in gelatin (GA fibers) to provide a surface for ECs to adhere to. In considering the co-culture application of these fibers, we quantified diameter by incubating the fibers in 100/0, 70/30, and 50/50 media (Fig. 4.3A). Image analysis showed no statistically significant differences in diameter between alginate and GA fibers at any

media composition, although there was a pronounced reduction in diameter ($425 \pm 14.27 \mu\text{m}$ in 50/50 media) in response to increased EGM-2 composition in the media (**Fig. 4.3B**). The decrease in equilibrium swelling of the fibers is likely due to increased divalent cation concentration in EGM-2 media, particularly of calcium ($\sim 1.6 \text{ mM}$), relative to RPMI B27 (0.42 mM), which results in a higher effective concentration in 70/30 and 50/50 media compositions.

We next developed a coating solution to specifically seed endothelial cells on the outer wall of the GA fibers to later form functional vascular structures in EHMs. Alginate fibers were embedded into the walls of PDMS molds to replicate our final EHM-casting procedure, and a variety of coating solutions were testing. A hydrogel-based solution was necessary because the GA fibers are suspended in the air and seeding endothelial cells in media alone resulted in the cells dripping off the fibers before they adhered. We first tested a 1 mg/mL collagen seeding solution, however due to the gelation time the coating was uneven and tended to ball up in areas forming nodes. After further iteration, we found that a multicomponent coating solution of $1\% \text{ w/w}$ gelatin, $0.25\% \text{ w/w}$ alginate, 1 mg/mL collagen enabled facile and even coating along the length of the fiber using a micropipette (**Fig. 4.3C**). The inclusion of gelatin was necessary to reliably create a smooth coating surface and minimize node formation, while a low concentration of alginate provided short-term crosslinking with residual calcium on the GA fibers prior to longer-term gelation of the collagen to form a stable gel (**Fig. 4.3D**). We then tested our optimized coating solution *in vitro* by incorporating ECs at a density of $2.5 \times 10^7 \text{ cells/mL}$ in coating solution, applying the solution to the fibers (approximately $1.25 \times 10^5 \text{ ECs per tissue}$), and encapsulating the coated GA fibers in a 2 mg/mL collagen gel. Culture over 5 days resulted in nearly full circumferential coverage (**Fig. 4.3E, F**) and conserved patency, demonstrating successful EC delivery and engineered vessel formation.

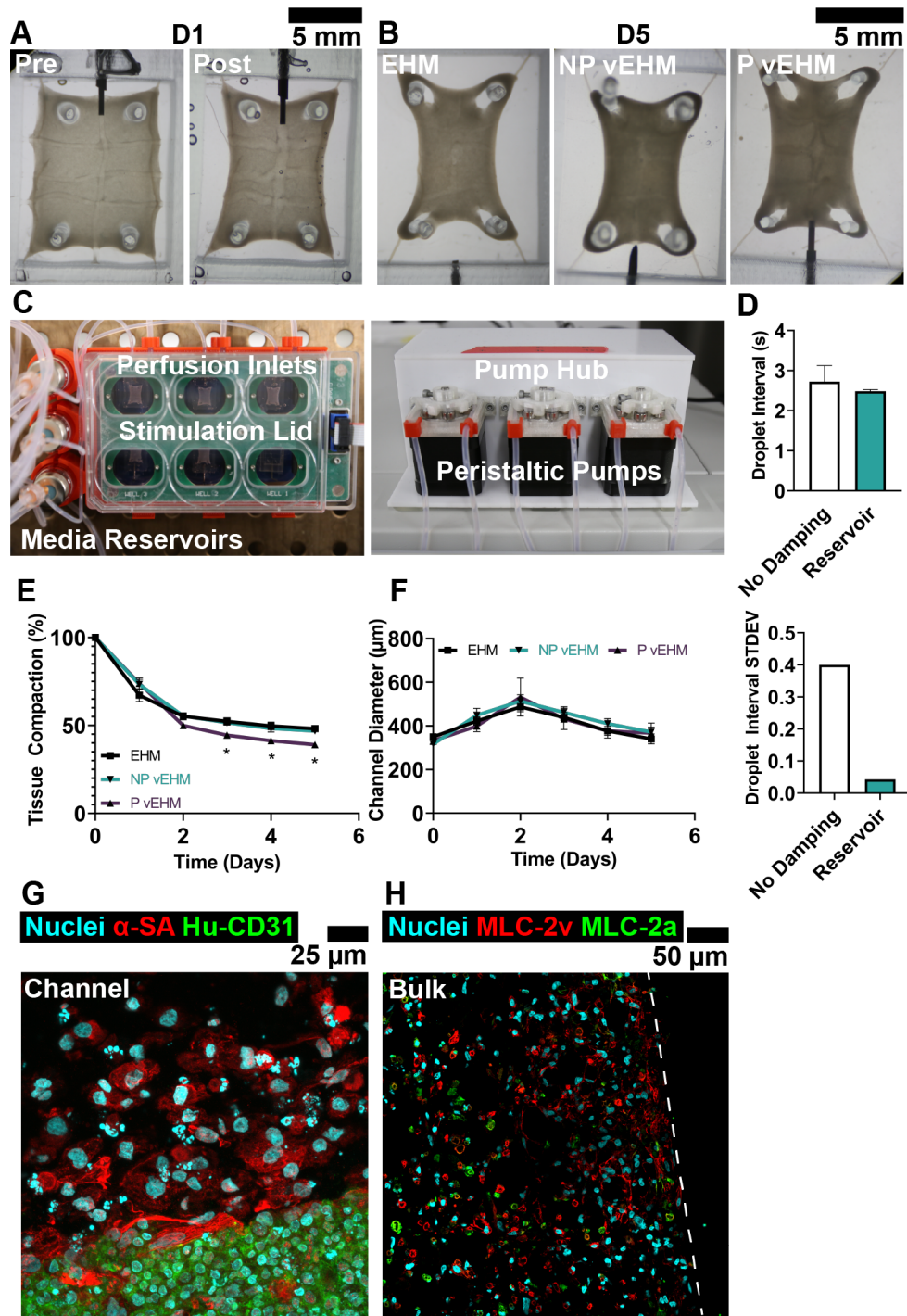


Figure 4.4: Fabrication and perfusion culture of vEHMs. (A) Fabricated EHMs and vEHMs before and after treatment with un-crosslinking solution. (B) EHMs and vEHMs prior to implantation after 5 days of culture. (C, D) P vEHMs are perfused using a custom peristaltic pump system. (E, F) Tissue compaction and channel diameter over culture period. $n = 11-14$ per group. (G) Histological evaluation of tissues adjacent to channels and (H) within the bulk.

4.4.3 Dynamic perfusion culture of EHMs

In order to develop larger constructs with perfusable vasculature for therapeutic effect in rodents, we scaled up our fabrication process to create 10 x 12 mm EHMs (prior to compaction). The PDMS molds included a thicker wall section on one end to accommodate the insertion of a stainless steel needle for dynamic perfusion culture of EHMs. GA fibers were embedded into the walls of the mold to create an array pattern of channels, and bundled collagen microfibers were embedded on longitudinal ends of the tissue to provide greater structure to the tissue, improved handleability during implant, and height alignment with the perfusion needle (**Suppl. Fig. B.3**).

To form tissues, GA fibers were coated with ECs (2.5×10^7 cells/mL; vEHMs) or without ECs (control EHMs) prior to casting a cell-collagen mixture containing hiPSC-CMs (1.5×10^6 cells/mL) with 5% primary hCFs and incubated overnight. The next morning, GA fibers were un-crosslinked to wash out alginate and establish patent vessels (**Fig. 4.4A, B**). After chelation, a subset of tissues with endothelialized channels were connected to a peristaltic pump-based perfusion system (**Fig. 4.4C**) for closed loop recirculation of media and paced under 1 Hz field stimulation. A reservoir system was incorporated to ensure that sufficient media was present to cover the vEHM and fill the entire length of tubing. This reservoir also acted to dampen the natural peristaltic flow of the pump and trap air bubbles to prevent embolisms. The pump speed was determined by rotational speed of the connected stepper motors, which was controlled by an Arduino. Peristaltic pump flow was experimentally measured to 7.28 mL/hr, and average shear stress was calculated to be 3.21 dyne/cm² assuming Poiseuille flow and average channel diameter of 400 μ m (**Fig. 4.4D**).

During 5 days of *in vitro* culture, significant remodeling occurred with tissues compacting to about 50% of initial size, with P vEHMs compacting significantly more ($39.08\% \pm 1.83\%$) than control ($48.24\% \pm 1.80\%$, $p = 0.014$) or NP ($46.89\% \pm 1.76\%$, $p = .038$) vEHMs (**Fig. 4.4E**). Diameter measurements during this time frame showed a transient

rise in diameter that re-stabilized to approximately 400 μm during the culture period, but otherwise remained similar across groups (**Fig. 4.4F**). This was due to the un-crosslinking treatment of fibers, which first caused swelling of the alginate chains prior to washout due to calcium chelation. Histological evaluation at 5 days revealed endothelialized vessels in vEHMs, with elongated hiPSC-CMs primarily located at the edges of tissues and more-rounded cardiomyocytes located in the bulk of tissues due to a lack of passive tension cues for alignment (**Fig. 4.4G, H**).

4.4.4 P vEHMs demonstrate semi-conserved pattern morphology and improved engraftment

Following *in vitro* fabrication and characterization of the vEHMs, we next sought to evaluate their ability to engraft onto infarcted hearts using a rodent model of ischemia-reperfusion MI. At a time point of two weeks, hearts were harvested and underwent OCT and microCT imaging prior to sectioning for histological evaluation. During microfil perfusion, it was noted that a distinct vessel array reminiscent of the original pattern geometry was visible in 1 of 3 hearts implanted with P vEHMs (**Fig. 4.5A**). Further, it was noted that EHM and NP vEHM implants were distinctly paler in color than P vEHMs, suggesting greater intra-implant perfusion in the latter group.

Morphometric analysis of hearts from picrosirius red – fast green stains showed that each group had similar infarct size and anterior wall thickness excluding implants (**Fig. 4.5B-D**), though no group showed improvements in heart function via echocardiography at the 2 week time point (**Suppl. Fig. B.4**). Implants were clearly visible (**Fig. 4.5B**, red dotted lines) and often surrounded by highly vascularized adhesion tissue from the chest wall (**Fig. 4.5B**, asterisks). Viable implant size was quantified from immunohistochemical cardiac stains but no difference was found between groups (EHM: $0.86 \pm 0.09 \text{ mm}^2$, NP

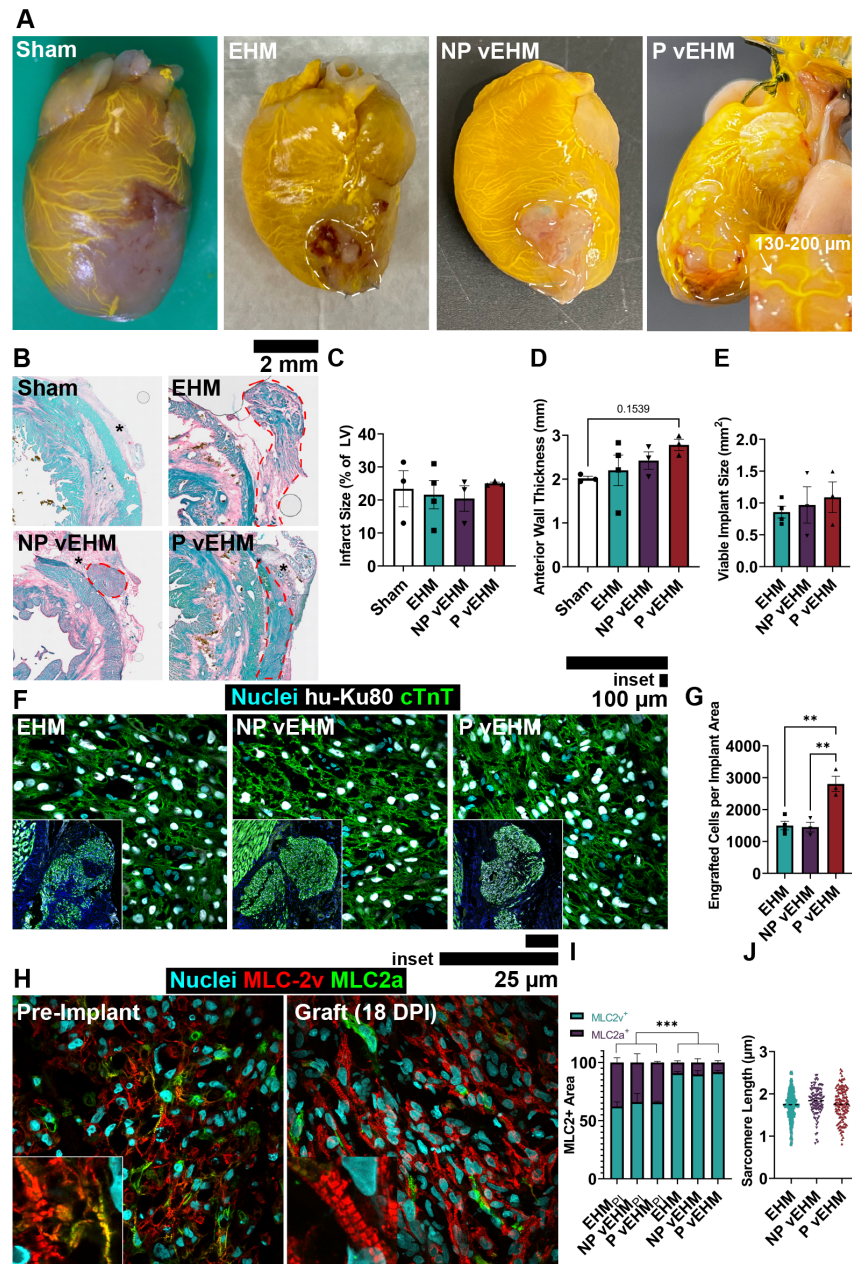


Figure 4.5: Implants mature *in vivo* and engraftment is best improved by *in vitro* perfusion. (A) Explanted hearts after perfusion with radiopaque dye Microfil show differential coloring of implant. Dotted lines indicate implants. Inset: Vasculature in implant with preserved array geometry. (B) Picrosirius red - fast green staining of transverse heart sections showing anterior free wall with infarct and implant (red dotted lines). Asterisks indicate adhesion tissue. (C) Quantification of infarct size, (D) anterior wall thickness, and (E) viable implant size. (F, G) Histological evaluation of human cell engraftment with human Ku80 staining. $n = 3-4$ animals per group. (H, I) Histological evaluation of graft maturation with atrial (MLC-2a) and ventricular (MLC-2v) myosin-heavy chain isoforms. $n = 3-4$ per group. (J) Quantification of hiPSC-CM sarcomere length in implants.

vEHM: $0.97 \pm 0.29 \text{ mm}^2$, P vEHM: $1.09 \pm 0.24 \text{ mm}^2$; **Fig. 4.5E**). However, implant staining for human-specific nuclei with Ku80 (Hu-Ku80) revealed significantly greater human cell density in P vEHMs as well as increased engraftment when normalized by implant sectional area ($2806 \pm 239.3 \text{ Hu-Ku80+ nuclei/mm}^2$) when compared to EHMs ($1499 \pm 132.0 \text{ Hu-Ku80+ nuclei/mm}^2$, $p = 0.0024$) and NP vEHMs ($1452 \pm 154.1 \text{ Hu-Ku80+ nuclei/mm}^2$, $p = 0.0029$; **Fig. 4.5F, G, Suppl. Fig. B.5A**). Further, quantification of total intra-implant cells showed no differences across implants, suggesting similar levels of host cell infiltration (**Suppl. Fig. B.5B**). To confirm that increased cell engraftment was not due to proliferation, we stained implants for human-specific Ki-67 (Hu-Ki67) and cTnI (**Suppl. Fig. B.5C, D**). Quantification revealed no significant differences in hiPSC-CM proliferation across implant groups, suggesting that the increased number of human cells found in P vEHMs was due to improved survival and engraftment relative to other groups. Staining for MLC2 isoforms as an indicator of maturation showed significantly greater ventricular isoform expression by area between implant groups (89.68% - 91.65% MLC2v+ by area) and in vitro tissues (62.16% - 65.75% MLC2v+ by area, all $p < 0.001$) pre-implantation, though there were no differences between implant groups (**Fig. 4.5H, I**). Similarly, measurements of sarcomere length in implants showed no significant differences between implant groups (EHM: $1.73 \pm .02 \text{ }\mu\text{m}$, NP vEHM: $1.82 \pm .03 \text{ }\mu\text{m}$, P vEHM: $1.75 \pm .03 \text{ }\mu\text{m}$; **Fig. 4.5J**). Together, these data suggest that perfused, patterned vasculature has the ability to be conserved *in vivo* and that *in vitro* perfusion improves implant engraftment.

