January 2017

Differentiation Control In Tissue-Engineered Vascular Grafts With Oxygen & Small Molecule Inhibitors

Joshua Morgan Arthur Siewert
Yale University, jma.siewert@gmail.com

DIFFERENTIATION CONTROL IN TISSUE-ENGINEERED VASCULAR GRAFTS WITH OXYGEN & SMALL MOLECULE INHIBITORS

JOSHUA MORGAN ARTHUR SIEWERT

A thesis submitted to the Yale University School of Medicine
in partial fulfillment of the degree of Doctor of Medicine

Mentor: Dr. Laura E. Niklason
March, 2017

ABSTRACT

Tissue-engineered vascular grafts (TEVGs) have the potential to provide life-saving arterial replacements to patients requiring vascular bypass, hemodialysis access, and pediatric coronary surgery. Recent years have seen impressive strides towards widespread clinical use, but significant work still remains in optimizing cell source and graft growth, including the suppression of unwanted calcification during culture.

In this study, I examine oxygen concentration and two small-molecule bone morphogenic protein (BMP) inhibitors, DMH-1 and LDN193189, as potential avenues of increased control over mesenchymal stem cell (MSC) differentiation of into vascular smooth muscle cells (SMCs). Applying BMP-inhibitor concentrations from 0.01 to 10 μM at oxygen tensions of 2 & 20% over two weeks of growth, I use reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to evaluate resultant expression levels of smooth muscle (SM22-α), bone (OCN), and cartilage (Col2a) marker genes. Via multiple linear regression, I demonstrate that low oxygen growth causes a statistically significant SM22-α downregulation ($\Delta\Delta C_q$ = 1.77 ± 0.22, mean ± standard error, p <0.0%) coupled with increased Col2a ($\Delta\Delta C_q$ = -2.85 ± 0.73, p =0.1%) and type-I collagen expression ($\Delta\Delta C_q$ = -1.75 ± 0.65, p = 1.2%), suggesting that physiological oxygen tensions increase the incidence of chondrogenic differentiation. In contrast, LDN193189 increases SM22-α expression ($\Delta\Delta C_q$ = -0.78 ± 0.27 per μM, p =0.8%) and reduces Col2a expression ($\Delta\Delta C_q$ = -2.17 ± 0.89, p =2.2%), seeming to usefully suppress chondrogenesis.

Additionally, I evaluate the effects of pulsatile vessel growth conditions on an attractive new cell source: MSCs derived from induced pluripotent stem cells (iPSCs). Using PicoGreen and modified Bradford assays, I demonstrate that pulsatile growth conditions significantly increase dry weights of double-stranded DNA (dsDNA) from 0.0780 ± 0.002% to 0.1414 ± 0.008% (mean ± standard error, p = 0.015%) and collagen from 25 ± 2% to 46 ± 2% (p = 2.3%), approaching a sample of native aorta at 55% collagen.

Overall, results suggest normoxic growth conditions remain superior for SMC differentiation, but that the BMP-inhibitor LDN193189 may have a future role in suppressing cartilage production during TEVG growth. Furthermore, iPSC-derived MSCs demonstrate similar responses to traditional bone marrow MSCs, and may well represent an attractive cell source in future TEVG production.

PUBLICATIONS

Some ideas and figures may have appeared previously in the following publications:

[1] Sumati Sundaram, Jennifer One, Joshua Siewert, Stephan Teodosescu, Liping Zhao, Sashka Dimitrievska, Hong Qian, Angela H. Huang, and Laura Niklason. "Tissue-Engineered Vascular Grafts Created From Human Induced Pluripotent Stem Cells." In: *STEM CELLS Translational Medicine* 3.12 (Dec. 2014), pp. 1535–1543. ISSN: 21576564. DOI: 10.5966/sctm.2014-0065. URL: http://doi.wiley.com/10.5966/sctm.2014-0065.

[2] Sumati Sundaram, Joshua Siewert, Jenna Balestrini, Ashley Gard, Kevin Boehm, Elise Wilcox, and Laura Niklason. "Tissue engineering and regenerative medicine." In: *Rossi's Principles of Transfusion Medicine*. Chichester, WestSussex: John Wiley & Sons, Ltd., Apr. 2016, pp. 488–504. DOI: 10.1002/9781119013020.ch42. URL: http://doi.wiley.com/10.1002/9781119013020.ch42.

A new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems...

— Leland R. Kaiser, Ph.D. [1]

ACKNOWLEDGMENTS

I would like to give sincere thanks to Dr. Laura Niklason for providing such a fantastic research environment, abundantly supplied with the tools, support, and community required to produce the innovative and pioneering work for which she is known. Beyond this, however, I'd like to thank her for providing guidance and inspiration to me and all others in her orbit.

I would like to further thank Dr. Sumati Sundaram for being both an excellent supervisor and friend, providing continual guidance, advice, assistance, and prodding throughout the summer—and hopefully many years to come. Stephan Teodosescu and the other members of the Niklason lab created a delightful summer, full of learning and laughter.

I'd also thank my dear friends, Leigh Johnson—for a long history of thesis editing and support, acknowledged and unacknowledged—and Daniel Bondeson, for all those coffee-filled afternoons of writing in bookshops. As ever, my family and brother for unwavering love and support, and the Hinksons, who've adopted me during my stay in New Haven and provided a constant background of warmth and prayers.

Thanks finally to the Yale Stem Cell Center for assistance with cell culture, and both the NIH and Connecticut Innovations for funding support of the Niklason lab, without which this work would not have been possible. Thanks finally to Dr. Paul Heerdt for feedback and approval of the thesis draft.

CONTENTS

I MOTIVATION AND HYPOTHESES 1

1 INTRODUCTION 2
- 1.1 Tissue engineering 2
- 1.2 The need for vascular grafts 3
  - 1.2.1 Dialysis access patients 6
  - 1.2.2 Pediatric cardiovascular surgery 7
  - 1.2.3 Peripheral and coronary bypass grafts 8
- 1.3 Current approaches to TEVG production 9
  - 1.3.1 TEVG structure 11
  - 1.3.2 TEVG cell sources 13
- 1.4 Calcification as a barrier to integrity 16
  - 1.4.1 The effect of oxygen tension 18
  - 1.4.2 Bone morphogenic protein signaling 19

2 AIMS 22
- 2.1 iPSC differentiation 22
- 2.2 Oxygen and small-molecule differentiation control 22

II METHODS AND RESULTS 24

3 METHODS 25
- 3.1 Laboratory techniques 25
  - 3.1.1 Media changes and sterile technique 25
  - 3.1.2 Cell passaging 25
  - 3.1.3 RNA isolation, quantification, and RT-qPCR 26
  - 3.1.4 Protein isolation and quantification 27
  - 3.1.5 Vessel collagen quantification 28
  - 3.1.6 Vessel DNA quantification 28
- 3.2 Static vs. flow culture conditions 29
- 3.3 Small-molecule and hypoxia inhibition 30

4 RESULTS 33
- 4.1 iPSC Pulsatile Growth 33
- 4.2 Differentiation control via O_2 and BMP-inhibition 34
  - 4.2.1 Differentiation into smooth muscle phenotypes 35
  - 4.2.2 Effects of hypoxia and BMP-inhibitors 36

5 DISCUSSION 44
- 5.1 iPSC Pulsatile Growth 44
- 5.2 Differentiation control via O_2 and BMP-inhibition 44
  - 5.2.1 Differentiation into smooth muscle phenotypes 45
  - 5.2.2 DMSO as a confounding factor 46
  - 5.2.3 Notes on qPCR analysis 47
  - 5.2.4 Effects of hypoxia and BMP-inhibitors 47

6 CONCLUSION 50

III APPENDIX 51
A APPENDIX 52

BIBLIOGRAPHY 58

Part I

MOTIVATION AND HYPOTHESES

1 INTRODUCTION

Below, I briefly introduce the origins of tissue engineering as a field (Section 1.1) and provide a succinct summary of the history of vascular surgery, providing an overview of the clinical need for tissue-engineered vascular grafts (TEVGs) as a vascular surgical option (Section 1.2). I trace major milestones in the TEVG field, and examine in particular research efforts in hemodialysis, pediatric coronary surgery, and bypass operations.

I then move on to examine the design requirements and specifications of a successful TEVG, alongside a description of the approaches undertaken by contemporary vascular tissue engineers in terms of both structure and cell source (Section 1.3). I focus in particular on mesenchymal stem cells and the novel potential of induced pluripotent stem cells, which this thesis briefly investigates.

Finally, I focus on the particular challenge to TEVG integrity posed by unwanted osteogenic and chondrogenic differentiation during cell culture, and the specific roles that oxygen concentration and hormonal signaling might play in driving these processes (Section 1.4). In (Chapter 2), I will go on to present my direct hypotheses as to effect of oxygen tension and BMP-inhibitors, which may play a role in the future TEVG development.

1.1 TISSUE ENGINEERING

The term "tissue engineering" first appears in the 1982 press release of the company funding Dr. Eugene Bell's laboratory at the Massachusetts Institute of Technology, who would go on to develop the world's first entirely lab-grown vessel [2]. A formal definition of the field emerged six years later, at an NSF-sponsored workshop in Granlibakken motivated by a growing shortage of donor organs [3]. As reiterated by Langer and Vacanti in their foundational early review of the field [4]:

> "Tissue Engineering" is the application of principles and methods of engineering and life sciences toward...the development of biological substitutes to restore, maintain, or improve tissue function.
>
> The basic point of the above definition is that tissue engineering involves the use of living cells plus their extracellular products in development of biological substitutes for replacements as opposed to the use of inert implants. The definition is intended to encompass procedures in

> which the replacements may consist of cells in suspension, cells implanted on a scaffold such as collagen and cases in which the replacement consists entirely of cells and their extracellular products. [5]

This definition remains accurate today, and highlights the important distinction between tissue-engineered products and the synthetic, polymer implants routinely used in today's surgeries: tissue engineering has its origins in cellular processes, and aims to produce biological tissue substitutes.