4.4.5 P vEHMs inosculate with host vessels and develop larger and more organized vasculature

We next sought to evaluate the impacts of patterned vasculature on *in vivo* vascular remodeling in our implants. Histological quantification of pan-vascular isolectin B4 and human-specific Ku80 (Hu-Ku80) was performed in the remote, infarct, and implant regions

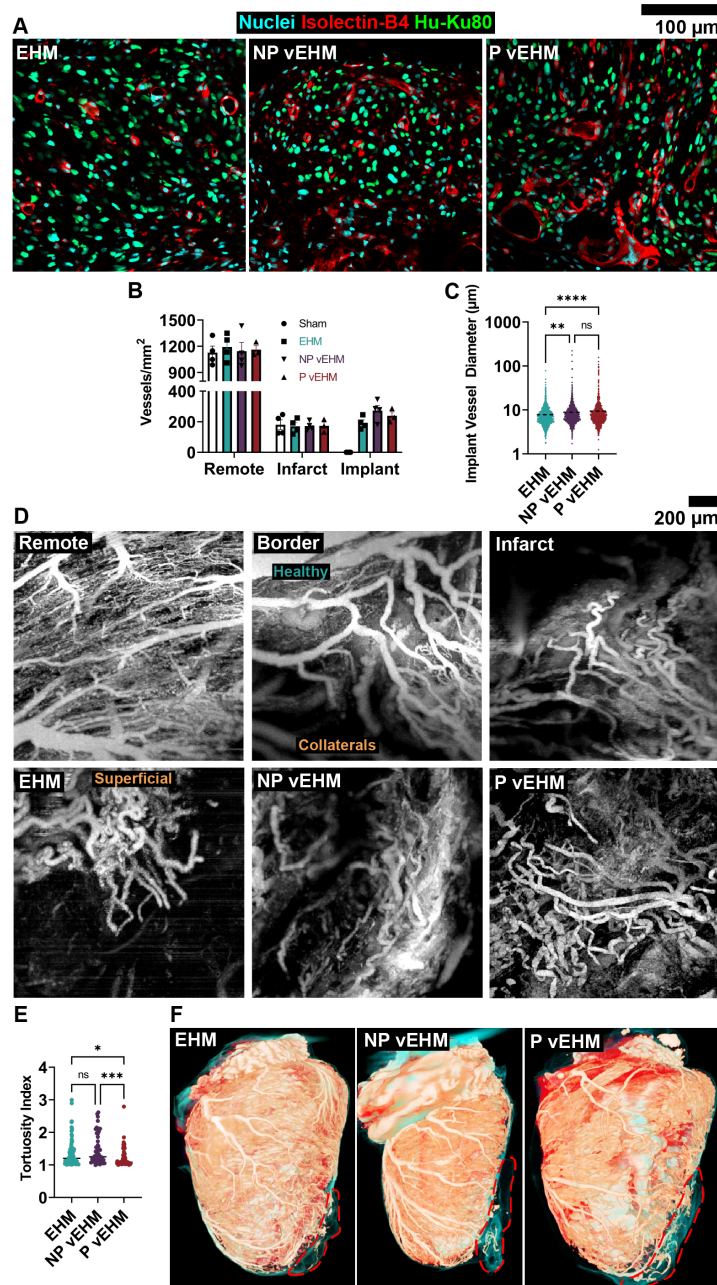


Figure 4.6: Patterned vasculature increases implant vessel diameter but only *in vitro* perfusion improves implant vessel organization. (A) Representative images of vessel development in implant groups. (B) Quantification of number of vessels across remote, infarct, and implants regions. $n = 3-4$ animals per group. (C) Distribution of intra-implant vessel diameters. $n = 3-4$ animals per group. (D) Representative images of perfused vasculature in remote, border, infarct, and implant regions. (E) Tortuosity index of perfused vasculature from OCT imaging. $n = 3-4$ animals per group. (F) MicroCT scans of cardiac and intra-implant vasculature (red-white) overlaid on tissue structure (teal). Red dotted lines indicate implants.

of each group for analysis (**Fig. 4.6A**, **Suppl. Fig. B.6A**). While no significant differences were found in the number of vessels across groups in either remote, infarct, or implant regions (**Fig. 4.6B**), we noted significantly increased vessel diameter in NP ($8.85 \pm 0.25 \mu\text{m}$ and range = $220.2 \mu\text{m}$, $p = 0.0024$) and P vEHMs ($9.41 \pm 0.30 \mu\text{m}$ and range = $155.2 \mu\text{m}$, $p < 0.0001$; **Fig. 4.6C**), with notably greater frequency of larger vessels than in control EHMs ($7.78 \pm 0.12 \mu\text{m}$ and range = $76.17 \mu\text{m}$). Costaining for human-specific CD31 (Hu-CD31) with isolectin B4 revealed two distinct phenotypes in NP and P vEHMs. Human ECs either appeared in capillary-like structures in NP vEHMs (**Suppl. Fig. B.6B**) or associated with vessel walls indicating a patterned vessel (**Suppl. Fig. B.6C**). While Microfil overlays were captured, the material was not reliably maintained in lumens during histological processing of the hearts after microCT scanning and thus could not be used as an accurate metric for perfusion. However, some microfil-filled lumens did contain Hu-CD31+ cells in NP and P vEHMs confirming inosculation occurred in both groups though remodeling reduced vessel diameter from the initial $400 \mu\text{m}$ at the time of implantation.

To better understand the 3D architecture and perfusability of intra-implant vasculature, we performed OCT imaging of the remote, infarct, and implant regions for each group (**Fig. 4.6D**, **Suppl. Fig. B.7**). Healthy remote regions were easily visualized with perfusion of 20% Intralipid solution, revealing hierarchically branched vascular architecture down to the capillary scale. Acquisition at border and infarct regions revealed increasingly tortuous vasculature and less visible capillary perfusion. Angiographs of EHMs revealed variable perfusable vessels, often with gaps that were lowly perfused. NP and P vEHM angiographs revealed greater perfusable vessels with typically larger sizes, and notably with the least tortuosity in the perfused group (1.2 ± 0.03 tortuosity index, compared to 1.35 ± 0.04 for EHMs, $p = 0.025$; and 1.46 ± 0.06 for NP vEHMs, $p = 0.0002$; **Fig. 4.6E**). Following OCT acquisition, we perfused the hearts with Microfil to enable microCT-assisted reconstructions of the cardiac vasculature, which revealed large ($>50 \mu\text{m}$) intra-implant vasculature in P vEHMs (**Fig. 4.6F**).

Together, these results demonstrate that patterned vasculature enables the development and maintenance of larger diameter vessels than control tissues, and *in vitro* perfusion enables greater vascular organization *in vivo*.

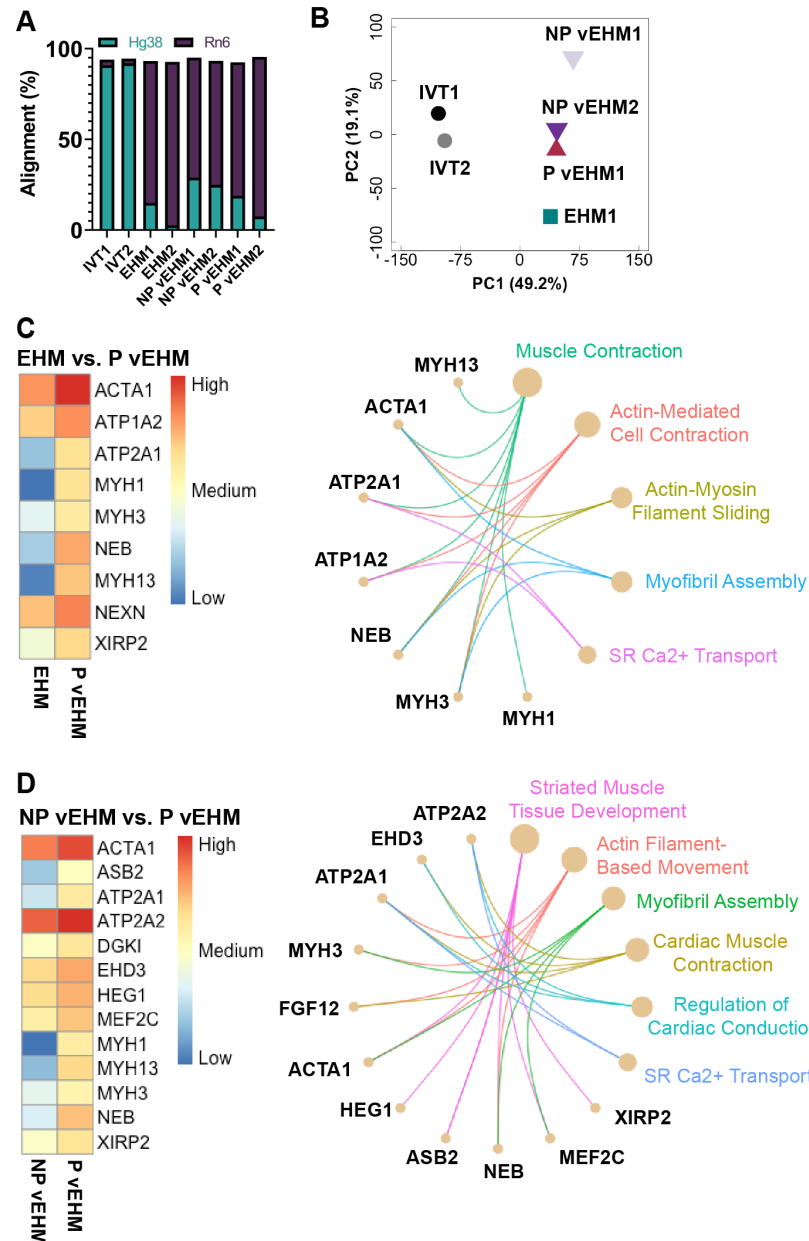


Figure 4.7: Exploratory RNASeq analysis of implanted tissues suggests perfusion improves cardiac development. **(A)** Alignment of sequenced samples to Hg38 (human) and Rn6 (rat) genomes. **(B)** Principle component analysis of human-aligned samples. **(C)** Heatmaps of differentially expressed genes and associated gene ontology terms for EHM vs P vEHM and **(D)** NP vEHM vs P vEHM comparisons.

4.4.6 Perfusion of vEHMs *in vitro* upregulates genes related to cardiomyocyte development and contractility

We lastly sought to further understand the effects of implantation and patterned, perfused vasculature on hiPSC-CM structure and function through exploratory bulk RNASeq analysis of explanted tissues. At the two-week timepoint, a subset of implanted tissues were manually debrided from the epicardial surface and processed for bulk RNA isolation and sequencing. Alignment analysis revealed an expected heterogeneous mixture of human and rat-derived reads in explanted tissues, and by comparison almost 100% human-aligned reads in *in vitro* control tissues (**Fig. 4.7A**). Notably, two samples had uncharacteristically low alignment scores to the human genome, and were thus excluded from further analysis. Principle component analysis of the revealed group stratification with implants segregated from *in vitro* control tissues primarily by principle component 1 (representing 49.2% of differences), and implant groups further separated across principle component 2 (representing an additional 19.1% of differences, **Fig. 4.7B**). This suggested that *in vitro* controls had very different transcriptomes as expected from *in vivo* microenvironmental signaling.

Next, we performed DGE analysis and gene ontology (GO) term analysis to isolate differences in expression as a result of vascular channel incorporation in the implanted tissues. We found 64 differentially expressed genes (DEGs) between EHM and NP vEHM, 23 DEGs between EHM and P vEHM, and 35 DEGs between NP vEHM and P vEHM, though with EHM and NP vEHMs mapping more closely together than P vEHM (**Suppl. Fig. B.8**). GO term analysis between EHM and P vEHM revealed upregulation of processes related to heart development(XIRP2), myofibril assembly (MYH1, MYH3, MYH13), and ion transport (ATP1A2, ATP2A1), indicative of improved hiPSC-CM structure and function relative to control EHMs (**Fig. 4.7C**, **Suppl. Fig. B.9A**) and NP vEHMs (**Fig. 4.7D**, **Suppl. Fig. B.9B**). Interestingly, there was little differential expression of genes related to vascularization between groups, and even downregulation of genes associated with vessel

development and angiogenesis (CD34, ANGPTL4, SERPINE1, CXCR2) in the P-vEHM group relative to NP-vEHM. We hypothesize this may be due to turnover of human ECs in patterned vasculature over the 2 week *in vivo* study, which may have been accelerated in the P vEHM group due to improved inosculation and perfusion of patterned vessels. However, we also noted GO term analysis related to downregulation of tumor necrosis factor (TNF) production, suggesting reduced inflammation. Together, these results suggest that *in vitro* perfusion of patterned channels substantially contributes to improved hiPSC-CM contractile development *in vivo*.

4.5 Discussion

Vascularization remains a primary concern in developing implantable cardiac tissues for proper engraftment and integration, and further it is unclear what the impact of vasculature is on hiPSC-CM development *in vivo*. In this study, we demonstrated a facile method for fabricating and culturing EHMs with patterned vasculature *in vitro*, and evaluated their ability to impact host vascular ingrowth, remodeling, and subsequent hiPSC-CM development. Compared to other techniques, which require expensive equipment and/or materials that cannot be autoclaved or otherwise easily sterilized [9, 10], our methodology provides a flexible foundation for creating perfusable cardiac tissues in standard 6-well tissue culture plates, and uses equipment that can easily be fabricated in-house using our open-source plans or otherwise easily commercially available materials and technologies.

Our study examined specific effects of mixed media on hiPSC-CMs and ECs in 2D and 3D, which has not been examined before. While negative effects on EC proliferation were unsurprising as EGM-2 media is optimized specifically for EC culture *in vitro*, the massive improvements seen in hiPSC-CM contractility and tissue structure were unexpected. Despite being the gold standard media for hiPSC-CM differentiation and culture, RPMI

B27 was originally created for hippocampal neuronal culture, and thus does not effectively recapitulate an optimal CM environment. For example, B27 media contains high glucose (10 mM), low calcium (0.42 mM), and no fatty acids for beta oxidation, whereas the *in vivo* CM microenvironment is relatively low in glucose, high calcium, and high reliance on fatty acid oxidation for ATP generation [27, 45]. A 50/50 mix of B27 and EGM-2 would contribute a slightly higher calcium concentration (EBM-2 basal media contains 1.6 mM Ca^{2+}), and growth factors including VEGF, bFGF, IGF, and EGF. Our group has shown that 3D culture of ECTs in physiological calcium levels results in improved calcium handling ability and contractility [45]. Groups have suggested that VEGF signaling induces an inotropic response via calcium channels [46, 47], and we have shown that incorporation of microparticles releasing VEGF resulted in increased tissue stiffness but not contractility at 4 weeks, suggesting a role in tissue remodeling [2]. IGF is a well known factor in cardiomyocyte biology, contributing to multiple pathways including fetal proliferation, hypertrophy, and ischemic protection [29, 41, 48]. Histology of our hiPSC-CMs in 2D and 3D revealed greater sarcomere development and hypertrophy when cultured in mixed media conditions relative to B27 alone, but further work will need to examine the contributions of individual media components.

More broadly, there is robust cross-talk between cardiomyocytes and endothelial cells in the native heart and which plays important roles *in vitro*. Nitric oxide (NO) is widely secreted by ECs and a known inotropic agent (though with concentration-dependent biphasic effects) [49] as well as roles in protection during ischemia-reperfusion [50]. Growth factors such as VEGF and FGFs are known to be secreted by cardiomyocytes and play central roles in angiogenesis [3, 51]. Future work will examine this cross-talk both in isolated 2D culture and 3D EHMs through the use of ELISA assays and RNA sequencing of purified populations to understand each cell type's contribution to overall vEHM structure and function, particularly in the presence or absence of perfusion. Understanding this signalling will also provide insight into how patterned vasculature can better recapitulate

arteriole-scale vessels through arteriogenic mechanisms [3].

Dynamic perfusion culture induced greater compaction than NP vEHMs or control EHMs, suggesting greater remodeling of the tissues. Further studies could pursue the mechanisms of perfusion-mediated remodeling and their implication on EHM structure and function. The estimated shear rate of 3 dyn/cm² set in our perfusion experiments is relatively lower than the average shear rates in human coronary arteries (approximately 5-12 Pa [52, 53]), though within reasonable limits of 1-10 dyn/cm² in comparable literature [12, 54–56]. In pilot experiments, greater flow rates and thus shear stresses proved to be deleterious to the structure of the entire tissue. However, lower shear stresses in arteriole-scale vessels such as those used in this study have been shown to be atherogenic *in vivo* [57, 58], though *in vitro* fabricated vessels displays a more naive phenotype than *in vivo* vasculature. Future work will examine shear stress training regimens to condition patterned vessel walls. Multi-layered vessels will be explored to recapitulate the tunica media and tunica adventitia by additional coating steps with smooth muscle cells and fibroblasts.