In the years after its conception, tissue engineering research quickly commenced in nearly all human organ systems [4]. The earliest successes, however, have occurred in skin, bone, and cartilage—for which FDA-approved products currently exist—as well as the cornea and bladder, for which research is also advancing towards clinical use [6]. Given the (superficially) simple structure of blood vessels, and the immense volume of vascular surgery worldwide (Section 1.2), it is unsurprising that vascular grafts also stand in this list an early pillar of tissue engineering success.

1.2 THE NEED FOR VASCULAR GRAFTS

The field of vascular surgery—and of organ transplantation, more generally—began in earnest with the work of French surgeon Alexis Carrel (1873-1944), recipient of the 1912 Nobel Prize for his work on for his vessel attachment [7]. His contributions included the use of cold storage for vessel grafts and the foundational three-point suturing technique that first allowed end-to-end anastomoses, joining two vessels for bypass or transplant [8, 9]. In 1948, building on Carrell's success, French surgeon Jean Kunlin performed the first bypass procedure of the modern era, using a patient's own (autologous) saphenous vein to bridge an obstruction in a patient's femoral artery [10, 11]. The now-ubiquitous coronary artery bypass graft (CABG) surgery emerged shortly thereafter, first with autologous arteries in 1960 [12], then with the now-familiar autologous saphenous vein in 1967 [13, 14].

Autologous saphenous vein quickly emerged as a widespread graft substrate, with relatively easy surgical access and acceptable levels of patency. Though it remains the gold standard in numerous vascular procedures to this day, it soon became evident veins were not ideal arterial grafts: they adapt to high-pressure arterial circulation imperfectly, and are prone to both intimal hyperplasia and accelerated atherosclerosis [15, 16]. Furthermore, a history of multiple operations and other patient factors often renders autologous vein unavailable, forcing surgeons to search for alternatives [17].

The world of vascular surgery first intersected with materials engineering in 1954, when American surgeon Michael DeBakey collabo-

POLYMER	TRADE NAME	ABBR.
Poly (ethylene terephthalate)	Dacron®	PET
Poly-tetraflouro-ethylene	Teflon®	PTFE
Expanded Polytetraflouroethylene	Gore-Tex®	ePTFE

Table 1: Common non-degradable polymers used for bypass grafts. Polyethelene (PE), polyurethane (PU), polydimethylsiloxane (PDMS), and polysulfone are also commonly used in stents; there also exists numerous biodegradable polymers, both single-component (e.g., polylactic acid [PLA], polyglycolide/polyglycolic acid [PGA], polyhydroxyalkanoate [PHS], and polydioxanone [PDS]) and multi-component (e.g., polyglactin/Vicryl [PG910], which is a mixture of a PGA & PLA copolymer). These prosthetics have the advantage of widespread use in high-flow regions like the aorta, but generally suffer from prohibitively high levels of thrombogenicity and occlusion in lower-flow vessels, leaving autologous veins or TEVGs as the preferable solution [15].

rated with textile engineer Thomas Edman to develop a new machine capable of knitting seamless tubes out of synthetic thread. Biological venous grafts could not produce the complex shapes required in aortic surgery, requiring something bespoke yet amenable to long-term residence in the patient. Settling on poly(ethylene terephthalate)—alternatively known as "Dacron," or PET—as the most biocompatible fibre, they successfully implanted synthetic aortic and iliac grafts in a variety of configurations [11, 18].

An additional category of vascular surgery was established after the 1960 development of the 'Scribner shunt,' external Teflon (ePTFE) tubing that permanently connected a cannula in the radial artery to another in the cephalic vein, providing continuous blood flow for the external hæmodialysis of renal patients [19–21]. The continuous flow reduced clotting within the cannula, which had plagued earlier intermittent attempts, and established overnight the field of vascular access. Though functional, the persistence of clotting and infection in the shunts soon led to the development of the completely internal Bresica-Cimino arteriovenous (AV) fistula in 1966, in which radial artery and cephalic vein were anastomosed together directly [22]. Three years later, surgeons found that connecting the two with an extra loop of saphenous vein newly enabled the surgery in a significant subset of patients with poor existing venous vasculature, including diabetics, the elderly, and those with prior fistula failures [21]. For those without suitable saphenous vein for harvest, surgeons quickly transferred DeBakey's pioneering work in aortic polymer grafts to this new site, using both PET and ePTFE (Table 1) [23].

Despite the half-century of intensive research into graft substitutes that followed, the materials used in today's vascular surgeries bear striking similarity to those of these early surgical pioneers. Unfortu-

nately, this has resulted in considerable unmet need, felt most keenly in small-diameter (< 6 mm) applications where non-degradable polymers are unacceptably thrombogenic, and no widely-accepted synthetic option yet exists [24].

The potential for a biological solution to this need emerged in 1986, when Weinberg and Bell developed the first tissue-engineered blood vessel [25]. Casting collagen, smooth muscle cells (SMCs), and culture medium in an annular mold, they produced a jellied tube they then placed around a central mandrel. In time, the collagen-embedded smooth muscle cells automatically contracted around the central support, generating a tubular lattice that could function as a vessel's middle layer (i.e., *media*). A Dacron mesh slipped overtop provided additional support, and they cast second layer of collagen on top—this time containing fibroblasts, rather than SMCs—to form the vessel's exterior layer (i.e., *adventitia*). Allowing this to contract down as well, they removed the mandrel and injected a suspension of endothelial cells into the newly-formed lumen, slowly rotating the vessel to grow a uniform endothelial coating (i.e., *intima*), thus successfully recreating the three layers of a normal artery.

The result was a functional vessel graft for in vitro study, but with a crucial failing. Lacking any substantial quantity of elastin, and producing collagen fibres oriented longitudinally instead of along physiological spirals, they were only able to generate vessels capable of withstanding pressures of ≈ 300 mmHg prior to bursting: less than a fifth of that of a saphenous vein. This was despite the added support provided by the Dacron mesh, which problematically represented a non-degradable element and inherently compromised the growth potential and biocompatibility of the resultant product.

Regardless, Bell & Weinberg's work offered an important proof-of-concept and sparked the cascade of milestones that followed. L'Heureux et al. manufactured the first TEVG free from all permanent synthetic components in 1993 [26]. Niklason et al. introduced pulsatile, radial strain into vessel growth in 1999, resulting in dramatically increased collagen production, which also mimicked native vessels in orientation [27]. Shin'oka et al. implanted the first TEVG in a human patient in 2001 [28], demonstrating unequivocally the clinical viability of the TEVG concept. In 2003, Dahl et al. decellularized their TEVGs to produce a cell-free, "off-the-shelf" graft product that pointed to future improvements in cost and convenience [29]. L'Heureux began the first adult, commercial clinical trials in 2007 [30], followed shortly by Humacyte's large-scale studies in 2011. Subsequent significant developments have included refinements in rapid post-implantation remodeling by Wu et al. [31] and the emergence of 3D-printed and electrospun graft foundations which may increase the range of TEVG shapes available [17].

Today, as TEVGs near the point of realistic clinical utility, there remain three key segments of clinical need, which I examine in turn below: vascular access in dialysis patients (Section 1.2.1), peripheral & coronary bypass operations (Section 1.2.3), and pediatric cardiac surgeries (Section 1.2.2). All three are common yet crucial procedures which traditionally rely on autologous vein or non-degradable polymer grafts, and all stand to be significantly improved by a reliable and cost-effective graft alternative.

1.2.1 *Dialysis access patients*

Over the past decade, over 1,000,000 North American patients initiated dialysis, primarily for end-stage renal disease (ESRD), with 383,992 undergoing hæmodialysis (HD) in 2010 alone [32]. Arteriovenous grafts (AVGs) remain the best access option for chronic hæmodialysis patients who are not candidates for direct AV fistulas, either due to poor existing vasculature—the case in many diabetic or elderly patients—or after the failure of an existing fistula. Claims data indicates that 28% of *all* American hæmodialysis patients are currently graft-reliant (2007, [33]), and, despite recent efforts to increase the proportion of American patients using fistulas, 5.9% of *new* hæmodialysis patients still end up relying on grafts by the fourth month of treatment (2013, [32]).

In patients without suitable saphenous vein, most AV grafts still rely on ePTFE (Table 1), which carries significant drawbacks. Impermeable to white blood cells, ePTFE grafts are prone to infection (4-20% annually [34]). Incapable of self-repair, repeated needle sticks lead to substantial mechanical damage ("coring"), which can result in pseudoaneurysm (2-10% over graft lifetime). The synthetic material also prompts a poorly-understood intimal hyperplasia reaction (50% at 3 years [35]), in which smooth muscle cells, macrophages, and microvessels proliferate to cause a narrowing of the lumen and potentially an occlusive thrombosis [36]. The surface of the ePTFE graft does not permit stable endothelialization, which likely contributes further to thrombus formation; the resulting need for angioplasty or stenting contributes to substantial graft lifetime costs and places a considerable burden on patients.

Biological alternatives to ePTFE have been studied, including bovine carotid arteries (Artegraft®; Artegraft Inc., North Brunswick, NJ [37, 38]), depopulated bovine ureters [39–41], bovine mesenteric vein (ProCol®; Hancock Jaffe Laboratories, Irvine, CA [42–44]), and human cryo-preserved cadaver saphenous vein (CryoVein®; CryoLife Inc., Kennesaw, GA [45]). None of these options, however, have gained any widespread acceptance, and there remains a substantial unmet clinical need for a superior alternative.

Hemodialysis grafts represent a particularly attractive target for TEVG research: operating sites are close to the body surface, occlusive complications are usually not life-threatening, and the highly-standardized operations take place in predictable and reproducible clinical settings. Perhaps unsurprisingly, many tissue engineering efforts have focused first on dialysis, with the intent of later transposing the technology to other vascular sites. This trend began with the L'Heureux group's small-scale clinical studies, reporting successful autologous (2009 [30]) and allogenic (2014 [46]) implantations of their endothelialized, sheet-based TEVGs (Lifeline™; Cytograft, Novata, CA). In 2016, acellular, off-the-shelf TEVGs from the Niklason group passed phase II trials in 60 ESRD patients with 89% secondary (i.e., still functional, but requiring a procedure to restore function) patency at 12 months (Humacyte, Durham, NC) [47]. Considerable optimism surrounds both engineering successes, and TEVGs look well-poised to reshape the landscape of dialysis access.

1.2.2 *Pediatric cardiovascular surgery*

Moderate to severe congenital cardiovascular malformations occur in approximately 6/1,000 live births [48]. Though recent decades have seen an encouraging improvement in outcomes, the burden of critical congenital heart defects (CHDs) remains high both in terms of associated healthcare costs and devastating patient morbidity and mortality [49, 50]. CHDs result in the deaths of twice as many children as all pediatric malignancies combined, and approximately half of CHD patients require surgical intervention [51].

Though numerous congenital heart defects exist, many—including hypoplastic left heart syndrome, tricuspid atresia, pulmonary atresia, double-inlet left ventricle, and unbalanced atrioventricular canal defects [52]—leave the child with only a single functional ventricle, rendering them potential candidates for modified versions of the famed Fontan procedure [53]. This staged surgery allows the single functional ventricle to supply the child's high-resistance systemic circulation, connecting the single ventricle directly to the aorta. Pulmonary circulation is then driven by central venous pressure alone, with the superior vena cava grafted directly into the pulmonary artery via an artificial conduit. Originally preferring PET for this graft, surgeons adopted ePFTE in a partially-successful attempt to reduce luminal narrowing. Unfortunately, as in Section 1.2.1, ePFTE conduit stenosis remains a grave source of long-term complications. Other pediatric cardiac surgeries (e.g., the Rastelli procedure connecting right ventricle to pulmonary artery) suffer from similar graft concerns [54, 55].

Pediatric grafts present the additional challenge of accommodating substantial patient growth, which currently necessitates replacement surgeries as patients age—neither synthetic grafts nor homo-

grafts accommodate expansion. These replacement operations themselves possess 5% peri-operative mortality [56], and strategies to delay surgeries or utilize oversized grafts result in substantial cardiac dysfunction [51]. The lack of an ideal graft material has spurred considerable interest in TEVGs as a single-surgery, growth-capable solution. This, coupled with the relatively modest mechanical demands of low-pressure pediatric pulmonary circulation, enabled what was to be the first successful implant of a tissue-engineered vascular graft in a human patient. In their landmark 1999 study on a four-year-old Fontan patient, Shin'oka et al. implanted a 2 cm biodegradable polyglycolic acid (PGA) tube, seeded with autologous cells expanded from a segment of the patient's vein. The patient suffered no major complications and the graft remained patent [28]. This encouraged a larger study of 25 patients; six-year follow-up demonstrated no major graft-related complications, and a considerable portion of patients even avoided the lifetime anticoagulation associated with synthetic grafts [57–60].

The first large-scale, FDA-approved clinical trial of CHD TEVGs began at Yale University in 2011[1], studying synthetic scaffolds seeded with bone-marrow-derived mesenchymal stem cells. Initial data is encouraging [61, 62], and is likely only the beginning of tissue engineering success in pediatrics.

1.2.3 *Peripheral and coronary bypass grafts*

Cardiovascular disease remains the leading cause of mortality globally [63]. In the United States alone, cardiovascular disease (CVD) and stroke cause an estimated $ 316.6 billion in annual direct and indirect costs; by 2030, 43.9% of the nation is projected to have some form of CVD. Between 2000 and 2010, the number of American inpatient cardiovascular procedures increased 28% from 5,939,000 to 7,588,000; given the aging demographic, this trend is likely to continue unabated [64]. Harmful arterial lesions arise in the blood supply of both the heart and the periphery, with cardiac circulation favouring foam-cell and lipid-core subtypes and fibrous plaques dominating elsewhere [65]. If lesion formation is not halted via medical management, both sites of atherosclerotic disease will see increasing stenosis and eventual interruption of blood flow, necessitating intervention.

Surgical treatment varies depending on lesion severity, with early-stage plaques often amenable to percutaneous coronary intervention (PCI), i.e., restoring the vessel lumen via angioplasty and potential stenting. More severe disease demands surgical graft implantation [66]—patients underwent 397,000 coronary artery bypass graft (CABG)

1 "A Pilot Study Investigating the Clinical Use of Tissue Engineered Vascular Grafts in Congenital Heart Surgery," sponsored by Dr. Christopher Breuer (NCT01034007); <https://clinicaltrials.gov/ct2/show/NCT01034007>.

procedures in 2010 alone, alongside similarly substantial rates of peripheral bypass grafts [64].

As in hæmodyalysis access, autologous saphenous vein remains the gold-standard of graft material, despite a vulnerability to accelerated atherosclerosis leading to only 50% 10-year patency [67]. Internal mammary and radial arteries provide superior patency, but are associated with increased risk of mediastinitis, angiospasm, and graft-site ischemia [68]. This, coupled with increased surgical difficulty during vessel harvest, significantly limits their use. Furthermore, as grafting does not address the underlying causes of atherosclerosis, patients often require subsequent grafts and their supply of usable autologous vessel quickly dwindles [66]. Unfortunately, when autologous vessel is unavailable, the small diameters of cardiac grafts often render synthetic polymers unacceptably thrombogenic, leading to extremely low long-term patency [15].

The staggering burden of CVD, coupled with the shortcomings of existing graft options, has attracted substantial interest in TEVGs for coronary and peripheral bypass procedures. Though peripheral, carotid, and coronary TEVGs have been successfully implanted in large animal models [6], the invasive nature of bypass procedures and the stringent design requirements of small-diameter vessels makes it likely that bypass trials will lag behind those in hæmodialysis patients.

1.3 CURRENT APPROACHES TO TEVG PRODUCTION

In his pioneering 1958 work, DeBakey carefully explains the considerations behind his choice of the first synthetic graft material. He settled upon Dacron as he found it to be:

- Flexible, durable, and elastic enough to withstand long-term implantation.
- Capable of being knit into a shape appropriate for any major arterial system.
- Easily handled by the surgeon: sterilized via autoclave without structural damage, cut by scissor or scalpel without fraying, and clamped during the procedure without damage.
- Porous—unexpectedly superior to waterproofed fabrics, as untreated Dacron allowed better attachment of fibrous tissue during intimal growth [18].

Despite the vast array of potential sites for a surgical graft described in prior sections, all share these initial, universal requirements. Engineers strive to create a robust, long-lasting, and readily-handled material which interacts harmoniously with the surrounding tissue,

be it destined for the arm of renal patients or the surface of an ischæmic heart. With tissue engineered approaches, cell products are often exquisitely biocompatible, but can be extremely fragile. The defining challenge of TEVG development has thus often been the considerable mechanical integrity that the arterial system demands.

Today, many groups formalize the essential properties of a successful graft as follows [69–71]:

A. BURST STRENGTH comparable to internal thoracic artery (> 3100 mmHg) or saphenous vein (>1700-2200 mmHg) for arterial applications, though some variation exists based on location within the circulatory system and the subsequent remodeling that will ensue upon implantation [70].

B. FATIGUE STRENGTH—as described by L'Heureux et al.—sufficient to maintain a stable diameter after 30 days of pulsatile loading, in vivo or in vitro, without susceptibility to aneurysm formation [69].

C. COMPLIANCE sufficient to prevent high stresses around the anastomosis site. By definition, compliance (C) measures the vessel's ability to deform under pressure, i.e.,

$$C = \frac{\Delta V}{\Delta P}$$

where ΔV is the incremental change in the vessel's volume upon the application of additional pressure, ΔP. Groups often report compliance in units of %-change per 100mmHg; values for saphenous vein and internal thoracic artery are approximately 12%/100 mmHg and 26%/100mmHg, respectively [71].

D. DURABILITY AND KINK RESISTANCE sufficient to allow easy handling by the surgeon, as well as the toughness necessary to retain sutures.

E. BIOCOMPATIBILITY, i.e., producing a stable, non-cytotoxic, non-thrombogenic, non-immunogenic, and infection-resistant endothelial layer. This also encompasses the vessel's potential for growth, remodeling, and self-repair in vivo.

F. ECONOMIC VIABILITY, albeit a secondary consideration to the preceding; an ideal graft will also be easily shipped, possess a readily-scalable manufacturing processes, and be capable of off-the-shelf storage to minimize costs.

To date, no single graft design has emerged as the optimal method of providing all of the above properties. Considerable progress has been made, however, since Bell's first vessel: an impressively diverse

variety of solutions have emerged that rely on vastly different combinations of cell sources, techniques, and materials.

Broadly, research efforts can be categorized based on (1) the method used to impart structure to the TEVG being constructed and (2) the sources and types of cells cultured in its creation. Both can have important ramifications for the vessel's ultimate performance.