The cellular composition of vEHMs played an active role in constraining the geometry of the tissue. Active remodeling of the ECM, particularly by hCFs, is necessary for proper syncytium formation and strong contractile properties of the tissue. However, this compaction also introduced several design compromises when introducing large, perfusable channels into the tissue. One such trade-off was the use of internal post geometries which can improve hiPSC-CM alignment and thus contractile potential [2, 24, 36]. Tiburcy et al. demonstrated that peak active force was reached when cardiac tissues were composed of approximately 60% cardiomyocytes [59]. However, inclusion of more fibroblasts would have increased compaction and led to the risk of greater channel closure during culture time. Thus, in combination with the increased size and relative lack of tension cues in the bulk of EHMs [60], hiPSC-CMs adopted a more rounded morphology compared to elongated and aligned morphology at the edges. Future directions will look at how GA fiber

patterning and endothelial coating may affect hiPSC-CM alignment, particularly in the case of alginate that is modified to contain RGD or other similar binding motifs.

Implantation of perfused vEHMs onto the infarcted LV of rodent hearts demonstrated improved patch engraftment, reduced vessel tortuosity, and improved hiPSC-CM contractile development. We also noted a trend towards increased free wall thickness, though this metric did not reach significance. We opted to exclude added thickness from the implant itself as this metric would also be inflated by variable levels of adhesion tissue that deposited between MI and implant surgery, as well as over the two week study. The lack of engrafted hiPSC-CMs in NP vEHMs was surprising, suggesting that channels alone were not sufficient to prevent hiPSC-CM death upon implantation but could be rescued by *in vitro* perfusion, which we hypothesized to be due to early inosculation and perfusion of the tissue. Our quantification revealed similar viable graft size and even higher engraftment than comparable studies [2, 11, 12, 61, 62]. Some studies have shown that incorporating microchannels into scaffolds improves host tissue ingrowth and directed vascular formation, suggesting that our non-endothelialized channels may still provide an indirect *in vivo* benefit in revascularization [63, 64].

In examining vascular development, implanted tissues all developed similar numbers of vessels irrespective of patterned channel condition. Vessel quantification similarly was not different from that in infarct regions. However, improved vessel diameters in NP and P vEHMs with some evidence of residual Microfil in Hu-CD31+ channels suggested that channel endothelialization and facilitated inosculation and perfusion of patterned channels as well as arteriogenic processes of host vasculature. Increased frequency of larger diameter vessels relative to capillary-scale ones could explain the non-significant results in vessel quantification. Future studies could incorporate bulk-seeded ECs to allow for hierarchical vascularization at the capillary coupled with large-scale channels. Redd et al. demonstrated that seeding endothelial cells into the bulk of constructs containing patterned vessels im-

proved graft perfusion and co-implanted hPSC-CM engraftment [12]. Our previous work demonstrated the benefits of angiogenic growth factor release from microspheres embedded in implants [2], and future work will explore coming this technique with engineered channels to further facilitate vascular inosculation and rapid perfusion upon implantation.

OCT imaging provided additional insight into intra-implant vascular structure that could not otherwise be adequately captured in histological images. While OCT has been demonstrated before in a cardiac context, it is a relative new technique and few other groups have performed OCT imaging of the heart [17] and of implanted vascular tissues [12]. To our knowledge only one other group has published the use of OCT on implanted cardiac tissue [11], though only as proof-of-concept. Our results show greatly improved image fidelity and comparisons across implant groups with differing vascularization conditions. In our work, we found more organized vasculature in P vEHMs compared to the relatively tortuous vessels in EHM and NP vEHMs, which was reminiscent of vessel collateralization in the infarct and border regions. It was particularly difficult to image perfused capillaries in all injured and implant regions of the heart, though it is unclear to what extent this was due to lower perfusion pressures (50-60 mmHg), relatively low vessel density, and capillary collapse. Further, adhesion tissue from the chest wall created an additional obstacles during imaging as dissecting this tissue away to prepare the heart for OCT acquisition resulted in occasional vascular leakages that obscured imaging capabilities. Future work will examine intra-implant perfusion velocity using dynamic light scattering OCT [65].

Our group has previously published on microCT analysis [2], however reconstructions necessitated a lot of manual reconnection of vessels due to air bubbles and partial vessel collapse, which created difficulties in robust analysis. Improvements in data processing workflows will be optimized to mitigate issues with automated vessel reconnection codes [42]. Future studies will seek to continue optimizing microfil perfusion to reduce issues including vessel blowout from high pressure and viscosity tuning in order to ensure full

perfusion of the entire coronary vascular tree [66].

Previous literature suggests that vascularization may have some impact on hiPSC-CM structural and functional maturation via paracrine signaling [7, 20, 67]. However, despite improved cardiomyocyte maturation from *in vivo* implantation we were unable to detect differences in hiPSC-CM maturation status across groups. We thus sought to determine any transcriptomic basis for cardiomyocyte maturation using exploratory RNASeq, which revealed upregulation of genes related to contractility and ion handling primarily in endothelialized channel groups, but particularly when perfused *in vitro*. Groups have previously shown RNASeq analyses of cardiomyocytes in 2D and 3D *in vitro* tissues for cardiac applications [12, 27, 59], as well as whole-heart bulk and single-cell RNA sequencing [68–70]. However, to our knowledge no group has previously published bulk RNA sequencing of explanted cardiac tissues.

Due to high alignment to the rat genome, we ran DGE and GO term analysis for each sample to explore host cell contributions to implant vascularization. However, our results noted upregulation of genes suggesting the presence of red blood cells (HBB, HBA, LGALS5) and "contaminating" rat cardiomyocytes (TNNT3, TNNI3, RYR1). We hypothesized this may be due to issues with sampling of host rat cells wherein we attempted to minimize collection of non-implant cells (e.g. underlying myocardium and adhesion tissue), which could have resulted in inconsistent rat cell population collection. This motivates future steps in single cell RNASeq where cell populations can be identified, quantified, and differential gene expression analyzed. Further, isolation of cell populations via single cell RNASeq will enable better assessment of human EC turnover as it relates to inosculation, particularly at multiple timepoints. RNASeq at different timepoints will allow studies in determining if hiPSC-CM maturation is facilitated by inosculation at earlier timepoints, or otherwise if long-term differences in *in vivo* maturation rate are revealed at later points. While there is published work on multi-species RNASeq particularly in the

area of oncology research, to our knowledge this has not been attempted in cardiovascular tissue engineering. However, techniques developed such as multi-species alignment deconvolution could be used in future studies to detect and filter out co-aligning human and rat reads to improve datasets for more robust analyses [71].

4.6 Conclusions

In summary, we developed a flexible *in vitro* system for fabrication and dynamic perfusion culture of vEHMs, and report the structural and functional effects of mixed-media culture on human ECs and hiPSC-CMs. Implantation of vEHMs in a rodent model of ischemia-reperfusion MI revealed that endothelialized channels both inoscultated with host vasculature and resulted in increased intra-implant vessel diameter. However, *in vitro* perfusion best improves hiPSC-CM engraftment, organization of vasculature for efficient hierarchical perfusion, and upregulation of genes related to cardiomyocyte development and maturation. Our study provides a strong foundation for future work in the field to understand the design criteria for fabricating complex heterocellular vEHMs in optimal culture conditions, and the benefits of vasculature on engineered tissue survival and maturation *in vivo* for therapeutic remuscularization of the heart after MI.

4.7 References

1. Laflamme, M. A. & Murry, C. E. Heart regeneration. *Nature* **473**, 326–335. ISSN: 0028-0836. <http://www.nature.com/doifinder/10.1038/nature10147> (2011).

2. Munarin, F., Kant, R. J., Rupert, C. E., Khoo, A. & Coulombe, K. L. Engineered human myocardium with local release of angiogenic proteins improves vascularization and cardiac function in injured rat hearts. *Biomaterials* **251**, 120033. ISSN: 01429612. <https://doi.org/10.1016/j.biomaterials.2020.120033> <https://linkinghub.elsevier.com/retrieve/pii/S0142961220302799> (Aug. 2020).
3. Kant, R. J. & Coulombe, K. L. Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues. *Acta Biomaterialia* **69**, 42–62. ISSN: 17427061. <https://doi.org/10.1016/j.actbio.2018.01.017> <https://linkinghub.elsevier.com/retrieve/pii/S174270611830028X> (Mar. 2018).
4. Dvir, T. *et al.* Prevascularization of cardiac patch on the omentum improves its therapeutic outcome. *Proceedings of the National Academy of Sciences* **106**, 14990–14995. ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.0812242106> (2009).
5. Koffler, J. *et al.* Improved vascular organization enhances functional integration of engineered skeletal muscle grafts. *Proceedings of the National Academy of Sciences* **109**, 1353–1353. ISSN: 0027-8424. <https://pnas.org/doi/full/10.1073/pnas.1120303109> (Jan. 2012).
6. Schaefer, J. A., Guzman, P. A., Riemenschneider, S. B., Kamp, T. J. & Tranquillo, R. T. A cardiac patch from aligned microvessel and cardiomyocyte patches. *Journal of Tissue Engineering and Regenerative Medicine* **12**, 546–556. ISSN: 19326254. <http://doi.wiley.com/10.1002/term.2568> (Feb. 2018).
7. Sun, X. *et al.* Transplanted microvessels improve pluripotent stem cell-derived cardiomyocyte engraftment and cardiac function after infarction in rats. *Science Translational Medicine* **12**, 1–11. ISSN: 19466242 (2020).

8. Mirabella, T. *et al.* 3D-printed vascular networks direct therapeutic angiogenesis in ischaemia. *Nature Biomedical Engineering* **1**, 0083. ISSN: 2157-846X. <http://www.nature.com/articles/s41551-017-0083> (June 2017).
9. Mirdamadi, E., Tashman, J. W., Shiwarski, D. J., Palchesko, R. N. & Feinberg, A. W. FRESH 3D Bioprinting a Full-Size Model of the Human Heart. *ACS Biomaterials Science & Engineering* **6**, 6453–6459. ISSN: 2373-9878. <https://pubs.acs.org/doi/10.1021/acsbiomaterials.0c01133> (Nov. 2020).
10. Kinstlinger, I. S. *et al.* Perfusion and endothelialization of engineered tissues with patterned vascular networks. *Nature Protocols* **16**, 3089–3113. ISSN: 1754-2189. <http://dx.doi.org/10.1038/s41596-021-00533-1> <http://www.nature.com/articles/s41596-021-00533-1> (June 2021).
11. Brady, E. L. *et al.* Guided vascularization in the rat heart leads to transient vessel patterning. *APL Bioengineering* **4**, 016105. ISSN: 2473-2877. <http://aip.scitation.org/doi/10.1063/1.5122804> (Mar. 2020).
12. Redd, M. A. *et al.* Patterned human microvascular grafts enable rapid vascularization and increase perfusion in infarcted rat hearts. *Nature Communications* **10**, 1–14. ISSN: 20411723. <http://dx.doi.org/10.1038/s41467-019-08388-7> (2019).
13. Vollert, I. *et al.* In vitro perfusion of engineered heart tissue through endothelialized channels. *Tissue engineering. Part A* **20**, 854–63. ISSN: 1937-335X. <http://www.ncbi.nlm.nih.gov/pubmed/24156346> (2014).
14. Laschke, M. W., Vollmar, B. & Menger, M. D. Inosculation: Connecting the Life-Sustaining Pipelines. *Tissue Engineering Part B: Reviews* **15**, 455–465. ISSN: 1937-3368. <https://www.liebertpub.com/doi/10.1089/ten.teb.2009.0252> (Dec. 2009).

15. Weyers, J. J. *et al.* Effects of cell grafting on coronary remodeling after myocardial infarction. *Journal of the American Heart Association* **2**, 1–13. ISSN: 20479980 (2013).
16. Weyers, J. J. *et al.* Sonic Hedgehog upregulation does not enhance the survival and engraftment of stem cell-derived cardiomyocytes in infarcted hearts. *PLOS ONE* **15** (ed Wang, M.) e0227780. ISSN: 1932-6203. <https://dx.plos.org/10.1371/journal.pone.0227780> (Jan. 2020).
17. Qin, W. *et al.* Depth-resolved 3D visualization of coronary microvasculature with optical microangiography. *Physics in Medicine and Biology* **61**, 7536–7550. ISSN: 0031-9155. <http://stacks.iop.org/0031-9155/61/i=21/a=7536?key=crossref.0731d2fb478dd8a1b0065814faec969b> (Nov. 2016).
18. Vinegoni, C., Aguirre, A. D., Lee, S. & Weissleder, R. Imaging the beating heart in the mouse using intravital microscopy techniques. *Nature Protocols* **10**, 1802–1819. ISSN: 1754-2189. <http://www.nature.com/doifinder/10.1038/nprot.2015.119> (Oct. 2015).
19. Merz, S. F. *et al.* Contemporaneous 3D characterization of acute and chronic myocardial I/R injury and response. *Nature Communications* **10**, 1–14. ISSN: 20411723. <http://dx.doi.org/10.1038/s41467-019-10338-2> (2019).
20. Gomez-Garcia, M. J., Quesnel, E., Al-attar, R., Laskary, A. R. & Laflamme, M. A. Maturation of human pluripotent stem cell derived cardiomyocytes in vitro and in vivo. *Seminars in Cell & Developmental Biology*. ISSN: 10849521. <https://doi.org/10.1016/j.semcdb.2021.05.022> <https://linkinghub.elsevier.com/retrieve/pii/S1084952121001312> (May 2021).

21. Guo, Y. & Pu, W. T. Cardiomyocyte Maturation. *Circulation Research* **126**, 1086–1106. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.119.315862> (Apr. 2020).
22. Dhahri, W. *et al.* In Vitro Matured Human Pluripotent Stem Cell-derived Cardiomyocytes Form Grafts With Enhanced Structure and Function in Injured Hearts. *Circulation*. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.121.053563> (Jan. 2022).
23. Abilez, O. J. *et al.* Passive Stretch Induces Structural and Functional Maturation of Engineered Heart Muscle as Predicted by Computational Modeling. *STEM CELLS* **36**, 265–277. ISSN: 10665099. <http://doi.wiley.com/10.1002/stem.2732> (Feb. 2018).
24. Querdel, E. *et al.* Human Engineered Heart Tissue Patches Remuscularize the Injured Heart in a Dose-Dependent Manner. *Circulation* **143**, 1991–2006. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.120.047904> (May 2021).
25. Sun, X. & Nunes, S. S. Maturation of human stem cell-derived cardiomyocytes in biowires using electrical stimulation. *Journal of Visualized Experiments* **2017**, 1–8. ISSN: 1940087X (2017).
26. Tohyama, S. *et al.* Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell* **12**, 127–137. ISSN: 19345909. <http://dx.doi.org/10.1016/j.stem.2012.09.013> (2013).
27. Feyen, D. A. *et al.* Metabolic Maturation Media Improve Physiological Function of Human iPSC-Derived Cardiomyocytes. *Cell Reports* **32**, 107925. ISSN: 22111247. <https://linkinghub.elsevier.com/retrieve/pii/S2211124720309062> (July 2020).

28. Miki, K. *et al.* ERR γ enhances cardiac maturation with T-tubule formation in human iPSC-derived cardiomyocytes. *Nature Communications* **12**, 3596. ISSN: 2041-1723. <http://dx.doi.org/10.1038/s41467-021-23816-3> <http://www.nature.com/articles/s41467-021-23816-3> (Dec. 2021).
29. Funakoshi, S. *et al.* Generation of mature compact ventricular cardiomyocytes from human pluripotent stem cells. *Nature Communications* **12** (2021).
30. Biagi, D. *et al.* In Situ Matured Early-Stage Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes Improve Cardiac Function by Enhancing Segmental Contraction in Infarcted Rats. *Journal of Personalized Medicine* **11**, 374. ISSN: 2075-4426. <https://www.mdpi.com/2075-4426/11/5/374> (May 2021).
31. Riegler, J. *et al.* Human Engineered Heart Muscles Engraft and Survive Long Term in a Rodent Myocardial Infarction Model. *Circulation Research* **117**, 720–730. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.115.306985> (Sept. 2015).
32. Lian, X. *et al.* Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/ β -catenin signaling under fully defined conditions. *Nature Protocols* **8**, 162–175. ISSN: 1754-2189. <http://www.nature.com/doi/10.1038/nprot.2012.150> (2012).
33. Burridge, P. W. *et al.* Chemically defined generation of human cardiomyocytes. *Nature Methods* **11**, 855–860. ISSN: 1548-7091. <http://www.nature.com/doi/10.1038/nmeth.2999> (2014).
34. Rupert, C. E., Irofuala, C. & Coulombe, K. L. K. Practical adoption of state-of-the-art hiPSC-cardiomyocyte differentiation techniques. *PLOS ONE* **15** (ed Guo, Y.) e0230001. ISSN: 1932-6203. <http://dx.doi.org/10.1371/journal.pone.0230001> <https://dx.plos.org/10.1371/journal.pone.0230001> (Mar. 2020).

35. Buikema, J. W. *et al.* Wnt Activation and Reduced Cell-Cell Contact Synergistically Induce Massive Expansion of Functional Human iPSC-Derived Cardiomyocytes. *Cell Stem Cell* **27**, 50–63. ISSN: 18759777. <https://doi.org/10.1016/j.stem.2020.06.001> (2020).
36. Munarin, F., Kaiser, N. J., Kim, T. Y., Choi, B.-R. & Coulombe, K. L. K. Laser-Etched Designs for Molding Hydrogel-Based Engineered Tissues. *Tissue engineering. Part C, Methods* **23**, 311–321. ISSN: 1937-3392. <http://www.ncbi.nlm.nih.gov/pubmed/28457187><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5444510> (2017).
37. Kaiser, N. J., Munarin, F. & Coulombe, K. L. Custom Engineered Tissue Culture Molds from Laser-etched Masters. *Journal of Visualized Experiments* **2018**, 1–7. ISSN: 1940-087X. <https://www.jove.com/video/57239/custom-engineered-tissue-culture-molds-from-laser-etched-masters> (May 2018).
38. Rupert, C. E., Kim, T. Y., Choi, B. R. & Coulombe, K. L. Human Cardiac Fibroblast Number and Activation State Modulate Electromechanical Function of hiPSC-Cardiomyocytes in Engineered Myocardium. *Stem Cells International* **2020**. ISSN: 16879678 (2020).
39. Kant, R. J., Bare, C. F. & Coulombe, K. L. Tissues with Patterned Vessels or Protein Release Induce Vascular Chemotaxis in an In Vitro Platform. *Tissue Engineering Part A* **00**, ten.tea.2020.0269. ISSN: 1937-3341. <https://waww.liebertpub.com/doi/10.1089/ten.tea.2020.0269><https://www.liebertpub.com/doi/10.1089/ten.tea.2020.0269> (Mar. 2021).
40. Kaiser, N. J., Bellows, J. A., Kant, R. J. & Coulombe, K. L. Digital Design and Automated Fabrication of Bespoke Collagen Microfiber Scaffolds. *Tissue Engineering*

- Part C: Methods* **25**, 687–700. ISSN: 1937-3384. <https://www.liebertpub.com/doi/10.1089/ten.tec.2018.0379> (Nov. 2019).
41. Laflamme, M. A. *et al.* Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature Biotechnology* **25**, 1015–1024. ISSN: 10870156 (2007).
42. Jiang, Y., Zhuang, Z. W., Sinusas, A. J., Staib, L. H. & Papademetris, X. in *Journal of Investigative Dermatology* **2**, 528–536 (Feb. 2011). https://linkinghub.elsevier.com/retrieve/pii/S0022202X15370834%20http://link.springer.com/10.1007/978-3-642-23626-6_65.
43. Afgan, E. *et al.* The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2018 update. *Nucleic Acids Research* **46**, W537–W544. ISSN: 0305-1048. <https://academic.oup.com/nar/article/46/W1/W537/5001157> (July 2018).
44. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**, 2114–2120. ISSN: 1460-2059. <https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btu170> (Aug. 2014).
45. Minor, A. J. & Coulombe, K. L. K. Stimulating Calcium Handling in hiPSC-Derived Engineered Cardiac Tissues Enhances Force Production. *Stem Cells Translational Medicine*, 1–10. ISSN: 2157-6564. <https://academic.oup.com/stcltm/advance-article/doi/10.1093/stcltm/szab002/6528832> (Feb. 2022).
46. Shi, L., Ko, S., Ko, M. L., Kim, A. J. & Ko, G. Y. Peptide Lv augments L-type voltage-gated calcium channels through vascular endothelial growth factor receptor 2 (VEGFR2) signaling. *Biochimica et Biophysica Acta - Molecular Cell Research*

- 1853**, 1154–1164. ISSN: 18792596. <http://dx.doi.org/10.1016/j.bbamcr.2015.02.007> (2015).
47. Rottbauer, W. *et al.* VEGF-PLC γ 1 pathway controls cardiac contractility in the embryonic heart. *Genes and Development* **19**, 1624–1634. ISSN: 08909369 (2005).
 48. Rupert, C. E. & Coulombe, K. L. IGF1 and NRG1 Enhance Proliferation, Metabolic Maturity, and the Force-Frequency Response in hESC-Derived Engineered Cardiac Tissues. *Stem Cells International* **2017**. ISSN: 16879678 (2017).
 49. Segers, V. F. M., Brutsaert, D. L. & De Keulenaer, G. W. Cardiac Remodeling: Endothelial Cells Have More to Say Than Just NO. *Frontiers in Physiology* **9**. ISSN: 1664-042X. <http://journal.frontiersin.org/article/10.3389/fphys.2018.00382/full> (Apr. 2018).
 50. Leucker, T. M. *et al.* Endothelial–cardiomyocyte crosstalk enhances pharmacological cardioprotection. *Journal of Molecular and Cellular Cardiology* **51**, 803–811. ISSN: 00222828. <https://linkinghub.elsevier.com/retrieve/pii/S0022282811002744> (Nov. 2011).
 51. Talman, V. & Kivelä, R. Cardiomyocyte—Endothelial Cell Interactions in Cardiac Remodeling and Regeneration. *Frontiers in Cardiovascular Medicine* **5**, 1–8. ISSN: 2297-055X. <https://www.frontiersin.org/article/10.3389/fcvm.2018.00101/full> (July 2018).
 52. Doriot, P.-A. *et al.* In-vivo measurements of wall shear stress in human coronary arteries. *Coronary Artery Disease* **11**, 495–502. ISSN: 0954-6928. <http://journals.lww.com/00019501-200009000-00008> (Sept. 2000).
 53. Dhawan, S. S. *et al.* Shear stress and plaque development. *Expert Review of Cardiovascular Therapy* **8**, 545–556. ISSN: 1477-9072. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5467309/pdf/nihms213759.pdf>

- 20<http://www.tandfonline.com/doi/full/10.1586/erc.10.28>
(Apr. 2010).
54. Debbi, L., Zohar, B., Shandalov, Y. & Levenberg, S. Integrating Engineered Macro Vessels with Self-assembled Capillaries in 3D Implantable Tissue for Promoting Vascular Integration In-vivo. *BioRxiv* (2020).
 55. Fang, Y. *et al.* Optimizing Bifurcated Channels within an Anisotropic Scaffold for Engineering Vascularized Oriented Tissues. *Advanced Healthcare Materials* **9**, 1–9. ISSN: 21922659 (2020).
 56. Kurokawa, Y. K. *et al.* Human Induced Pluripotent Stem Cell-Derived Endothelial Cells for Three-Dimensional Microphysiological Systems. *Tissue Engineering Part C: Methods* **23**, 474–484. ISSN: 1937-3384. <http://online.liebertpub.com/doi/10.1089/ten.tec.2017.0133> (2017).
 57. Cunningham, K. S. & Gotlieb, A. I. The role of shear stress in the pathogenesis of atherosclerosis. *Laboratory Investigation* **85**, 9–23. ISSN: 0023-6837. <http://www.nature.com/articles/3700215> (Jan. 2005).
 58. Resnick, N. *et al.* Fluid shear stress and the vascular endothelium: for better and for worse. *Progress in Biophysics and Molecular Biology* **81**, 177–199. ISSN: 00796107. <https://linkinghub.elsevier.com/retrieve/pii/S0079610702000524>
(Apr. 2003).
 59. Tiburcy, M. *et al.* Defined Engineered Human Myocardium With Advanced Maturation for Applications in Heart Failure Modeling and Repair. *Circulation* **135**, 1832–1847. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.116.024145> (May 2017).
 60. Wang, H. *et al.* Necking and failure of constrained 3D microtissues induced by cellular tension. *Proceedings of the National Academy of Sciences* **110**, 20923–20928.

- ISSN: 0027-8424. <http://www.pnas.org/cgi/doi/10.1073/pnas.1313662110> (2013).
61. Gerbin, K. A., Yang, X., Murry, C. E. & Coulombe, K. L. K. Enhanced electrical integration of engineered human myocardium via intramyocardial versus epicardial delivery in infarcted rat hearts. *PLoS ONE* **10**, 1–20. ISSN: 19326203 (2015).
62. Weinberger, F. *et al.* Cardiac repair in guinea pigs with human engineered heart tissue from induced pluripotent stem cells. *Science Translational Medicine* **8**, 148–363. ISSN: 1946-6234. <http://stm.sciencemag.org/cgi/doi/10.1126/scitranslmed.aaf8781> (2016).
63. Tang, F. *et al.* Microchannels are an architectural cue that promotes integration and vascularization of silk biomaterials in vivo. *ACS Biomaterials Science and Engineering* **6**, 1476–1486. ISSN: 23739878 (2020).
64. Jiang, Z. *et al.* Construction of chitosan scaffolds with controllable microchannel for tissue engineering and regenerative medicine. *Materials Science and Engineering: C* **126**, 112178. ISSN: 09284931. <https://doi.org/10.1016/j.msec.2021.112178>
<https://linkinghub.elsevier.com/retrieve/pii/S0928493121003179> (July 2021).
65. Lee, J., Wu, W., Jiang, J. Y., Zhu, B. & Boas, D. A. Dynamic light scattering optical coherence tomography. *Optics Express* **20**, 22262. ISSN: 1094-4087. <https://www.osapublishing.org/oe/abstract.cfm?uri=oe-20-20-22262> (Sept. 2012).
66. Redenski, I. *et al.* Microcomputed Tomography-Based Analysis of Neovascularization within Bioengineered Vascularized Tissues. *ACS Biomaterials Science & Engineering* **8**, 232–241. ISSN: 2373-9878. <https://pubs.acs.org/doi/10.1021/acsbiomaterials.1c01401> (Jan. 2022).

67. Cruz-Moreira, D. *et al.* Assessing the influence of perfusion on cardiac microtissue maturation: A heart-on-chip platform embedding peristaltic pump capabilities. *Biotechnology and Bioengineering* **118**, 3128–3137. ISSN: 0006-3592. <https://onlinelibrary.wiley.com/doi/10.1002/bit.27836> (Aug. 2021).
68. Quaife-Ryan, G. A. *et al.* Multicellular transcriptional analysis of mammalian heart regeneration. *Circulation* **136**, 1123–1139. ISSN: 15244539 (2017).
69. McLellan, M. A. *et al.* High-Resolution Transcriptomic Profiling of the Heart During Chronic Stress Reveals Cellular Drivers of Cardiac Fibrosis and Hypertrophy. *Circulation* **142**, 1448–1463. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.119.045115> (Oct. 2020).
70. Wang, Y. *et al.* Single-cell analysis of murine fibroblasts identifies neonatal to adult switching that regulates cardiomyocyte maturation. *Nature Communications* **11**, 2585. ISSN: 2041-1723. <http://dx.doi.org/10.1038/s41467-020-16204-w> <http://www.nature.com/articles/s41467-020-16204-w> (Dec. 2020).
71. Kluin, R. J. C. *et al.* XenofilteR: computational deconvolution of mouse and human reads in tumor xenograft sequence data. *BMC Bioinformatics* **19**, 366. ISSN: 1471-2105. <https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-018-2353-5> (Dec. 2018).

Chapter 5

A novel approach to cardiac revascularization

5.1 Synergy of Specific Aims

CVD and HF are leading causes of global morbidity and mortality. Conventional standards of healthcare treatment have not kept up with the demands of CVD progression, necessitating further innovations to improve heart function after injury such as MI, and ultimately improve patient prognosis and quality of life. Despite a significant amount of ongoing research in hPSC-CM-based therapeutics for post-MI treatment, we noticed a distinct dearth of literature regarding the role of vascularization, particularly at the large-animal and clinical stages. These works focused instead on contractile and electrophysiological maturation, with little consideration as to the potential benefits of providing vascular conduits on EHM development *in vivo*.

We thus identified vascularization as a key factor for study in this dissertation work, and proposed that robust EHM prevascularization would enable greater engraftment, perfusion,

and maturation. We designed a set of cohesive and far-reaching Specific Aims intended to develop a greater understanding of dynamic *in vivo* vascularization in the context of engineering a cellular therapeutic and its impact on improving EHM engraftment and development. Our central hypothesis was that patterning large, arteriole-scale vascular channels in an EHM would provide greater avenues for bulk tissue inosculation and perfusion, thereby enabling greater cardiomyocyte engraftment and maturation.

Together, our Aims allowed for the development of a sophisticated and cohesive system for studying the role of patterned vasculature in directing host vascular migration (Aim 1), implementation of the technique in a cardiac regenerative engineering context to determine structural and functional consequences of tissue formation (Aim 2), and *in vivo* application to investigate impact in a complex, physiologically relevant environment and validate *in vitro* findings (Aim 3). This pipeline allowed for iterative improvement on the patterning design and technique informed by additional considerations and constraints as the project transitioned from an *in vitro* vascularization study to *in vivo* cardiac tissue implantation research. We propose our findings as an effective dual remuscularization-revascularization treatment of MI that serves as a foundational basis for critical study and translational application of vascularization techniques in concert with epicardial delivery of hiPSC-CMs for therapeutic benefit. Novel contributions of our work to the field will support further scientific contributions towards the goal of biomanufacturing vascularized hPSC-CM patches for clinical scale-up and applications.

5.2 Broader impacts on heart regeneration

This dissertation work contributes to the fields of cardiovascular tissue engineering and heart regeneration a number of novel techniques for both experimental design and analysis of *in vitro* and *in vivo* vascular phenomena, contextualized by the needs of cardiac tissue

engineering applications. Vascular considerations in the field remain a vastly understudied area despite its foundational importance in supporting cell viability, and this research addresses this critical need across multiple scales. By leveraging existing assays and clinically available technologies for use in novel contexts, our innovations are relevant and easily adaptable for benchside, pre-clinical, and translational applications in cardiac tissue engineering.

Our work in Aim 1 greatly improved upon the already robust aortic ring assay, which examines vascular outgrowth in response to angiogenic agents [1]. Analysis has typically relied on manual measurement and counting of vascular branches, which while appropriate for studying drug and molecule-based interventions, was not easily implementable in a tissue engineering context. Modification of our group's PDMS molding system [2] allowed for co-culture of aortic rings with engineered tissues [3]. This change widened the possible applications of the assay, importantly allowing the study of angiogenic signaling interplay between surrogate host and implanted tissue, lending the assay more relevance to an *in vivo* environment and to the tissue engineering field. To support this change we developed a suite of unbiased and open-source analysis tools to evaluate vascular migration from the aortic ring using histological stains or micrographs. More broadly, these tools are widely applicable for other tissue engineering analyses, such as the use of the nearest neighbors algorithm to study density or infiltration into a tissue. Together, these innovations provide new options for tissue engineers to evaluate host-implant vascular migration in a controlled but complex *in vitro* environment to study vascular efficacy of a tissue engineered therapeutic before *in vivo* implementation.

Leading translational research in large animal studies [4–6] and even upcoming clinical research involving cellular therapeutics with hiPSC-CMs such as the BioVAT HF trial (NCT04396899) and HECTOR trial (NCT05068674) are as of yet unable to determine true engraftment efficiency or its impact on long-term function due to a lack of focus on a vas-

cular component to their studies. Higher-ordered vascular organization is fundamentally incompatible with intramyocardial injection-based therapeutics, with the only option being co-injection of endothelial cells or other microvessel-based sources [7]. Even some epicardial patch-based methods, such as those by the Eschenhagen group [6] and our own [2, 8], have used internal post geometries to direct internal tissue alignment, allowing for greater contractile alignment but similarly preventing vascular design considerations. Our current studies have eschewed this design format to accommodate larger-order vascular channels for robust prevascularization, though we noted its effects on bulk cellular alignment *in vitro*. However, literature including our work has shown the benefits of a patterned vasculature on patch survival and engraftment, even if only transiently present [9–11]. While there is published literature on the effects of *in vitro* prevascularization [12–14] on *in vivo* inosculation, our study developed further contributions both in accessibility and scientific merit.