1.3.1 *TEVG structure*

In standard culture, cells form two-dimensional sheets on their growth surfaces. To generate the three-dimensional shape of a blood vessel, groups have explored three primary approaches:

a. CONSTRUCTING ENGINEERED SCAFFOLDS using some combination of synthetic and/or natural materials, then seeding them with cells. Scaffold materials are generally selected to degrade after implantation and facilitate biological remodeling.

   Synthetic polymers are a widely- and immediately-available choice, inexpensive, and easily tailored to specific applications. Shin'oka et al.'s pediatric grafts, for instance, employed a porous, degradable mesh of poly-L-lactide (PLLA) & poly-ϵ-captolactone (PCL) reinforced with polyglycolide (PGA), in which they then cultured bone-marrow-derived mononuclear cells (BM-MNCs). Explant analysis from clinical trials indicated that, as expected, the mesh disappeared months after implantation [72]. Niklason and Langer have also used PGA and poly(lactide-co-clycolide) (PLGA); other commonly-employed materials include PEUU, P4HB, and PCL [71]. Though most groups seed these scaffolds in vitro, others have also explored the implantation of completely acellular polymer scaffolds to allow rapid host cell invasion and remodeling, effectively "growing" the vessel in the patient [73]. Niklason et al. combined both approaches: scaffolds were seeded and grown in culture, but decellularized prior to implantation to allow host cell invasion.

   As established in Bell's 1986 work, another approach is to begin with *natural* polymers—often exquisitely biocompatible, but generally possessing poorer mechanical strength than synthetic alternatives. Work continues on collagen-only scaffolds, with improved structural integrity provided by cyclic strain during culture [74]. Multiple groups have examined fibrin as a potential base material, condemning traditional synthetics (i.e., PGA and PLGA) for creating an acidic in vivo environment as they hydrolyze, and producing byproducts that purportedly cause the de-differentiation of smooth muscle cells (SMCs) [75–77]. Thus far, fibrin vessels do not match the mechanical strength of autologous saphenous vein, but work continues in large an-

imal models. Other investigated materials include electrospun fibroin—the chief constituent of silk and spider webs [78]—and chitosan, a derivative of the chitin comprising the shells of crustaceans, which closely mimics the glycosaminoglycans common in the human extracellular matrix (ECM) [79]. Both have shown acceptable biocompatibility, but have not yet demonstrated mechanically robust performance in large animals. Groups have also explored combinations of natural and synthetic materials, aiming to create a tunable, hybrid biomaterial possessing both the biocompatibility of the former and the mechanical integrity of the latter; it remains unclear if this approach can outstrip the performance of natural matrix laid down by cells cultured in synthetic-only scaffolds [71].

B. DECELLULARIZING NATURAL MATRICES: An alternative to *building* tubular structure is to use that already present in nature, i.e. by decellularizing allogeneic or xenogeneic tissues both vascular and extra-vascular (e.g., mucousal or ureteric). As discussed in Section 1.2.1, this has already gained some traction in hæmodialysis applications, with commercial grafts based on decellularized human saphenous vein as well as bovine carotids, ureters, and mesenteric vein. Farther from clinical application, groups have also explored human amniotic membranes [80, 81], porcine bladder [82], and small intestinal submucosa from a variety of animal sources [83, 84].

   Decellularization aims to remove all antigenic and cellular material from the original substance while maintaining the structure and strength of the extracellular matrix, generally by combining surfactants/detergents with physical agitation. Though the natural matrix architecture can produce mechanically robust and biocompatible grafts, starting with a biological source also introduces considerable expense and complications surrounding reproducibility and quality control. Substantial variation exists in decellularization techniques, and incomplete antigen removal has been linked to graft failure; risk is compounded when xenogeneic sources are employed [85, 86].

C. SELF-ASSEMBLY: Finally, tissue-engineering by self-assembly (TESA) builds structures "bottom-up," entirely from cultured cells and their products. As pioneered by L'Heureux et al., this initially took the form of thick cell sheets, supplemented with ascorbate to encourage ECM formation, rolled in layers around mandrels to form the desired tubular shape [87]. To avoid mechanical trauma while detaching the sheets, many groups culture cells on a thermoresponsive polymer, such as poly-*N*-isopropylacrylamide (PIPAAm), which becomes effectively hydrophobic above a certain temperature [88]. Combining multiple sheet lay-

ers can yield impressive burst pressures, and forms the basis of the Lifeline™ graft, but requires a lengthy maturation period to fuse cell layers together. A more recent method uses cultured threads, rather than cultured sheets—grown via similar techniques, these can then be woven into three-dimensional structures to the fusion time requirement while retaining comparable mechanical strength [89]. Even smaller units of tissue have also been explored, including the use of three-dimensional bioprinting techniques to precisely deposit spherical, multicellular aggregates, which fuse together in bioreactor culture [90, 91]. These have the exciting potential of generating custom-shaped grafts to suit complex and branching vascular systems, but have as yet failed to match the mechanical strength of more standard approaches.

The approach chosen to produce a vessel has enormous influence on its integrity. In their 2015 review, Pashneh-Tala et al. graphically summarize the burst pressures, compliance, and suture retention strengths reported in all recent literature grafts [71]; no TEVG reported parity with native vessel across all three metrics, yet each of the approaches outlined above have produced extremely promising candidates.

The approach taken in this thesis—decellularizing the ECM produced by cells cultured on synthetic biodegradable scaffolds—combines the advantages cell-deposited matrix architecture the ready and inexpensive availability of biodegradable synthetics, and the off-the-shelf potential of ultimately cell-free products. This also avoids the complexities of decellularizing harvested biological materials: growing the initial vessel from well-characterized cell lines enables complete and repeatable control over the vessel's properties and antigenicity.

1.3.2 *TEVG cell sources*

Native blood vessels consist of three layers [92]:

- The intima, on the luminal surface of the vessel, is responsible for the prevention of thrombosis and the regulation of contractility. It is comprised of endothelial cells (ECs), which also play a role in regulating the phenotype of cells in the media.

- The media forms the bulk of the vessel wall, and contains multiple layers of contractile smooth muscle cells (SMCs), which in turn produce and maintain the thick extracellular matrix (ECM) that surrounds them. This ECM is largely comprised of collagen and elastic fibres, which in turn consist of an elastin core and an outer layer of fibrillin-rich microfibrils [93]. These elastic fibres are particularly important at low pressures, where they exhibit a linear, spring-like stress-strain response; as pressures increase,

collagen fibres increasingly contribute to generate high ultimate tensile strength [94]. In the physiological media, these collagen fibres are predominately circumferential and parallel to SMCs, with helically-oriented fibres providing additional strength in both axial and circumferential directions [95]. The luminal extent of the media contains the internal elastic lamina, which is especially rich in elastin, and the basal lamina, to which the intima adheres.

- THE ADVENTITIA, the outermost connective tissue layer of the vessel, is comprised primarily of collagen and fibroblasts (FBs) and provides substantial structural support to the vessel. Here, collagen fibres are largely axial. Increasingly, adventitial fibroblasts are thought to play an important role in remodeling and wound repair [95].

In tissue-engineered grafts, the thin intimal layer of the vessel can be produced readily via endothelial seeding and seems to form quickly in vivo even on acellular grafts. As such, most of the focus in TEVG development has been on the adventitial and medial layers and their thick ECM, produced by vascular SMCs and FBs. Of these two cell types, SMCs are of particular utility as they are more capable of making mature elastin for the all-important medial layer [96, 97]. Most TEVGs rely on SMCs at some point in their construction, and the source and culture of these SMCs has become a second key design element.

Unfortunately, the direct harvest of adult vascular cells as sources for graft production can be invasive, and yields somatic cells of limited replicative potential; these differentiated cells quickly cease division and become senescent. Attempts to artificially increase their active lifespan with human telomerase reverse transcriptase (hTERT) raises concerns for malignant transformation [98]. Furthermore, cells taken from older, atherosclerotic patients demonstrate still more rapid senescence—particularly concerning given the patient demographics of arterial bypass and HD graft recipients [99]. Together, these factors encourage the use of younger (i.e., capable of more division) cells in vascular graft design.

Mesenchymal stem cells (MSCs, often semi-synonymously termed mesenchymal *stromal* cells [101]) are one such entity. These multipotent cells have the long, thin, spindled, plastic-adherent appearance of fibroblasts and, as shown in Figure 1, are capable of producing not only the smooth muscle cells of vasculature but also adipocytes (fat), chondroblasts (cartilage), osteoblasts (bone), cardiomyocytes, and possibly even neuronal cell lines [102]. Though the International Society for Cellular Therapy developed specific MSC antigen and differentiation criteria in 2006, these have not been uniformly adopted and "MSCs" may well represent a heterogenous collection of cells with