As part of Aim 2, we enabled broader adoption of dynamic perfusion culture of engineered tissues through our open-source peristaltic pump using common Arduino-based electronics and fabrication methods that enables low-flow perfusion of vEHMs in a sterile environment that is compatible with standard tissue culture equipment and disposables. These designs are approximately 10-20% of the cost of a commercial equivalent and are freely accessible online and can be further customized by the end user to lower the barrier to entry and accelerate research in the area of cardiovascular tissue engineering. Additionally, we showed for the first time the effects of cell-type specific medias on heterocellular tissue culture in a 2D and 3D context and motivate specific mixture ratios based on structural consequences at the cellular level. The apparent contractile maturation of hiPSC-CMs seen in mixed media culture parallels the field's more recent pushes to understand and apply maturation methodologies to hiPSC-CMs [15, 16]. Our work joins a growing volume of literature demonstrating methods of maturing cardiomyocytes, but importantly places it in context of a heterocellular, and thus more physiologically relevant, microenvironment. However, while maturation mediums are optimized for hiPSC-CM development, our work

illustrates the need for a defined medium to maximize heterocellular co-culture and cell-cell interactions.

Implantation of our EHMs in Aim 3 advanced the field's understanding of vascularization and its effect on implant development *in vivo* through advancements in imaging applications and implant genomic analysis. In particular, our advancements in imaging modalities using microCT-based reconstructions and OCT imaging allowed us to capture perfused vessel analyses across micro and macro scales and more accurately determine the post-implant vascular remodeling that occurs *in vivo*. Our results demonstrate the benefits of coupled 3D perfused vascular analyses that are unable to be captured from histological staining alone that reveal nuances of vascular remodeling initiated by *in vitro* vessel patterning and perfusion. Further, our work is pioneering the workflow for performing these precise and comprehensive analyses, which can be implemented into the existing methodologies for terminal *in vivo* analysis as they are more widely adopted by the field.

Finally, our RNASeq analyses of explanted cardiac tissue represents a large leap in the field, as to our knowledge it has never been performed before on engrafted hiPSC-CMs. Multi-species RNASeq in this context has similarly not been performed, as existing literature focuses primarily on xenografts in the context of cancer [17]. Next generation sequencing has become a fairly ubiquitous characterization technique owing to a greater understanding of the human genome and continual technological innovations that improve the power of these analyses and reduce costs. Our results demonstrate that transcript-level analyses reveal nuanced differences in treatment groups that would not be identified by histological staining alone. These analyses also consider the contribution of the host to implant remodeling over time, and shows the value of bulk RNASeq as a powerful analysis tool, particularly as a more accessible alternative to single cell RNASeq for widespread adoption and further advancements in analyzing implant development.

5.3 Future directions

As the cardiovascular tissue engineering field advances towards translational implementations of hPSC-CM-based therapeutics, a number of barriers remain to be addressed. Safety is a cornerstone of phase 1 and 2 trials, and ongoing trials typically center primary outcomes around incidence of adverse events or arrhythmias (e.g., NCT04982081, NCT03763136, NCT04696328). Pre-clinical research, particularly at the large-animal scale, thus focuses primarily on hPSC-CM contractile maturation, electrical coupling, and arrhythmia susceptibility. However, as subsequent waves of clinical trials begin to more rigorously examine clinical efficacy through functional or surrogate endpoints for efficacy (including some ongoing trials, e.g. NCT05068674, NCT04396899, NCT03759405, and NCT04696328), the importance of vascular contributions to hPSC-CM engraftment, maturation, and thus efficacy of cellular therapeutics will become more apparent. This dissertation research demonstrates the essential role of revascularization co-therapy with remuscularization and is visionary in leveraging the host vascular response to improve hiPSC-CM engraftment. Further, this research provides necessary infrastructure for future large animal and clinical work in determining the effect of *in vitro* prevascularization in functional dosing studies. In the shorter term, our work provides opportunities for further research in both *in vitro* and small animal *in vivo* aspects.

Our *in vitro* host-implant inosculation model based on the aortic ring assay provides a particularly flexible platform for further study by modulating variables including the aortic ring itself, geometry via the PDMS mold, and the co-cultured engineered tissue. Recent literature has demonstrated that harvested vessels show distinct angiogenic phenotypes dependent on the location they were harvested from (e.g. carotid or femoral artery) [18]. For applications in cardiovascular tissue engineering, use of the left or right coronary artery would be appropriate to better recapitulate the microenvironment of vascular cells that an implanted cardiac tissue would be interacting with. Co-culture of aortic rings with vEHMs

would provide an additional suite of further studies in host-implant interactions such as in CM-EC cross-talk [19]. ECM remodeling such as by cardiac fibroblasts in vEHMs would provide further insight into how implant-mediated remodeling cues may influence vascular chemotaxis, such as by creating "highways" of oriented fibrils for directed migration towards vEHMs.

Our work combined the use of 3 different cell types of interest for our applications in cardiac tissue engineering to create complex and robust EHMs. Future studies will be focused on incorporating hiPSC-ECs and hiPSC-CFs, as autologous implants are an ultimate translational goal of this work. Further, in-house derivation provides additional flexibility in using specific cellular sub-types. Recent literature has demonstrated the ability to differentiate mature compact ventricular myocardium, which is associated with the outer ventricular wall for efficient contractile function, as opposed to trabecular myocardium associated with the inner lumen [20]. Coronary artery ECs would be an appropriate cell type to populate our arteriole-scale channels, as EC sub-types show differing angiogenic properties [12, 21]. More broadly, the use of hypoimmune hiPSC-derived cells [22] may also be beneficial in clinical interventions where personalized autologous therapeutics are not feasible. While this work is relatively new, pre-clinical work in immune-competent allogeneic mice recipients has already demonstrated rescued perfusion in a hind limb ischemia model with implanted hypoimmune hiPSC-ECs and alleviation of functional parameters in infarcted mice hearts with injected hypoimmune hiPSC-CMs and hiPSC-ECs [23].

Our work also considered the *in vitro* media needs of distinct cell types when in heterocellular culture. However, our proposed media mixture does not represent an optimal solution, and future work must identify new formulations to best ensure individual cell type survival without compromised function. This aspect is particularly challenging considering the current research emphasis on metabolic maturation of cardiomyocytes to push a more adult-like phenotype [15, 16], primarily by relying on fatty acids to shift the hPSC-CM

metabolic profile towards beta oxidation of lipids. While research in this area is extremely promising, it's unclear how hiPSC-CM structure and metabolism shift if only transiently exposed to such maturation media, or the effects of such media on other key cell types including endothelial cells and fibroblasts. Thus, developing an optimal co-culture medium will be necessary in future work. This may necessitate individual testing of components to best understand their individual contribution, and lead to the formulation of bespoke medias based on application.

In advancing vascularization techniques for hiPSC-CM-based therapeutics towards clinical translation, scaling up tissues to sizes appropriate for large animal and eventually clinical trials remains a challenge. Querdel et al. proposed a therapeutic threshold for improving cardiac function to be approximately 3.3×10^6 CMs engrafted per gram of heart, or effectively 1×10^9 cells for an average 300 g human heart [6]. However, current research including our own suggests average engraftment rates of 10-20% of infarct area, suggesting significant acute hiPSC-CM death post-implantation which can currently only be overcome by delivering far more cells than the target threshold [6, 10, 24, 25]. This greatly motivates further work in vascularization as patch sizes necessary to accommodate such a high number of hiPSC-CMs will necessitate solutions to further mitigate hypoxic stress, particularly once implanted. Thus, future studies must focus on upscaling epicardial patch sizes and densities, incorporating appropriate vasculature for nutrient delivery, and integrating these needs with more sophisticated automation technologies. Automation of these processes will greatly facilitate scale-up both from an efficiency and GMP perspective. Efforts in bioprinting are poised to become leaders in this regard within the next 5-10 years, with groups including the Feinberg lab at Carnegie Mellon University [26] and the Miller Lab (now Volumetric Biotechnologies) [27] already developing intricate technologies and protocols for complex vascular and cardiac scaffold fabrication. However, integration with the specific considerations of a translational hiPSC-CM implant such as with our work will be necessary in implementing automation into clinical workflows.

Beyond the novelties to the field established from our work, continued development of *in vivo* implant evaluation remains an ongoing focus of preclinical work. In particular, RNASeq has potential to better understand exact mechanisms of implanted cardiac tissue remodeling and maturation *in vivo*. Future work could use transcriptional RNASeq data to understand key factors dictating *in vivo* cardiac maturation, and particularly any interplay with vascular cross-talk. This data could be used to determine appropriate maturation pathways to upregulate *in vitro* prior to implantation such as through CRISPR-Cas9-mediated gene insertion or peptide/small molecule treatments. There is also a need to move to single cell RNASeq for more robust analyses of cell populations [28]. The goals of future work should be to understand each population's individual contributions to implant development and remodeling, particularly as they relate to vascularization and its effects on engraftment and cardiac maturation. These analyses combined with epigenetic profiling via ATAC sequencing will allow for distinct cell subtype specification in the implant and heart and visualization of population shifts across the initial inosculation stages post-implantation and subsequent remodeling across months. Our work also demonstrated the effects of perfusion on reducing implant vessel tortuosity, and exploratory RNASeq may be beneficial in determining exact mechanisms for inducing or otherwise maintaining vascular organization. On an organ level, 3D echocardiographic strain analysis should be performed to understand regional mechanics in the implant and underlying infarct/myocardium, and how vascular tortuosity and perfusion may play a role in remodeling, vascular and ECM alignment, and thus pumping function over time. Lastly, temporal considerations must be addressed to better understand how implants remodel and persist at longer timepoints. Our two week study demonstrated more robust engraftment of hiPSC-CMs when perfused *in vitro*, but studies out to 4 weeks or greater must be performed to understand if long-term engraftment is maintained, and importantly the impact of prevascularization on maintenance of the implant. Further, our work represents relatively acute intervention, but further studies must understand how implant integration is impacted in patient populations with chronic HF.

5.4 Conclusions

This dissertation research accelerates hiPSC-CMS therapies for remuscularizing the heart post-MI. Our innovations across each of the 3 Specific Aims are advancing areas of cardiovascular tissue engineering not yet adequately explored at the large animal and clinical levels and will greatly impact the field. Future work spurred by our foundational studies will enable greater study of mechanisms how of vascularization improves engraftment and thus mechanical benefits in the post-infarct heart, and drive further clinical applications of cellular therapeutics for HF using dual revascularization-remuscularization treatments.

5.5 References

1. Nicosia, R. F. The aortic ring model of angiogenesis: a quarter century of search and discovery. *Journal of Cellular and Molecular Medicine* **13**, 4113–4136. ISSN: 15821838. <http://doi.wiley.com/10.1111/j.1582-4934.2009.00891.x> (Oct. 2009).
2. Munarin, F., Kaiser, N. J., Kim, T. Y., Choi, B.-R. & Coulombe, K. L. K. Laser-Etched Designs for Molding Hydrogel-Based Engineered Tissues. *Tissue engineering. Part C, Methods* **23**, 311–321. ISSN: 1937-3392. <http://www.ncbi.nlm.nih.gov/pubmed/28457187><http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC5444510> (2017).
3. Kant, R. J., Bare, C. F. & Coulombe, K. L. Tissues with Patterned Vessels or Protein Release Induce Vascular Chemotaxis in an In Vitro Platform. *Tissue Engineering Part A* **00**, ten.tea.2020.0269. ISSN: 1937-3341. <https://waww.liebertpub.com/doi/10.1089/ten.tea.2020.0269><https://www.liebertpub.com/doi/10.1089/ten.tea.2020.0269> (Mar. 2021).

4. Romagnuolo, R. *et al.* Human Embryonic Stem Cell-Derived Cardiomyocytes Regenerate the Infarcted Pig Heart but Induce Ventricular Tachyarrhythmias. *Stem Cell Reports* **12**, 967–981. ISSN: 22136711 (2019).
5. Dhahri, W. *et al.* In Vitro Matured Human Pluripotent Stem Cell-derived Cardiomyocytes Form Grafts With Enhanced Structure and Function in Injured Hearts. *Circulation*. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.121.053563> (Jan. 2022).
6. Querdel, E. *et al.* Human Engineered Heart Tissue Patches Remuscularize the Injured Heart in a Dose-Dependent Manner. *Circulation* **143**, 1991–2006. ISSN: 0009-7322. <https://www.ahajournals.org/doi/10.1161/CIRCULATIONAHA.120.047904> (May 2021).
7. Sun, X. *et al.* Transplanted microvessels improve pluripotent stem cell-derived cardiomyocyte engraftment and cardiac function after infarction in rats. *Science Translational Medicine* **12**, 1–11. ISSN: 19466242 (2020).
8. Munarin, F., Kant, R. J., Rupert, C. E., Khoo, A. & Coulombe, K. L. Engineered human myocardium with local release of angiogenic proteins improves vascularization and cardiac function in injured rat hearts. *Biomaterials* **251**, 120033. ISSN: 01429612. <https://doi.org/10.1016/j.biomaterials.2020.120033> <https://linkinghub.elsevier.com/retrieve/pii/S0142961220302799> (Aug. 2020).
9. Mirabella, T. *et al.* 3D-printed vascular networks direct therapeutic angiogenesis in ischaemia. *Nature Biomedical Engineering* **1**, 0083. ISSN: 2157-846X. <http://www.nature.com/articles/s41551-017-0083> (June 2017).
10. Redd, M. A. *et al.* Patterned human microvascular grafts enable rapid vascularization and increase perfusion in infarcted rat hearts. *Nature Communications* **10**, 1–14.

- ISSN: 20411723. <http://dx.doi.org/10.1038/s41467-019-08388-7> (2019).
11. Brady, E. L. *et al.* Guided vascularization in the rat heart leads to transient vessel patterning. *APL Bioengineering* **4**, 016105. ISSN: 2473-2877. <http://aip.scitation.org/doi/10.1063/1.5122804> (Mar. 2020).
 12. Kant, R. J. & Coulombe, K. L. Integrated approaches to spatiotemporally directing angiogenesis in host and engineered tissues. *Acta Biomaterialia* **69**, 42–62. ISSN: 17427061. <https://doi.org/10.1016/j.actbio.2018.01.017> %20<https://linkinghub.elsevier.com/retrieve/pii/S174270611830028X> (Mar. 2018).
 13. Laschke, M. W. & Menger, M. D. Prevascularization in tissue engineering: Current concepts and future directions. *Biotechnology Advances* **34**, 112–121. ISSN: 07349750. <http://dx.doi.org/10.1016/j.biotechadv.2015.12.004> %20<http://linkinghub.elsevier.com/retrieve/pii/S0734975015300598> (Mar. 2016).
 14. Chen, X. *et al.* Prevascularization of a Fibrin-Based Tissue Construct Accelerates the Formation of Functional Anastomosis with Host Vasculature. *Tissue Engineering Part A* **15**, 1363–1371. ISSN: 1937-3341. <http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2008.0314> (June 2009).
 15. Feyen, D. A. *et al.* Metabolic Maturation Media Improve Physiological Function of Human iPSC-Derived Cardiomyocytes. *Cell Reports* **32**, 107925. ISSN: 22111247. <https://linkinghub.elsevier.com/retrieve/pii/S2211124720309062> (July 2020).
 16. Guo, Y. & Pu, W. T. Cardiomyocyte Maturation. *Circulation Research* **126**, 1086–1106. ISSN: 0009-7330. <https://www.ahajournals.org/doi/10.1161/CIRCRESAHA.119.315862> (Apr. 2020).

17. Kluin, R. J. C. *et al.* XenofilteR: computational deconvolution of mouse and human reads in tumor xenograft sequence data. *BMC Bioinformatics* **19**, 366. ISSN: 1471-2105. <https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-018-2353-5> (Dec. 2018).
18. Vaghela, R. *et al.* Vessel grafts for tissue engineering revisited—Vessel segments show location-specific vascularization patterns in ex vivo ring assay. *Microcirculation* **29**, 1–8. ISSN: 1073-9688. <https://onlinelibrary.wiley.com/doi/10.1111/micc.12742> (Feb. 2022).
19. Leucker, T. M. *et al.* Endothelial–cardiomyocyte crosstalk enhances pharmacological cardioprotection. *Journal of Molecular and Cellular Cardiology* **51**, 803–811. ISSN: 00222828. <https://linkinghub.elsevier.com/retrieve/pii/S0022282811002744> (Nov. 2011).
20. Funakoshi, S. *et al.* Generation of mature compact ventricular cardiomyocytes from human pluripotent stem cells. *Nature Communications* **12** (2021).
21. Sieveking, D. P., Buckle, A., Celermajer, D. S. & Ng, M. K. C. Strikingly Different Angiogenic Properties of Endothelial Progenitor Cell Subpopulations. Insights From a Novel Human Angiogenesis Assay. *Journal of the American College of Cardiology* **51**, 660–668. ISSN: 07351097 (2008).
22. Deuse, T. *et al.* Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nature Biotechnology* **37**, 252–258. ISSN: 1087-0156. <http://www.nature.com/articles/s41587-019-0016-3> (Mar. 2019).
23. Deuse, T. *et al.* Hypoimmune induced pluripotent stem cell–derived cell therapeutics treat cardiovascular and pulmonary diseases in immunocompetent allogeneic mice. *Proceedings of the National Academy of Sciences* **118**, e2022091118. ISSN:

- 0027-8424. <http://www.pnas.org/lookup/doi/10.1073/pnas.2022091118> (July 2021).
24. Gerbin, K. A., Yang, X., Murry, C. E. & Coulombe, K. L. K. Enhanced electrical integration of engineered human myocardium via intramyocardial versus epicardial delivery in infarcted rat hearts. *PLoS ONE* **10**, 1–20. ISSN: 19326203 (2015).
25. Shiba, Y. *et al.* Electrical integration of human embryonic stem cell-derived cardiomyocytes in a guinea pig chronic infarct model. *Journal of Cardiovascular Pharmacology and Therapeutics* **19**, 368–381. ISSN: 19404034 (2014).
26. Lee, A. *et al.* 3D bioprinting of collagen to rebuild components of the human heart. *Science* **365**, 482–487. ISSN: 0036-8075. <https://www.sciencemag.org/lookup/doi/10.1126/science.aav9051><https://www.science.org/doi/10.1126/science.aav9051> (Aug. 2019).
27. Kinstlinger, I. S. *et al.* Perfusion and endothelialization of engineered tissues with patterned vascular networks. *Nature Protocols* **16**, 3089–3113. ISSN: 1754-2189. <http://dx.doi.org/10.1038/s41596-021-00533-1><http://www.nature.com/articles/s41596-021-00533-1> (June 2021).
28. Hegenbarth, J.-C., Lezzoche, G., De Windt, L. J. & Stoll, M. Perspectives on Bulk-Tissue RNA Sequencing and Single-Cell RNA Sequencing for Cardiac Transcriptomics. *Frontiers in Molecular Medicine* **2**, 1–14. ISSN: 2674-0095. <https://www.frontiersin.org/articles/10.3389/fmmed.2022.839338/full> (Feb. 2022).

Appendix A

Supplementary Information Related to Chapter 3

Material in Appendix A is reproduced with permission from R. J. Kant, C. F. Bare, and K. L. K Coulombe, “Tissues with patterned vessels or protein release induce vascular chemotaxis in an *in vitro* platform”, *Tissue Eng. Part A*, 27 (19-20), 1290-1304, Oct 2021.

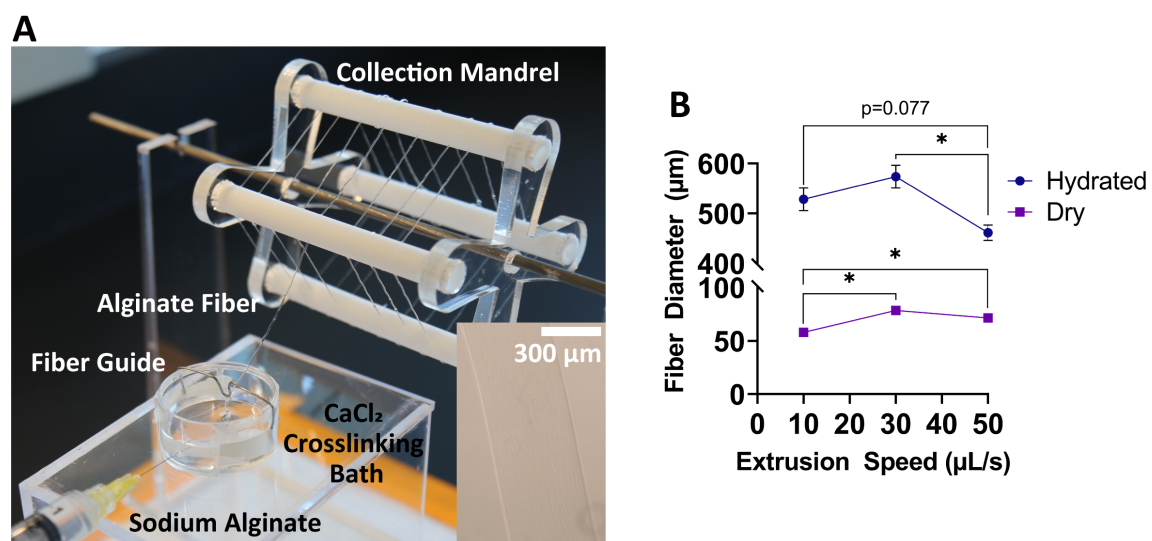


Figure A.1: Wet spinning of sacrificial alginate fibers. **(A)** Wet spinning apparatus to create and dry alginate fibers. **(B)** Diameter of dry and hydrated fiber as a function of alginate extrusion speed. Hydrated ($n = 25-30$), Dry ($n = 7-10$). *: $p < 0.05$.

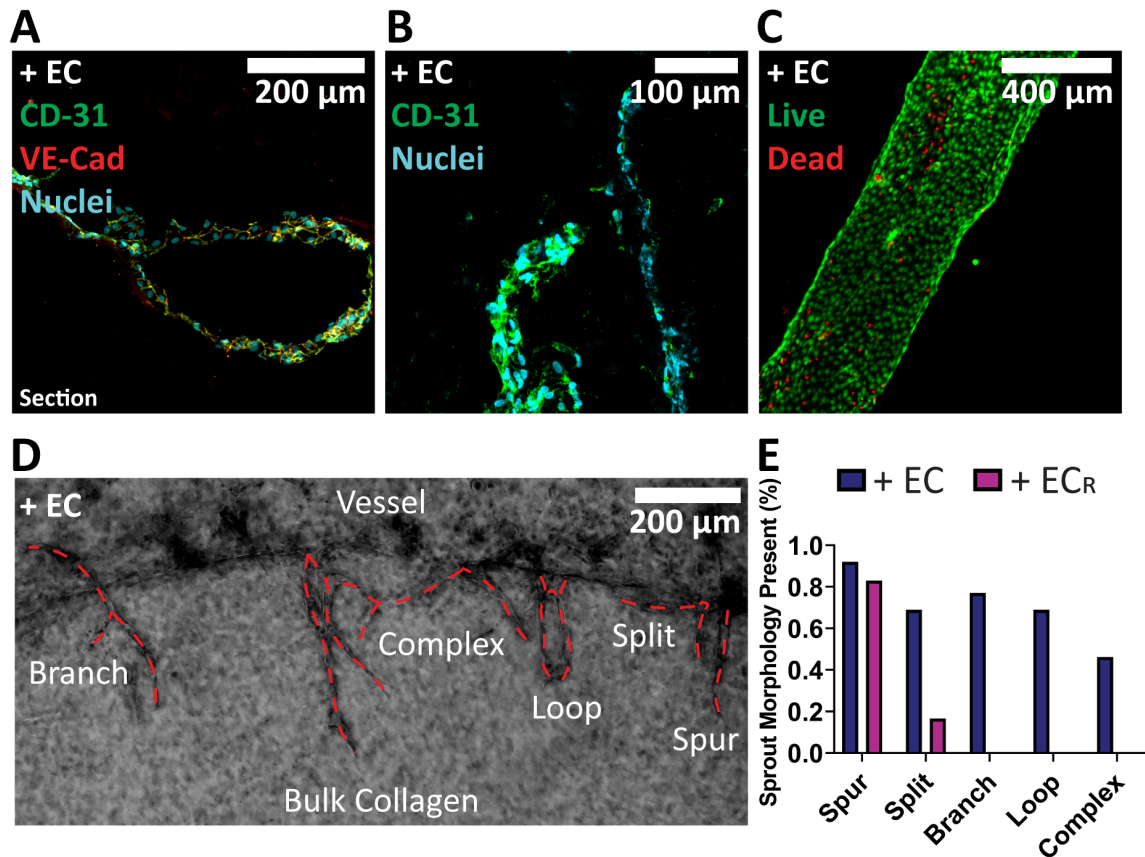


Figure A.2: Engineered vessel cross-section and regression. (A) Cross-section of engineered vessel with a sprout showing full wall coverage (20x magnification). (B) Angiogenic sprout adjacent to a regressed sprout (30x magnification). (C) Live/dead stain of an engineered vessel at day 7 exhibiting a non-angiogenic phenotype with regressed sprouts (10x magnification). (D) Phase contrast image (4x magnification) of a channel describing observed sprouting morphologies and (E) quantification of sprout morphologies that arose throughout the experiments. + EC (n = 13), + EC_R (n = 6).

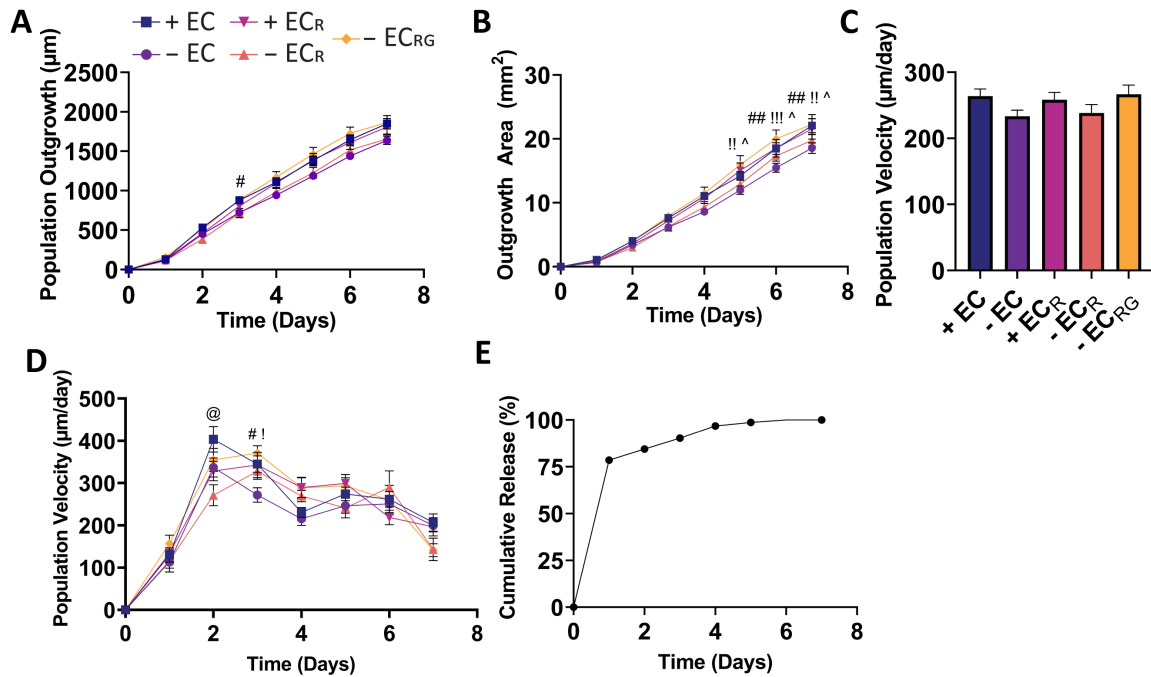


Figure A.3: Population outgrowth metrics. (A) Average outgrowth distance and (B) area per day. (C) Average and (D) per-day population velocity (days 2 and 3 emphasized in Fig. 3D). (E) Cumulative release of model protein from construct gels. + EC (n = 17), - EC (n = 15), + EC_R (n = 11), - EC_R (n = 11), - EC_{RG} (n = 8). *: p < 0.05, **: p < 0.01, ***: p < 0.001. #: Significance between + EC and - EC. !: Significance between - EC_{RG} and - EC. ^: Significance between - EC and + EC_R. @: Significance between + EC and all groups except - EC_{RG}.

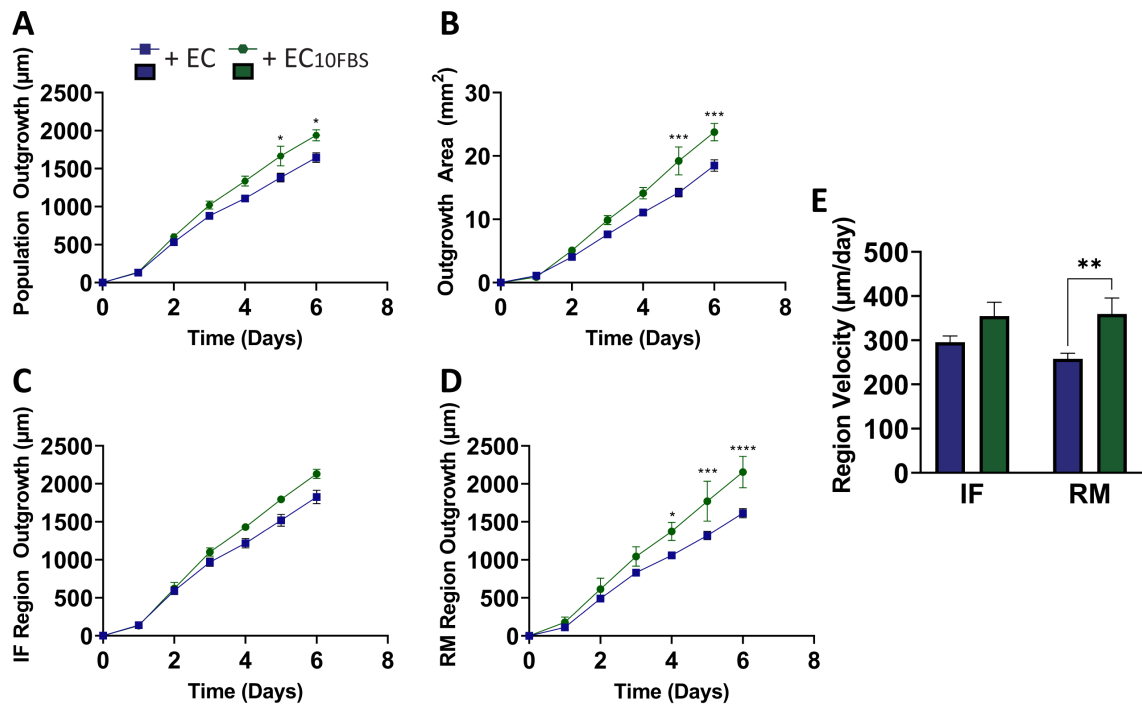


Figure A.4: Outgrowth metrics comparing + EC group to setups containing engineered vessels and EGM-2 media supplemented to 10% FBS (+ EC_{10FBS}). (A) Total outgrowth, (B) area, (C) IF region outgrowth, and (D) RM region outgrowth per day. (E) Regional velocity. + EC (n = 17), + EC_{10FBS} (n = 4). *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

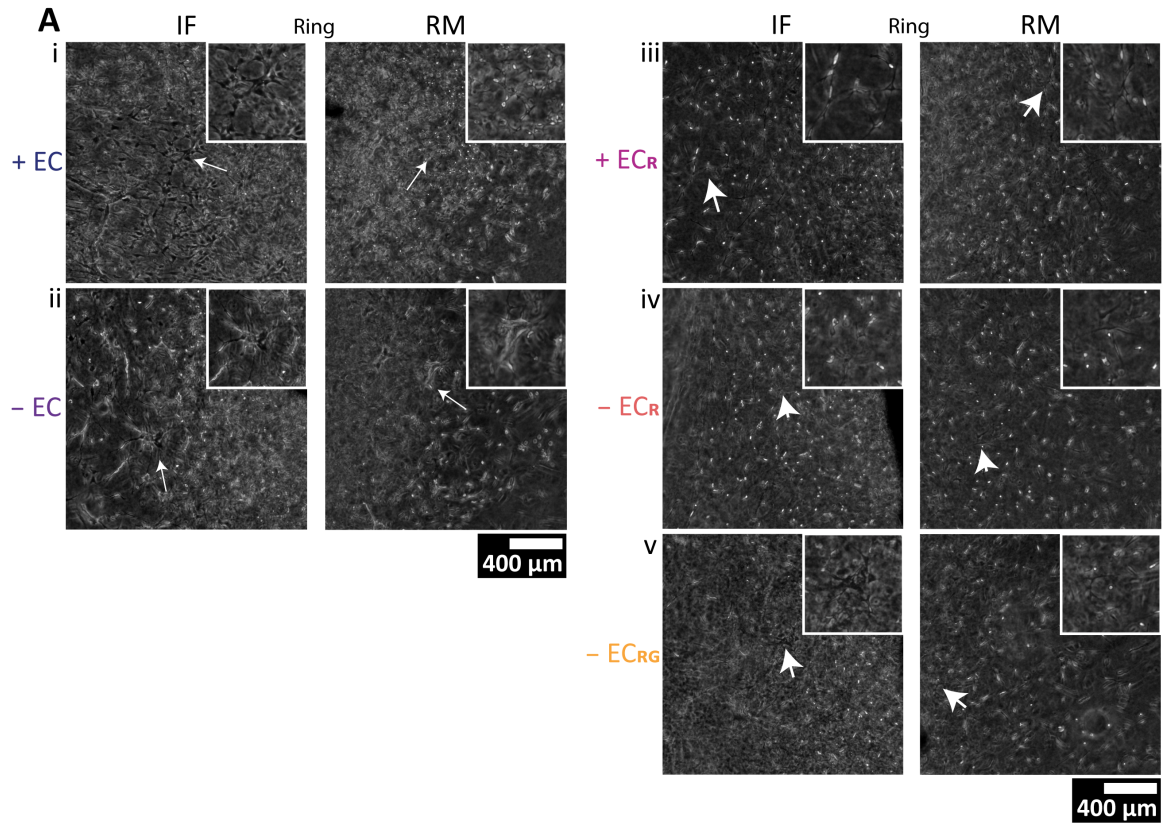


Figure A.5: Representative images of vascular outgrowth from distinct interface (IF) and remote (RM) regions adjacent to the aortic ring for each condition at day 7, with general outgrowth angled towards the left for IF regions and to the right in RM regions (4x magnification). White arrows indicate networked structures.