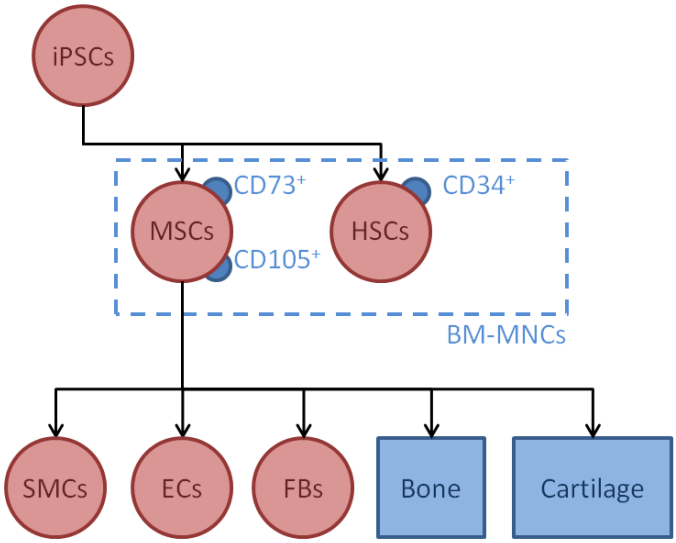


Figure 1: An *extremely* abbreviated map of the differentiation of the stem/progenitor cells relevant to vascular graft engineering. Pluripotent stem cells (either embryonic or induced) give rise to numerous progenitor lines, including CD34+ hæmatopoietic stem cells—which generate the myeloid and lymphoid lineages of the blood—and mesenchymal stem cells (MSCs). Both of these are constituents of bone marrow mononuclear cells (BM-MNCs). MSCs are rare (1 in 10,000 BM-MNCs), and though not immortal, are capable of manyfold expansion in culture, and have been shown to be broadly differentiable into numerous tissue types, including all three of the primary components of vascular tissue [100].

varying degrees of potency [103, 104]. Regardless, functional populations are widely studied in literature and can be extracted not only from bone marrow (BM-MSCs), but also adipose tissue [105], liver [106], and even human hair follicles [107]. They have been shown to be both antithrombogenic [108] and assistive of EC colonization in vivo [109]—both extremely enticing properties in vascular engineering. Readily available and possessing a high proliferative capacity, MSCs are a widely-used cell source for TEVGs, and an important foundation for future research and graft manufacture [110–114].

Since their 2006 discovery, induced pluripotent stem cells (iPSCs) have also attracted immense interest from the vascular engineering community, potentially allowing the growth of entirely autologous grafts from transformed adult cells [88, 115]. This is of obvious utility for cell-containing grafts, expanding the replicative potential of non-immunogenic autologous cells precisely personalized for each patient. Even in the construction of decellularized grafts, however, iPSCs could represent a superior source of cells capable of *indefinite* expansion, permitting the creation of human cell banks for predictably uniform graft production. Multiple groups have demonstrated the generation of MSCs (and subsequently SMCs) from iPSCs [116], and

patent iPSC-derived TEVGs were demonstrated in mouse models in 2012 [117]. Human iPSC TEVGs followed in 2014 both on nanofibrous poly(l-lactic acid) [118] and PGA [110] scaffolds, to which the research in this thesis contributes in small part.

1.4 CALCIFICATION AS A BARRIER TO INTEGRITY

As described, this diverse set of approaches has produced several candidates that satisfy the basic mechanical requirements of a functional graft, and, as discussed above, numerous clinical trials are in progress. To truly realize the promise of tissue engineering approaches, however, attention is turning to the large-scale reproducibility and biocompatibility of the final TEVG product. One significant obstacle faced by most cell-culture approaches—and a crucial factor in mechanical performance in the longer term—is the prevention of calcification in the graft wall both during vessel construction and after implantation, when in vivo remodeling exerts considerable influence on vessels' mechanical properties.

Evidence suggests that in vivo vascular calcification is an active, cell-driven process mediated by osteocytes and chondrocytes [119, 120]. These arise from both progenitors and SMCs themselves, which retain some phenotypic plasticity [121, 122]. Myocardial tissue engineering efforts have also been hindered by such calcification, with both BM-MNCs and BM-MSCs demonstrating substantial and unwelcome chondrogenesis when injected into damaged heart tissue [123, 124]. Similarly, studies of vascular graft cell sheets produced from MSC-derived SMCs demonstrated a significant increase in calcification over SMCs taken directly from human aorta [125]. Preventing calcification during graft construction thus requires careful control over differentiation.

Thus far, encouraging the growth of smooth muscle phenotypes in vitro has involved mimicking conditions seen in native vessels, including

- SOLUBLE GROWTH FACTORS: transforming growth factor β 1 (TGF-β1) and platelet-derived growth factor (PDGF) are both released in vivo by platelets in response to vascular injury [125, 126], and both are widely used to induce SMC differentiation in vitro. Other groups have also driven SMC phenotypes with ascorbic acid (i.e., vitamin C) [127] and retinoids (i.e., vitamin A), both implicated in embryonic vasculogenesis.

  TBG-β1 is part of a broader TGF-β superfamily, a collection of related extracellular growth factors that control large portions of tissue development. Forming homo- or hetero-dimers, the ligands bind to Type I and Type II subfamilies of transmembrane serine/threonine kinase receptors. Binding co-localizes

the two corresponding receptor types, principally activating a set of highly-conserved SMAD proteins with numerous and varied transcriptional effects in the nucleus [2]. Other notable superfamily members include Müllerian inhibiting substance(MIS), an essential signal in male embryogenesis, and bone morphogenic proteins (BMP) 2 through 7, which are crucial signals in cartilage and bone development. TBG-β1 is ubiquitous and multifunctional in vivo [130]; its differentiation effects can also be strongly dose-dependent. In vasculature, TBG-β1 is particularly responsible for vasculogenesis in embryos and vascular repair after platelet recruitment [131, 132]. At low concentrations, it can also promote differentiation by inducing PDGF-A [128].

PDGF has four mammalian forms (PDGF-A, PDGF-B, PDGF-C and PDGF-D), all of which also produce biologically-active, disulfide-linked homo- and hetero-dimers (e.g., $PDGF_{AB}$, $PDGF_{BB}$, etc.). These react with two distinct PDGF receptors, PDGFRα and PDGFRβ, both transmembrane receptor tyrosine kinases with differing affinities for the PDGF subtypes and different, overlapping downstream signaling effects. The $PDGF_{BB}$ subtype binds to both receptors and is commonly used for SMC differentiation, though its potency seems to depend on the ratio of PDGFRα and PDGFRβ on the MSC surface. This ratio is in turn influenced by multiple local micro-environmental factors—notably including mechanical stress, which drives the system towards PDGFRα predominance and an SMC endpoint [133].

- MECHANICAL STIMULATION: in vivo vessels see pulsatile forces throughout growth due to the flow of blood, which can be replicated during cell culture to improve the mechanical strength of grafts [134]. Mechanisms of this effect include the upregulation of both collagen and elastin production by SMCs [74, 135], and the increased production of matrix metalloproteinase 2 (MMP-2), which hastens the remodeling and realignment of already-deposited collagen to improve integrity [136]. Cyclic strain also directly promotes the SMC phenotype, however, as demonstrated by increased expression of the SMC markers smooth muscle α-actin, calponin-1, and smooth muscle myosin heavy chain (SMMHC) [94]. TGFβ-1 signaling increases, further promoting differentiation and ECM synthesis. Notably, *equiaxial* strain (i.e., the cell sheet stretched in all directions, as if on the surface of a balloon) was less effective than *uniaxial* strain in encouraging SMC differentiation, which pleasingly corresponds with the circumferential strain one would expect to see in a pulsing vessel [137].

2 TGF-β1 also sends signals via SMAD independent pathways; MAPK and Wnt pathways can be activated and push MSCs to a chondogenic endpoint [128, 129].

- DIRECT CELLULAR AND MATRIX CONTACT: Though normally separated from SMCs by a thick elastic lamina in vivo, endothelial cells are brought into contact with deeper vascular layers during both injury and development, and have been shown to direct SMC behaviour. Similarly, data suggests that direct cellular contact with ECs can induce MSC differentiation into SMC phenotypes, specifically increasing SM α-actin expression [138]—effects are mediated at least in part via activation of latent TGF-β [139]. Contact with glycosaminoglycans in the extracellular matrix produced by ECs causes similar differentiation into vascular phenotypes—an effect not seen with soluble factors produced by the EC, nor with fibronectin, collagen IV, or laminin alone [140].

Most TEGV efforts use various combinations of these factors to produce an imperfect yet serviceable population of SMCs from MSC progenitors. The partial overlap of these signals with osteogenic and chondrogenic pathways, however, coupled with multipotent progenitors' immense flexibility, means that the complete suppression of off-target differentiation remains an important technical challenge, and the focus of this thesis.

Further increases in differentiation control and consequent reduction in chondrogenesis and osteogenesis may require the consideration of as-yet less-studied factors. This includes both the effect of the oxygen tension (i.e., oxygen partial pressure) surrounding cells during culture—often very different than that seen by SMCs in vivo—and the effects of a number of small-molecule inhibitors shown to arrest the signaling pathways associated with chondro/osteogenesis.

1.4.1 *The effect of oxygen tension*

For convenience, much of in vitro cell/tissue culture is conducted at near-atmospheric oxygen tensions, typically at a partial pressure of $pO_2 = 21\%$ (dissolved oxygen concentration near $193\,\mathrm{nmol\,mL^{-1}}$). This is starkly different from physiological conditions; arterial smooth muscle cells in the aortic wall, for instance, see O_2 concentrations of 11.2% at the lumen, falling rapidly to 2.2% at a depth of $150\,\mu\mathrm{m}$ [141]. It is perhaps not surprising, then, that oxygen tension may be impacting MSC and SMC behaviour.

Huang et al., intrigued by endocardial ossification after BM-MSC injection in damaged hearts, studied the role that hypoxia plays in guiding MSC differentiation [142]. Comparing rat BM-MSCs incubated at 2% and 20% O_2, they noted significant inhibition of calcification at lower oxygen tensions, as measured by the gene expression of osteogenic markers (i.e., Collagen-I, osteocalcin, and alkaline phosphatase [ALP]). At the same time, however, chondrocytic differentiation seemed to increase. Similarly, Gawlitta et al. studied MSC

differentiation with a view to improving cartilage tissue engineering efforts. Comparing 5% and 20% O_2 concentrations on human BM-MSCs, they noted that normoxic (i.e., 20% O_2) conditions significantly enhanced the hypertrophic maturation of chondrocytes, the transformation that in bone development allows the cells to begin mineralization [143]. Based on both of these studies, it may be that hypoxic conditions can prove useful in the suppression of mineralization in TEVG applications, where chondrogenesis and subsequent hypertrophy have negative effects on mechanical performance.