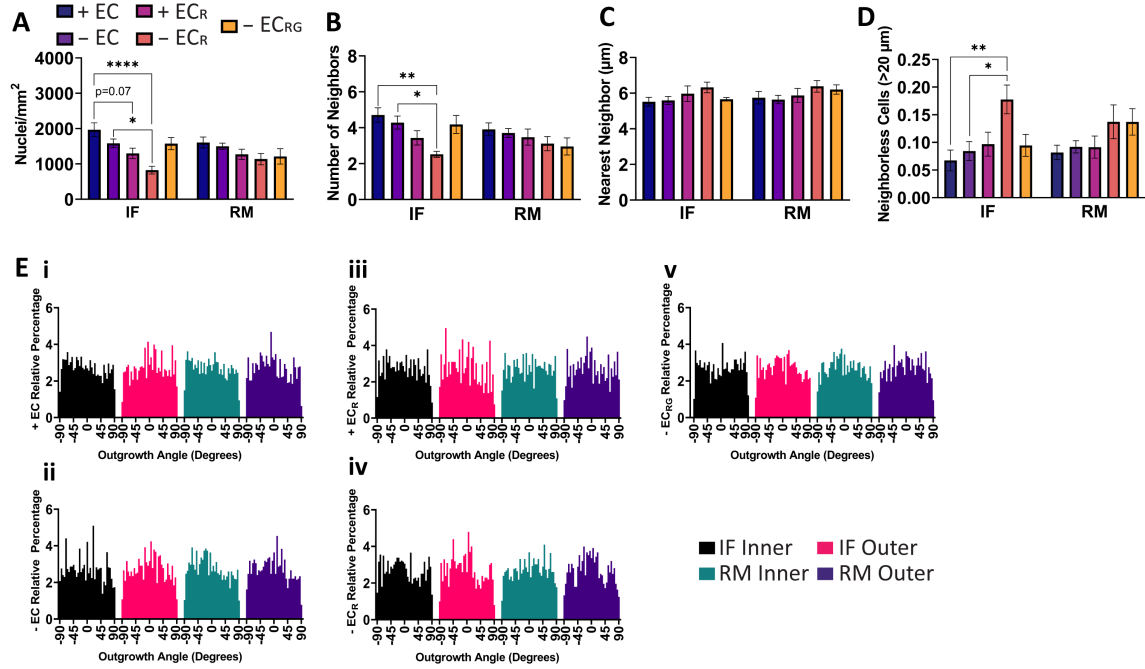


Figure A.6: Regional nuclear characterization. Nuclear analysis for (A) density, (B) number of nearest neighbors, (C) nearest neighbor, and (D) number of neighborless cells. (E) Nuclear orientation histograms for each group, with 0° indicating perpendicular orientation relative to edge of the ring and ± 90° indicating tangent orientation to the ring. + EC (n = 8), - EC (n = 8), + EC_R (n = 6), - EC_R (n = 6), - EC_{RG} (n = 5). *: p < 0.05, **: p < 0.01, ***: p < 0.001, ****: p < 0.0001.

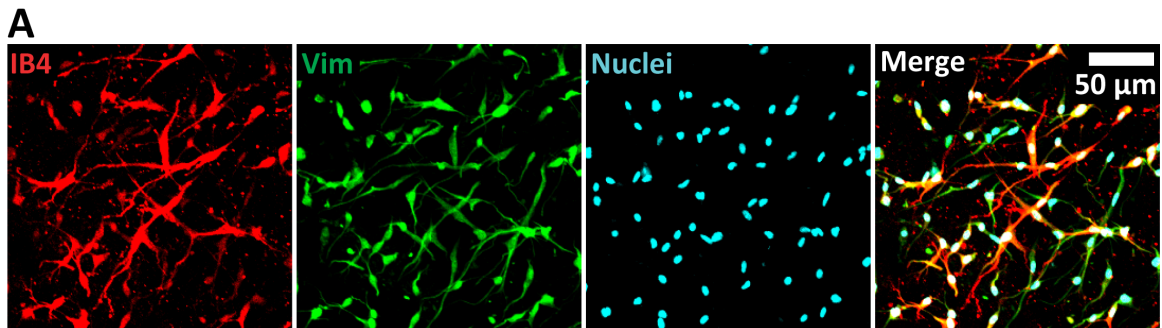


Figure A.7: Representative histological stain of vascular outgrowth marking endothelial cells with isolectin B4 (IB4) and all other cells with the intermediate filament protein vimentin (10x magnification).

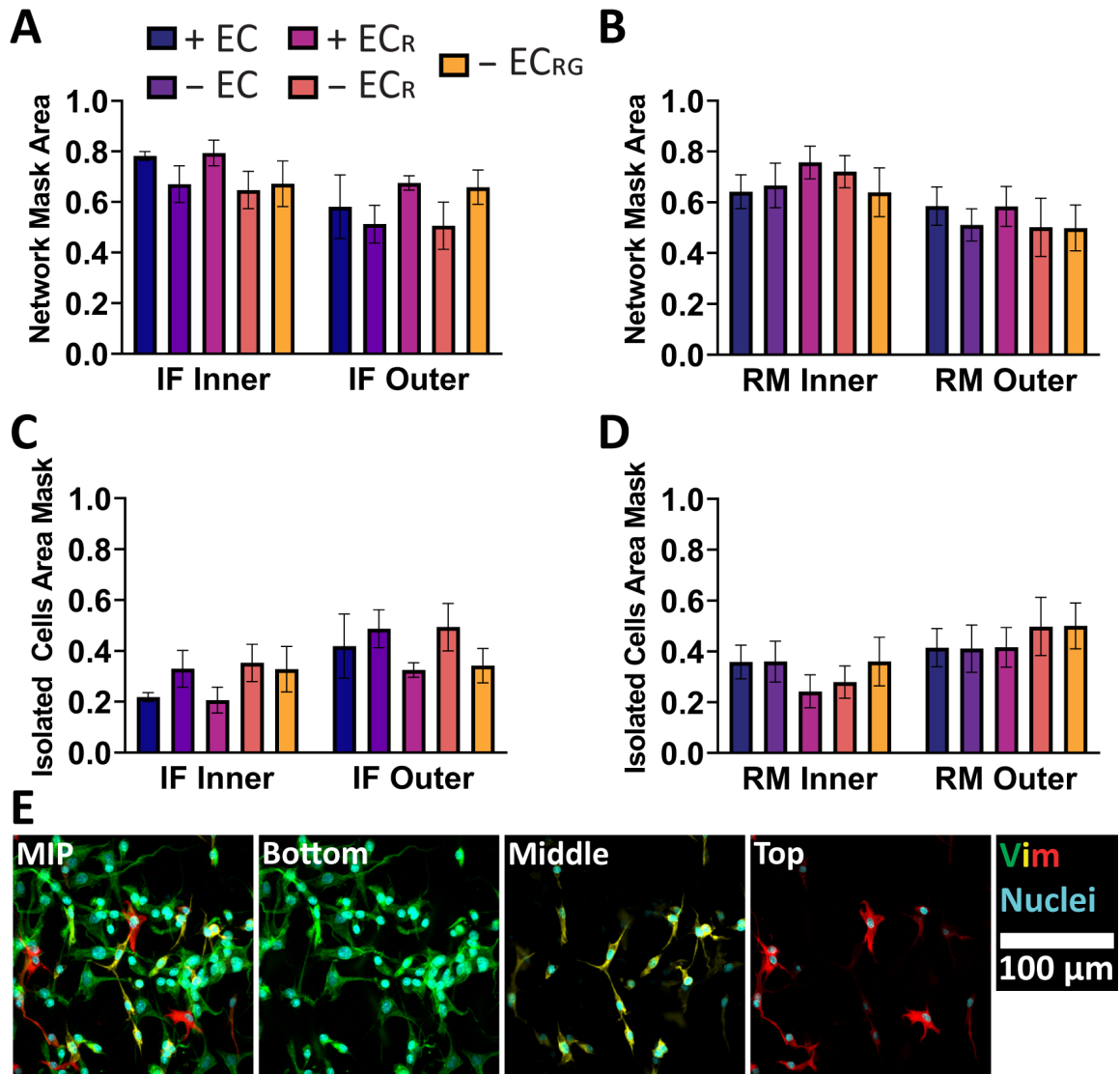


Figure A.8: Subpopulation mask analysis of vimentin stains. Network mask quantification for (A) interface and (B) remote areas. Single cell mask analysis for (C) interface and (D) remote areas. (E) Vascular outgrowth from aorta separates into distinct layers (60x magnification). + EC (n = 5), - EC (n = 7), + EC_R (n = 6), - EC_R (n = 6), - EC_{RG} (n = 5).

Appendix B

Supplementary Information Related to Chapter 4

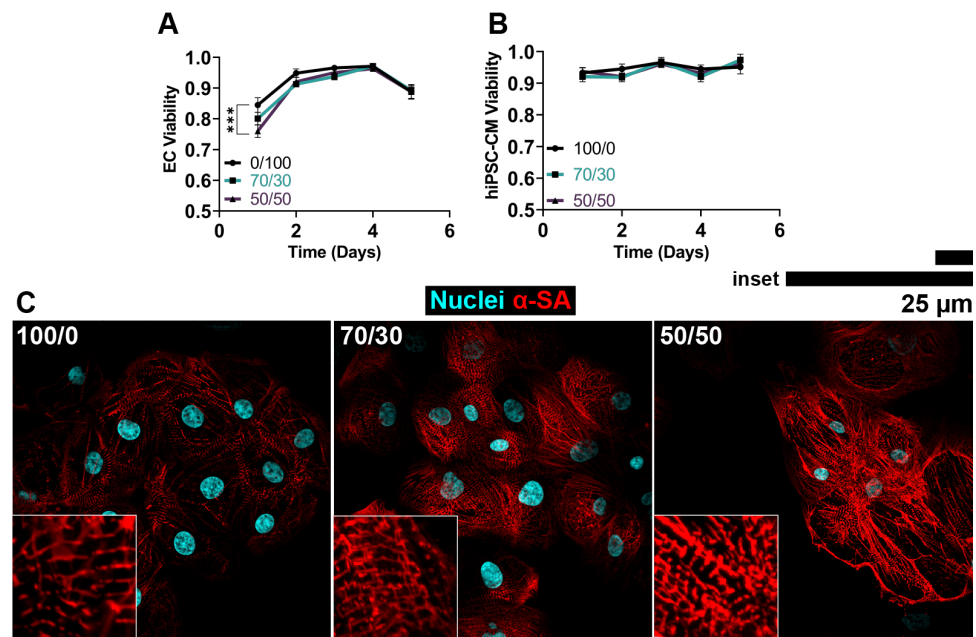


Figure B.1: Viability and morphology of ECs and CMs. (A) Viability of ECs and CMs (B) over 5 days of mixed media culture. n = 6-9 per group. (C) Histological staining of CMs using α sarcomeric actinin (α -SA).

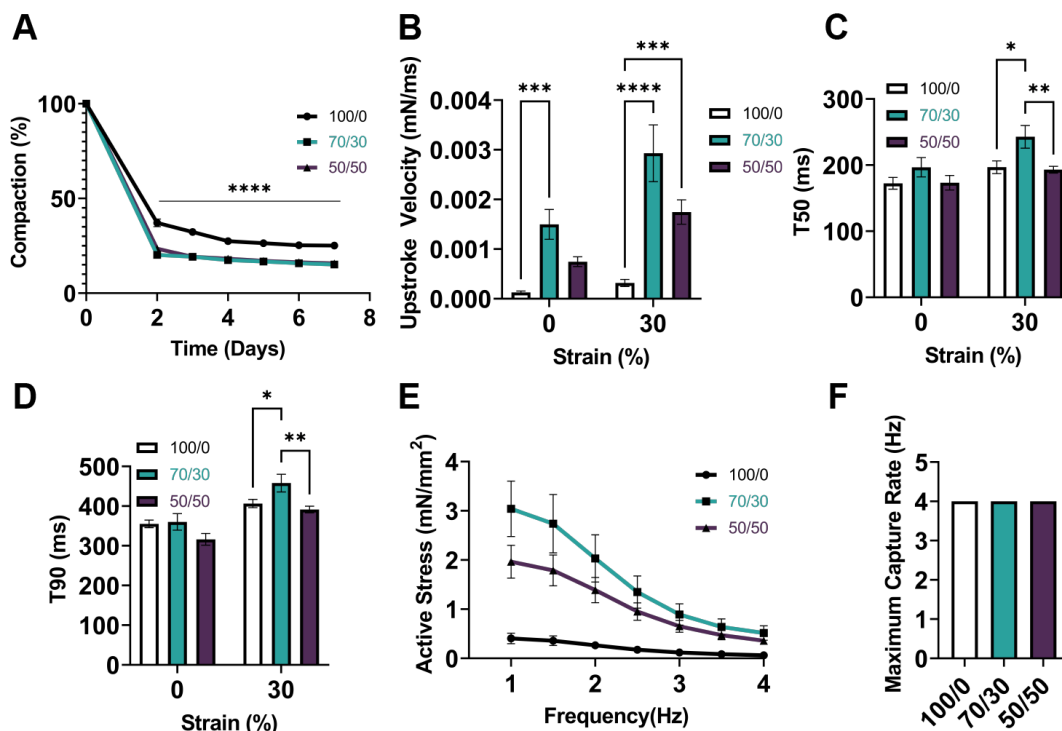


Figure B.2: ECT compaction and contractile kinetics. (A) ECT compaction over one week of mixed media culture. ECT contractile kinetics of (B) upstroke velocity, time to (C) 50% and (D) 90% relaxation (T50 and T90), (E) force frequency response, and (F) maximum capture rate. n = 18-21 samples per group.

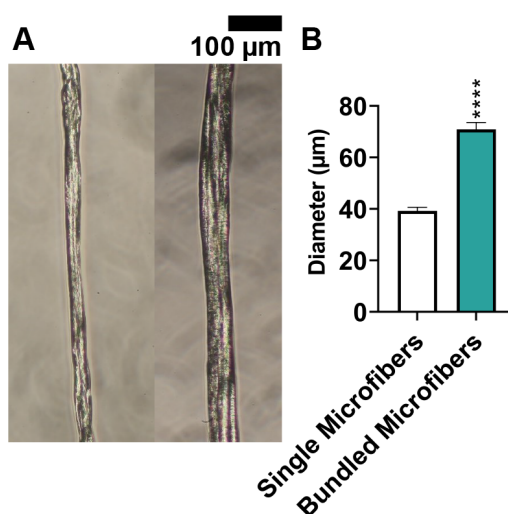


Figure B.3: Wetspun collagen microfiber bundles. (A) Wetspun collagen microfibers are wrapped around themselves four times to create bundled fibers. (B) Collagen microfiber diameter compared to individual microfibers. n = 16-18 fibers per group.