Hypoxic growth may also have vascular graft benefits independent of chondrogenesis suppression. Bjork et al., studying neonatal dermal fibroblasts seeded onto fibrin-based tubular scaffolds, studied the effect of low oxygen tensions (2%) and insulin supplementation in an attempt to more closely mimic physiological conditions [144]. Hypoxia resulted in a threefold increase in tensile strength and elasticity, with a twofold increase in collagen weight per cell. Collagen fibril formation was enhanced by increased production of collagen prolyl-4-hydroxylase, an essential enzyme in the post-translational biosynthesis of collagen.

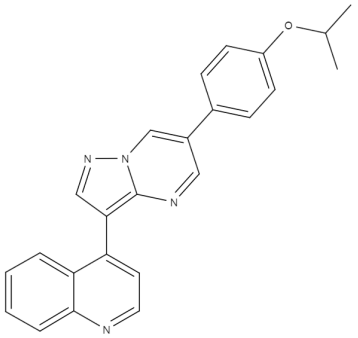
Together, these results suggest oxygen tension as an important lever in the future optimization of MSC-based TEVGs, and motivates further investigation into its effects.

1.4.2 *Bone morphogenic protein signaling*

TGF-β1 is an extremely common tool in differentiating MSCs to SMCs in vascular tissue engineering [114, 125, 145–148], often in combination with ascorbic acid or PDGF, as described above. It may have additional benefits beyond differentiation: TGF-β1 enhances SMC contractility [149] and, when combined with insulin, enhances elastin deposition [71] and thus membrane integrity.

TGF-β1's differentiation is not specific to SMC outcomes, however, when applied to MSCs—cells that possess an innate tendency toward bone [150] and cartilage [151] differentiation. While unable to initiate osteogenesis on its own, the factor amplifies osteoprogenitor proliferation in the early stages of BMP-induced osteogenesis [130]. TGF-β1 can also directly induce MSC chondrogenesis in vitro, and is actually specified in combination with other members of the TGF-β superfamily (TGF-β3 and BMP-2, -4, -6, and -7) in chondrogenesis protocols [151, 152]. Adenoviral-mediated transfer of TGF-β1 into MSCs resulted in robust chondrogenic differentiation [153].

SMC and chondrogenic differentiation seem to rely on SMAD and non-SMAD pathways, both of which TGF-β1 can induce in MSCs. Whether not TGF-β1 ultimately pushes them towards SMC or chondrogenic endpoints in vitro ultimately seems to depend on the specific microenvironment of the cell, including the presence of other

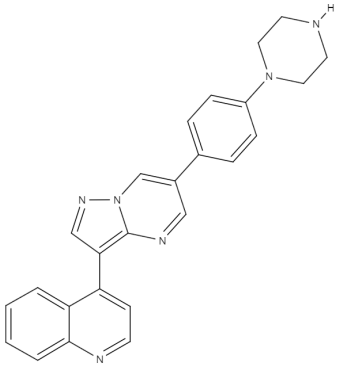


LDN193189	
Formula	$C_{25}H_{22}N_6$
Mol. weight	406.693u
PubChem ID	25195294
IC_{50}	$< 50nM$

Figure 2: Structure and physical properties of LDN193189. Structure generated at <molview.org> based on PubChem database; IC_{50}data from [154]. LDN193189 selectively blocks ALK2 and ALK3 receptors, thus inhibiting BMP4-mediated SMAD activation. Data suggests 200-fold selectivity for BMP signaling versus TGF-β, and a greater potency than dorsomorphin analogues [154].

growth factors [129]. This suggests that preventing osteo- and chondrogenesis requires strict control of all growth factors present in TEVG culture.

One potential suppressor of unwanted calcification could be BMP-specific inhibitors, specifically targeting and eliminating a major contributor to osteo- and chondrogenic signaling. Small molecules like LDN193189 (Figure 2) and DMH-1 (Figure 3) have been successfully used to suppress unwanted ossification [154] and chondrogenesis [155] in multipotent progenitors, albeit not yet for the purposes of TEVG development. Ideally, however, substances such as these could be additive with existing methods of encouraging SMC outcomes, helping to further tip the balance in favour of calcification-free grafts in future large-scale TEVG implementations.



DMH-1	
Formula	$C_{24}H_{20}N_4O$
Mol. weight	380.451 g/mol
PubChem ID	50997747
IC_{50}	$\approx$ 500nM

Figure 3: Structure and physical properties of dorsomorphin homologue 1 (DMH-1). Structure generated at <molview.org> based on PubChem database. Similar to LDN, dorsomorphin selectively blocks ALK2, ALK3, and ALK 6 activity, thus also preventing BMP signaling with a high degree of selectivity over TBG-β pathways [154].

2 AIMS

The rapid progression of TEVGs towards clinical impact in hæmodialysis, pediatric surgery, and bypass operations intensifies the need for highly-reproducible, off-the-shelf grafts with predictable properties, amenable to mass production. This in turn calls for flexible cell lines capable of generating multiple generations of vessels with minimal calcification or other defects.

To this end, this thesis attempts to answer two questions: do iPSC-derived SMCs still respond to the same signals and controls as BM-SCMs? And, can varying oxygen tensions and small-molecule growth factor inhibitors be used to suppress SMC tendencies towards osteo- and chondrogenesis?

2.1 IPSC DIFFERENTIATION

Our early studies in the derivation of MSCs from iPSCs were fraught with unexpected behavior and unusual osteogenic phenotypes, even when biomarker analysis indicated that the bulk of cells were differentiating correctly (Sumati Sundaram, personal communication). Conclusively demonstrating that existing BM-MSC TEVG techniques and research can be transferred to these new cell populations thus requires further study. With the ultimate goal of contributing to future iPSC-based TEVG construction, I examine the influence of pulsatile mechanical strain on iPSC-derived SMCs to determine whether they mirror their BM-derived counterparts in their increased proliferation and collagen production.

> *Hypothesis 1*: iPSC-derived MSCs will still demonstrate useful increases in proliferation and collagen production when exposed to pulsatile pressures, as has been shown in BM-MSCs ("Mechanical Stimulation" in Section 1.4).
>
> Specifically, vessels grown in pulsatile conditions will show a statistically-significant increase in both collagen and DNA weight versus non-pulsatile vessel controls.

2.2 OXYGEN AND SMALL-MOLECULE DIFFERENTIATION CONTROL

As discussed in Section 1.4.1 and Section 1.4.2, both oxygen tension and BMP-signal inhibition are potentially promising avenues of increased differentiation control. To determine whether either have useful potential in the reproducible production of TEVGs, I test the impact on MSC osteo- and chondrogenesis of various concentrations of

BMP inhibitors, LDN193189 and DMH-1 (Figures 2 & 3, under both high- and low-oxygen conditions.

If successful, results would guide future culture conditions and improve TEVG mechanical performance.

> *Hypothesis 2a*: BMP-inhibitors LDN193189 and DMH-1 will both suppress unwanted osteo- and chondrogenesis in both marrow-derived and iPSC-derived MSC populations during SMC differentiation.
>
> Specifically, treatment with both substances at optimal concentrations should result in a statistically-significant decrease in the expression of bone and cartilage markers (collagen II [Col2a] and osteocalcin [OCN]) without affecting the expression of SMC markers (collagen Ia [col1a1] and SM22-α [156, 157]).

> *Hypothesis 2b*: Hypoxic conditions (2% oxygen tension) that more closely mimic those seen physiologically by SMCs will improve the specificity of MSC differentiation into SMCs in vitro.
>
> Specifically, marrow-derived and iPSC-derived MSCs will see a statistically significant increase in the expression of SMC markers (col1a1 and SM22-α) in 2% O_2 vs. 20% O_2, with a concomitant decrease in bone and cartilage markers (Col2a and OCN).

Part II

METHODS AND RESULTS

The below summarizes research work conducted from May to August, 2013 towards the satisfaction of the requirements of the MD degree.

Comparisons between iPSC-derived and BM-derived MSCs are a piece of a larger study with Sundaram et al., as referenced in "Publications" above, and directed by Dr. Sumati Sundaram; studies on hypoxia and inhibitors involved greater independence. Division of labour was as follows, where SS indicates Sumati Sundaram and JS indicates Joshua Siewert:

iPSC Collagen Study	
Experimental design	SS
TEVG culture & growth	SS
Collagen & DNA assays	**JS**
Analysis & interpretation	**JS**

Differentiation Study	
Experimental design	SS & **JS**
Cell culture & growth	**JS**
qPCR & other assays	**JS**
Analysis & interpretation	**JS**

3

METHODS

3.1 LABORATORY TECHNIQUES

Individual procedures are outlined here; experimental design and rationale is outlined below in Sections 3.2 and 3.3.

3.1.1 *Media changes and sterile technique*

I performed all cell culture in an isolated, dedicated culture room using aseptic technique in laminar flow hoods, sterilized with continuous UV light in-between sessions. I kept all cell cultures in incubators maintained at 37°C when not in use. I changed aspirator tips between interactions with each well, and took care to minimize time between medium aspiration and replenishment of a given well. I heated all media to 37°C in a water bath prior to addition. I cultured all MSC cells in MesenCult™ [1] medium unless otherwise indicated. For all SMC differentiation, I used "4-20 medium," a mixture of high glucose Dulbecco's modified Eagle medium (DMEM) with 20% FBS (fetal bovine serum), penicillin G (10000 U/mL), copper sulfate (3 ng/mL), L-proline (50 ng/mL), L-alanine (40 ng/mL), glycine (50 ng/mL), and 50 µg/mL ascorbic acid as used in prior TEVG studies [110, 158]. In all cases, I changed media every 2-3 days.

To support iPSC cell line growth, I applied Matrigel coatings[2] to all plates during culture, proceeding per published manufacturer instructions.

3.1.2 *Cell passaging*

During cell culture, when cells reached $90-100\%$ confluence as confirmed by optical microscopy, I aspirated existing media under sterile conditions, and adding enough trypsin (warmed from frozen to 37 °C in a water bath) to cover cells; this would generally require 2 ml of trypsin for a T-75 flask. I returned the flasks to the 37 °C incubator for 5 minutes to facilitate detachment, confirmed detachment via optical microscopy, and diluted the cells 5:1 with low-glucose DMEM.

To determine the density of cells in solution, I mixed 10 µl of suspended cells with 10 µl of Trypan blue dye, then transferred 10 µl of the resultant mixture to a hæmocytometer consisting of five ($1 \times 1 \times$

1 STEMCELL Technologies Inc., Vancouver, BC, Canada, <http://www.stemcell.com>.

2 BD Biosciences, San Diego, CA, <http://www.bdbiosciences.com>.

GENE	FORWARD	REVERSE
OCN	GGACTGTGACGAGTTGGCTG	CCGTAGAAGCGCCGATAGG
Col2a	CTGCAAAATAAAATCTCGGTGTTCT	GGGCATTTGACTCACACCAGT
GENE	**MANUFACTURER**	**CAT. NO.**
GAPDH/G3PD	Qiagen	PPH00150F
SM22α/TAGLN	Qiagen	PPH19531F
Col1a1	Qiagen	PPH01299F

Table 2: Details of primers used for real-time quantitative reverse-transcriptase polymerase chain reaction throughout. In addition, I used the commercial primers from Qiagen, Inc. as indicated.

$0.1)\mathrm{mm}$ sections crisscrossed with $(0.1 \times 0.1)\mathrm{mm}$ demarcations. Under an optical microscope, I counted viable (i.e., non-staining) cells in the $5 \times (1 \times 1 \times 0.1)\mathrm{mm} = 0.5\,\mu\mathrm{l}$ region. With a manual count of N viable cells, and given the 1:1 dilution in dye, the original density was thus calculable as $2\mathrm{N}/(0.5\mu\mathrm{l}) = 4\mathrm{N}/\mu\mathrm{l}$ to provide a total cell count.

Centrifuging the suspended cells at 1000 RPM for 5 minutes and discarding the supernatant, I diluted the resultant pellet in enough growth medium to seed the new plate at 4000 cells/cm^2, then supplemented wells with growth medium to recommended working volumes[3].

I calculated all seeding densities based on existing hSMC work by Williams et al. [125].

3.1.3 *RNA isolation, quantification, and RT-qPCR*

I purified RNA from lysed cells using the RNeasy® Mini Kit (Qiagen cat. nos. 74104 and 74106), following the manufacturer protocol[4]. After RNA extraction, I quantified it via 260 nm absorbance measurements using a Thermo Scientific NanoDrop™ Spectrophotometer, blanking and cleaning the stage with RNAase-free water between each sample.

To analyze gene expression, I relied on real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) using the iScript™ cDNA Synthesis Kit (Bio-Rad, cat. no. 170-8891) to synthesize first-strand cDNA, then thermally cycled the reaction per kit protocol[5] using either an Eppendorf Mastercycler® or a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System coupled with a C1000 Thermal Cy-

3 See "Surface areas and recommended medium volumes for Corning cell culture vessels," available at <http://csmedia2.corning.com/LifeSciences/media/pdf/an_surface_areas_reco_med_vol_for_cc_vessels.pdf>, accessed 2013-07-15.

4 See published RNeasy® Quick-Start Protocol (Quiagen, January 2011), available for download at <https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24>.

5 See Bio-Rad iScript™ cDNA Synthesis Kit product insert, available at <http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4106228.pdf>.

cler, both loaded with custom cycling patterns. For samples with sufficient available RNA, I targeted 500 ng of RNA in each reaction based on NanoDrop quantification. Each sample and condition was plate in duplicate, with 20 µl volumes in all RT-PCR wells. I used iQ™ SYBR® Green Supermix (Bio-Rad cat. no. 170-8882) as the fluorescent probe, and the forward/reverse primer sequences given in Table 2. Accompanying BioRad CFX Manager™ software (version 3.0.1224.1015) calculated a quantitation cycle (C_q)[6] based on a multivariable, non-linear regression model.

I used the glyceraldehyde 3-phosphate dehydrogenase (GAPDH, also called G3PD) housekeeping gene as a reference to normalize average target quantification cycles, producing ΔC_q and allowing calculation of $\Delta\Delta C_q$ between two conditions. Thus, fold-change of target transcript levels between Condition α and Condition β would be given as $2^{\Delta\Delta C_q}$, where

$$\Delta C_{q\alpha} = C_{q\alpha} - C_{qGAPDH}$$

and

$$\Delta\Delta C_q = \Delta C_{q\beta} - \Delta C_{q\alpha}$$

as previously described by Livak et al. [160].

3.1.4 *Protein isolation and quantification*

I initially attempted to quantify protein mass in lysates using an A280 assay on the Thermo Scientific NanoDrop ™ Spectrophotometer, measuring absorbance at 280 nm without requiring standard curves or additional reagents[7]. A280 measurements on a commercial seven-concentration bovine serum albumin (BSA) standard set (Bio-Rad, # 500-0207) demonstrated expected accuracy, but measurements of actual cell lysates did not produce repeatable results when compared to Bradford assays of the same, possibly due to undesirable absorption by non-protein components.

For all subsequent protein quantification, I thus relied on a commercial Bradford/Coomassie Protein Assay (Bio-Rad, # 500-0002), measuring absorbance using a Beckman Coulter DU730 Spectrophotometer and the supplied "950 Bradford" program.

6 C_q and C_t (i.e., "threshold" cycle) are used interchangeably herein, "quantification" being the preferred term based on MIQE guidelines[159].

7 Per Thermo Scientific T010 Technical Bulletin for the NanoDrop 1000 & 8000 "Protein measurements", available at <http://www.nanodrop.com/Library/T010-NanoDrop%201000-&-NanoDrop%208000-Protein-Measurements.pdf>.

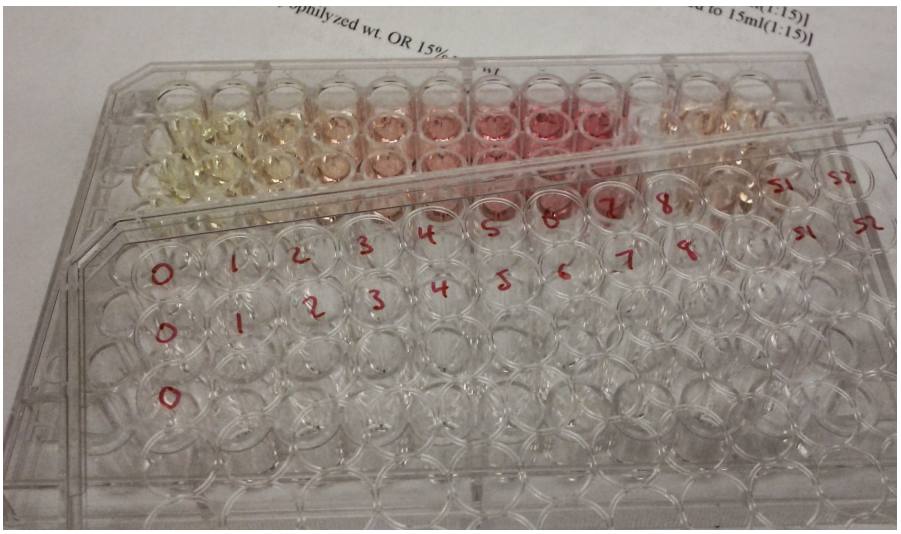


Figure 4: Series of collagen standards (left) ranging from $0\,\mu g\,mL^{-1}$ to $10\,\mu g\,mL^{-1}$ of hydroxyproline, with wells growing increasingly red as $\lambda = 550\,nm$ absorbance increases. Absorbance varies linearly with concentration.

3.1.5 *Vessel collagen quantification*

I quantified collagen in vessel samples with a standard colorimetric assay detecting hydroxyproline based on a modification of Stegemann's protocol [161]: digesting samples overnight in papain (140 µg) at 60 °C, incubating the result in 6N HCl at 115 °C for 18h, neutralizing, oxidizing with chloramine-T, then reacting with *p*-dimethylaminobenzaldehyde and converting hydroxyproline to collagen using a 1:10 w/w ratio as previously described [162]. I compared absorbance at 550 nm to a standard set of known concentrations to determine concentration, as shown in Figure 4.

3.1.6 *Vessel DNA quantification*

To quantify DNA in grown vessels, I used a Quant-iT™ PicoGreen® dsDNA Assay Kit (ThermoFisher Scientific, cat. no. P7589)[8] on samples digested with papain as above. A BioTek Synergy spectrofluorometer excited samples at 485 nm and measured fluorescence measured at 535 nm, which I then compared against a set standard DNA dilutions.