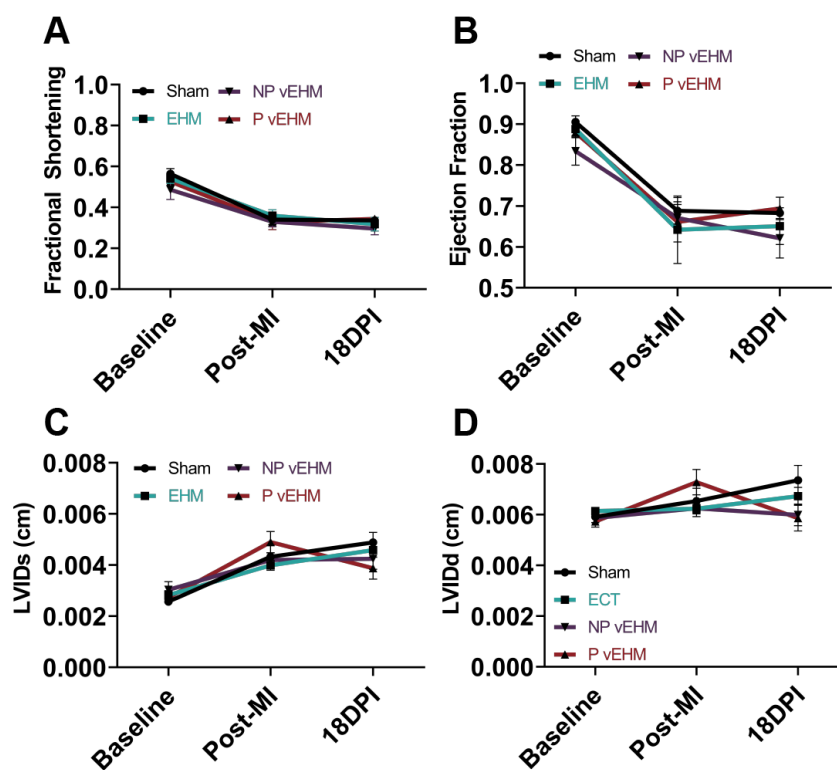


Figure B.4: Echocardiographic metrics of heart function. (A) Measurements of fractional shortening, (B) ejection fraction, and left ventricular inner diameter in (C) systole and (D) diastole. n = 4-6 animals per group.

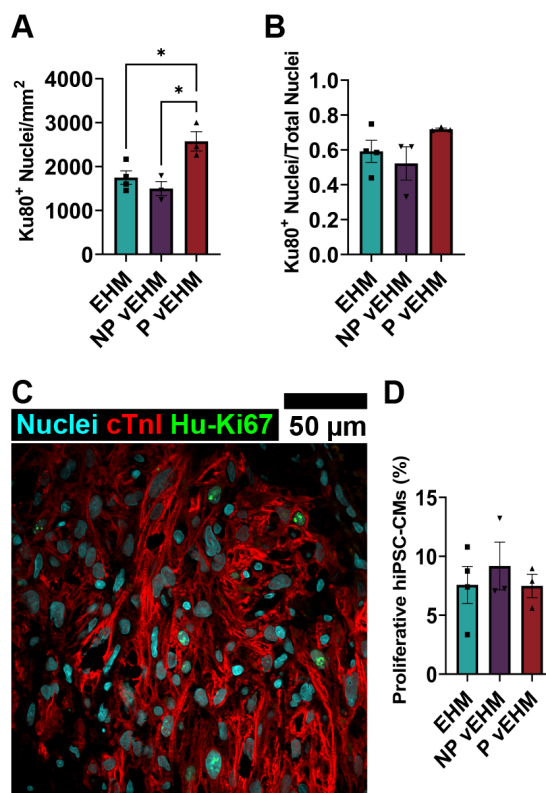


Figure B.5: Identification of human cells in implants. (A) Density of human cells in implants by human-specific Ku80+ staining. (B) Ratio of human-Ku80+ to total nuclei. (C, D) Proliferation assessment of hiPSC-CMs by staining for human-specific Ki67 and cTnI. n = 3-4 animals per group.

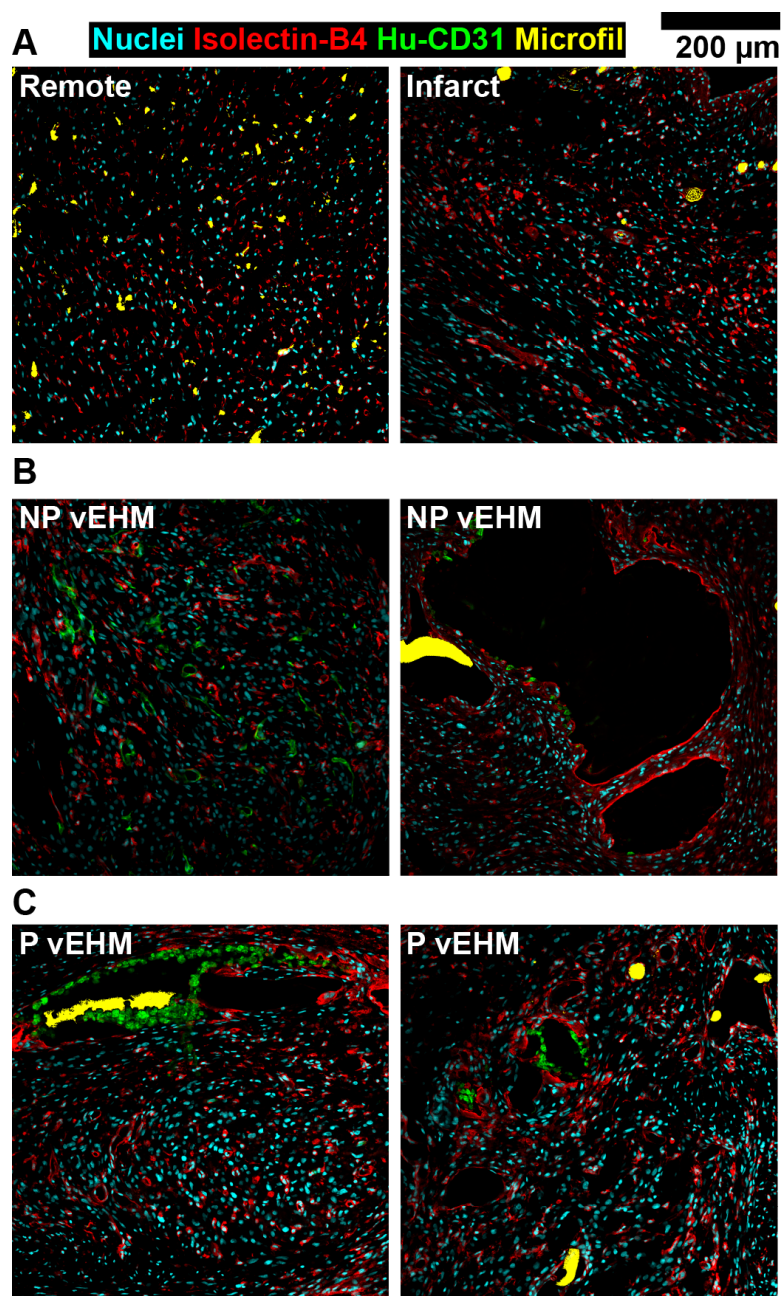


Figure B.6: Implant vascularization and human vascular morphology. (A) Representative images of vascularization in the remote and infarct regions of the heart. (B, C) Human endothelial cell morphology varies between implants and groups.

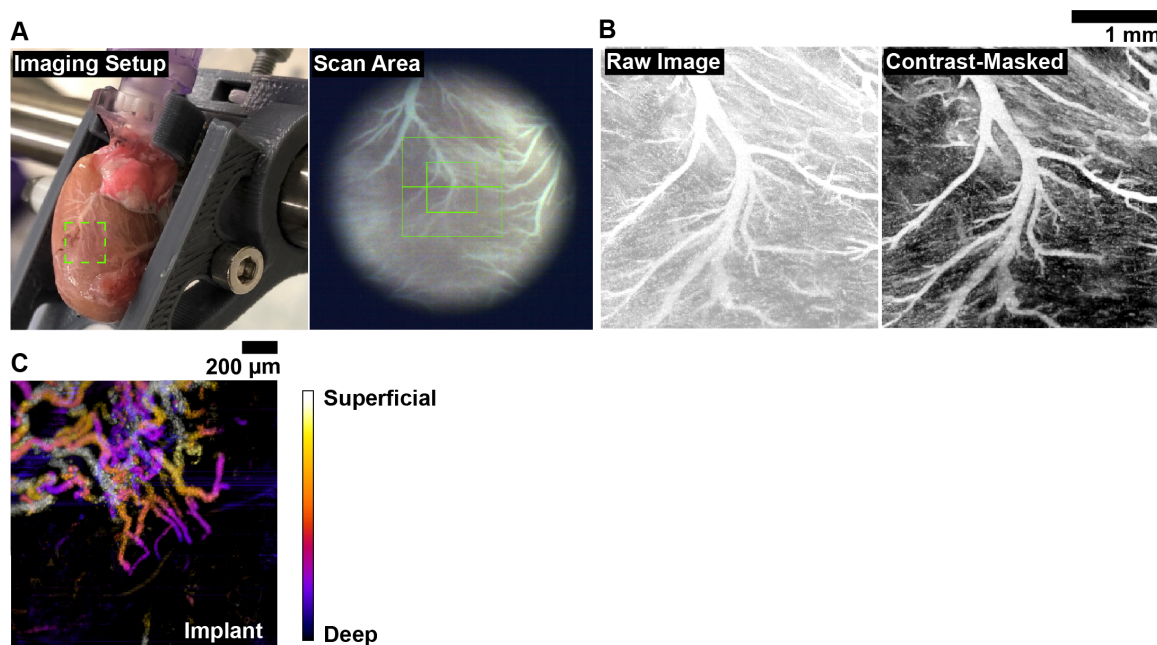


Figure B.7: Optical coherence tomography imaging workflow. (A) Heart imaging setup during intralipid perfusion for OCT acquisition and example scan area using the 5x lens. (B) Corresponding preview image after acquisition and final image after contrast-mask averaging of multiple volumes. (C) False-colored image of implant vasculature demonstrating vessel depth.

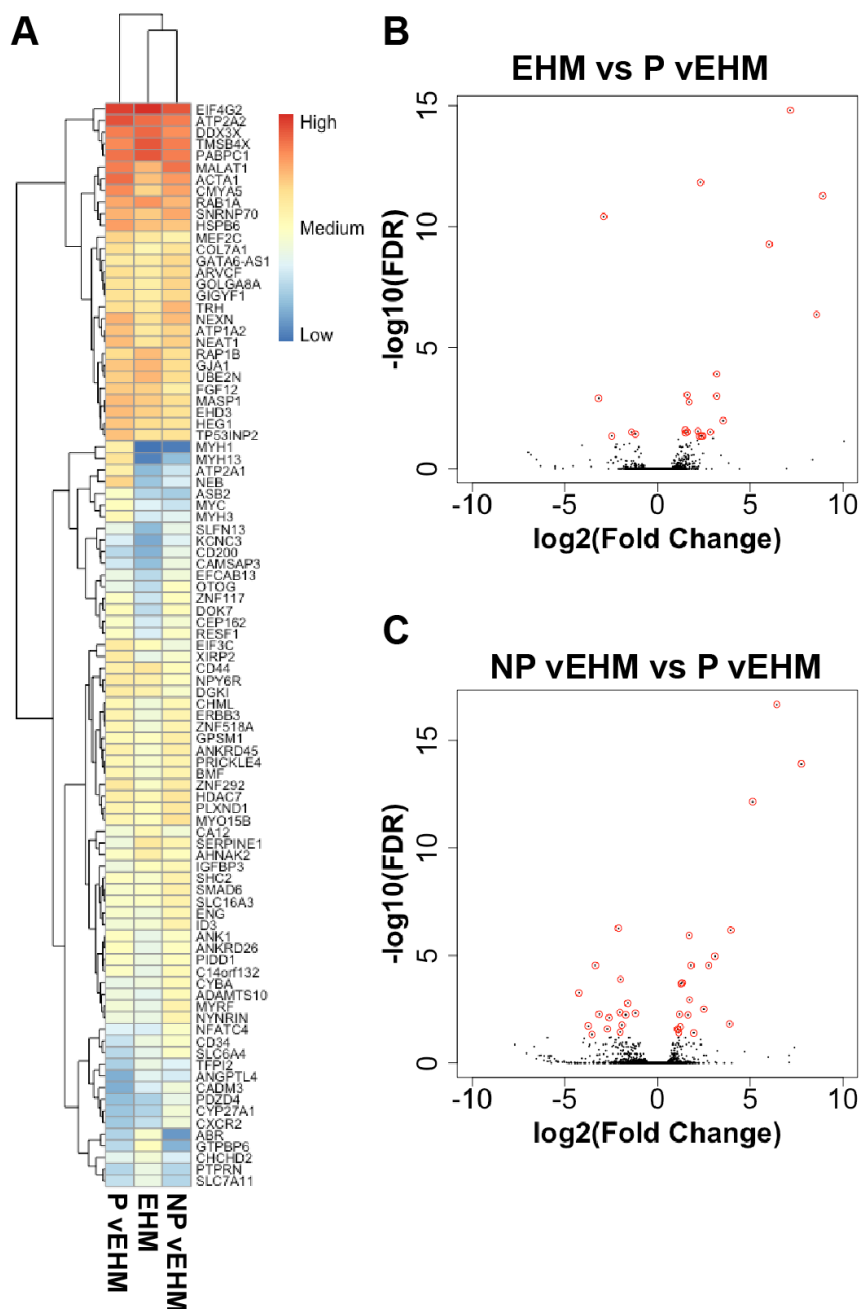


Figure B.8: Supplemental RNASeq analysis of implants. **(A)** Heatmap of differentially expressed genes across all implant groups. **(B)** Volcano plots showing significantly differentially expressed genes for EHM vs. P vEHMs and **(C)** NP vEHM vs P vEHMs.

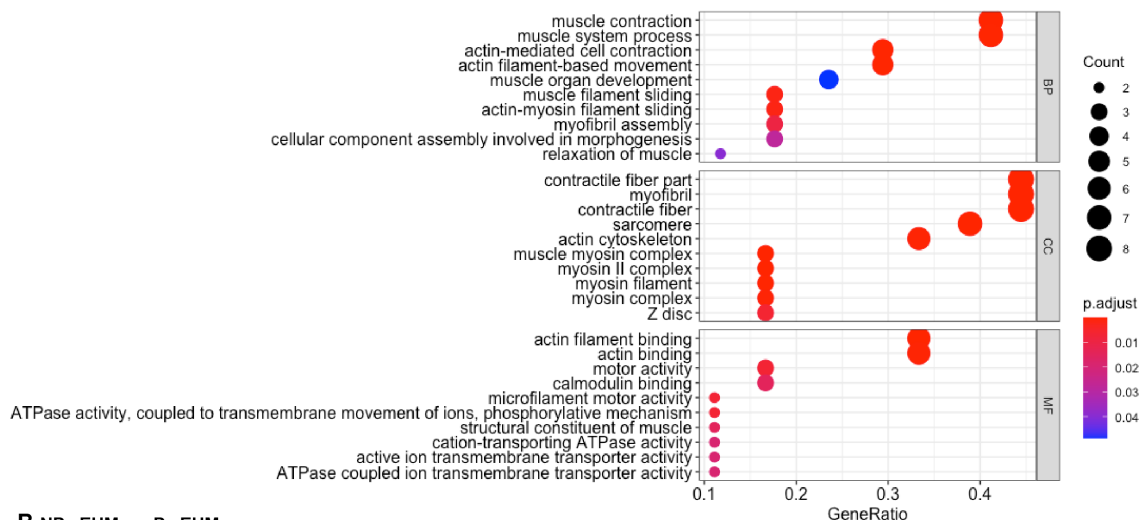
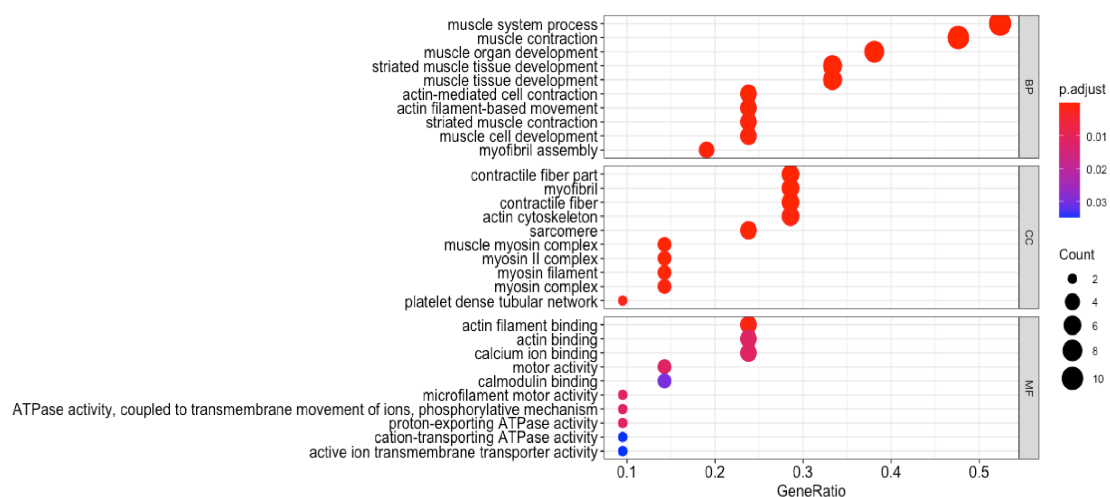
A EHM vs. P vEHM**B NP vEHM vs. P vEHM**

Figure B.9: Gene ontology term analysis. (A) Biological processes, cellular component, and molecular function term analysis for EHM vs. P vEHMs and (B) NP vEHM vs P vEHMs.