8 Published kit protocol is available at <https://tools.thermofisher.com/content/sfs/manuals/mp07581.pdf>.

3.2 STATIC VS. FLOW CULTURE CONDITIONS

To address the effects of pulsatile flow on iPSC vessels (Hypothesis 2.1), vessels were grown as described by Sundaram et al. [6], summarized here:

Prior to my arrival, iPSCs derived from human lung (IMR90-1) and human foreskin were provided by Dr. James Thomson (University of Wisconsin) [154] and cultured on Matrigel-coated plates [9] in commercially-available mTeSR™ culture medium [10]. Cells were passaged as described in Section 3.1.2 above; with these iPSC lines, confluence occurred every 5–6 days, and cells were dissociated using dispase. Methods were based on the feeder-free technique described by Xu et al. [163]. Cells were shown to express pluripotency markers Oct4, SSEA4, and Tra-1-60 via immunostaining and were confirmed to be karyotypically normal.

To differentiate iPSC cell lines into MSCs, cells first underwent neural crest induction for 10-12 days in a medium containing the TGF-β1 inhibitor SB 431542 (10μM, TOCRIS Cat. No. 1614), FGF2 ($10\,\mathrm{ng\,ml^{-1}}$, R& D Systems), and recombinant Wnt3a (25-50 $\mathrm{ng\,ml^{-1}}$, R& D Systems) as described by Menendez et al. [164]. The resultant neural crest cells were trypsinised and cultured in MesenCult™ growth medium (STEMCELL) for 4-7 days. MSC phenotype was confirmed via flow cytometry (positive for CD73, CD90, & CD105; negative for CD45) and successful differentiation into adipogenic, osteogenic, and chondrogenic lineages using the commercially available Human MSC Functional Identification Kit (Cat. No. # SC006, R& D Systems).

Vascular grafts were grown as described by Sundaram et al.:

> ...iPS-MSCs were seeded by directly pipetting cells onto sutured tubular polyglycolic acid (PGA) scaffolds (4 cm length, 3 cm diameter) over silicone tubing and then tied to the reactor arms using Dacron sleeves. The reactor was rotated every 10-15 minutes to ensure even distribution of cells into the PGA scaffold. The medium for growth of the TEVGs during the first half of the culture period was supplemented with $\mathrm{PDGF_BB}$ ($10\,\mathrm{ng\,mL^{-1}}$). For the remainder culture period, medium composition was switched to a low glucose DMEM with 10% FBS.
>
> In addition, L-ascorbic acid ($50\,\mathrm{\mu g\,mL^{-1}}$) was added to the medium three times a week to the cultures throughout the entire culture period. Additionally, vessels were cultured in a static mode for the initial 4 weeks to promote cell growth and differentiation.

9 BD Biosciences, San Diego, CA, <http://www.bdbiosciences.com>.

10 STEMCELL Technologies Inc., Vancouver, BC, Canada, <http://www.stemcell.com>.

After the completion of this static flow period, one bioreactor was left static as the non-pulsatile control, and another was provided with cyclic mechanical strain identical to previous studies on BM-MSCs: 2.5% strain at 2.75 Hz for a second 4 week period.

Taking 1 cm portions of both the pulsed vessel and the static control, I measured the dry weight of the vessel segments before performing papain digests and the collagen & DNA quantification assays described above in Sections 3.1.5 & 3.1.6, facilitating comparison of pulsatile and non-pulsatile conditions.

3.3 SMALL-MOLECULE AND HYPOXIA INHIBITION

To investigate the influence of small-molecule BMP signal inhibitors and hypoxia, I began with iPS-MSCs originally derived from the human lung (IMR90-1; clone 1, E8) iPSC line described in Section 3.2, alongside a separate population of BM-MSCs all seeded at approximately 4000 cells/cm^2.

Using standard 12-well plates (Falcon™ Polystyrene Microplates, Thermo Fisher Scientific), I plated 9 wells for each line in biological triplicate, aiming to produce a set each for both normoxic and hypoxic conditions, treated with either of two BMP inhibitors, LDN193189 hydrochloride (Sigma-Aldrich, Cat. No. SML0559) or DMH-1 (Tocris, Cat. No. 4125). This demanded a total of $2 \times 2 \times 3 \times 9 = 108$ individual wells for each of the two MSC lines. A limited supply of BM-MSCs prevented me from plating LDN193189 BM-MSCs in triplicate, however; the final experimental plate layout is thus as diagrammed in Figure 6.

At t=0, I replaced MesenCult™ medium with control and growth factor mixtures as indicated in Figure 5. Controls included:

A. The MesenCult™ medium used to culture the MSCs.

B. The "4-20 medium" (Section 3.1.1) used to culture SMCs. As in previous TEVG studies, I added fresh ascorbic acid weekly to counteract its breakdown when exposed to light [158]; after dissolution, I pushed the medium through a 0.2 μm filter to ensure no particulate matter remained.

C. A combination of the 4-20 medium and the growth factors used to encourage SMC differentiation: 1 ng/mL TGF-β1, 10 ng/mL $PDGF_BB$, and 50 μg/mL ascorbic acid, added from powder.

D. A combination of the 4-20 medium, the growth factors used to encourage SMC differentiation, and DMSO—the carrier used to dissolve the BMP inhibitor DMH-1—to control for the carrier's effect on the cells. I added DMSO volumes calculated to match the concentration present in the strongest DMH-1 wells.

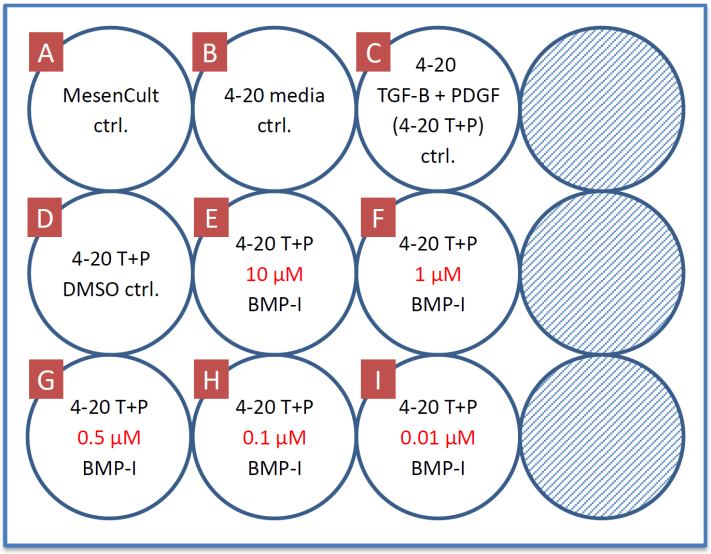


Figure 5: Layout of each experimental plate; shaded wells remained unfilled. Wells included media controls consisting of both (A) MesenCult™ and (B) 4-20 medium. SMC differentiation controls included (C) 4-20 medium + 1 ng/mL TGF-β1 + 10 ng/mL $PDGF_BB$ (i.e., "T+P") and (D) 4-20 medium + TGF-β1 + $PDGF_BB$ + 50 µg/mL ascorbic acid + DMSO (at the highest concentration used in the BMP-inhibitor treatments).
Remaining plates (E-I) included reactor media + TGF-β1 + $PDGF_BB$ + the studied inhibitor (i.e., "BMP-I")—either LDNLDN193189 or DMH1—at the concentrations indicated in red.

In the remaining wells (E through I), I added either LDN193189 or DMH1 at various concentrations to test the impact of BMP signaling inhibition. The wide range of concentrations (10 µM, 1 µM, 0.5 µM, 0.1 µM, and 0.01 µM) reflected the lack of direct IC_{50} data on the particular cell lines studied, hopefully including near-optimal concentrations somewhere in the series.

I placed one set (i.e., LDN193189- and DMH1-treated plates grown from iPSC-derived and BM-MSCs) in a 37°C incubator at room levels of oxygen, and another—the "hypoxic" set—in a separate incubator outfitted with N_2 canisters (Praxair) attached to a flow meter, oxygen sensor, and low-flow alarm; I set the flow meter to retain oxygenation rates of 2% through the duration of the experiment, and replaced N_2 canisters when required.

As described in Section 3.1.1, I changed media every 2-3 days with the media and growth factor specific to each well. I allowed cells to grow for 14 days prior to aspirating media, adding lysis buffer (Qiagen), and freezing at -80°C in Eppendorf tubes prior to RT-qPCR analysis of SMC, bone, and cartilage markers per the protocols in Section 3.1.3.

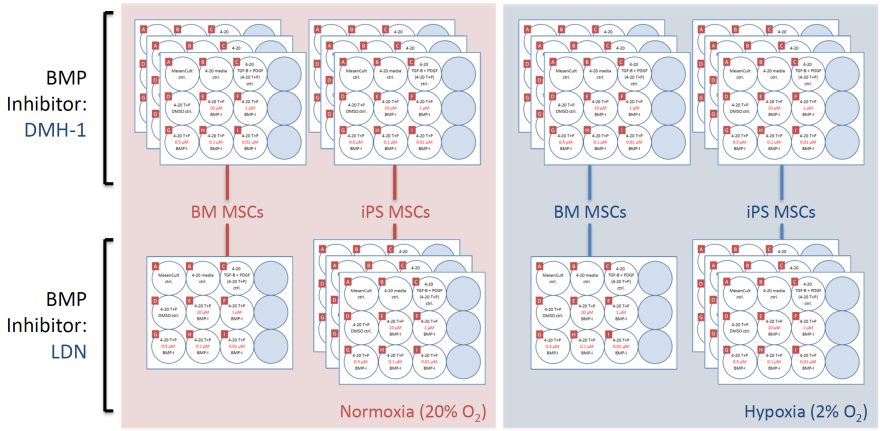


Figure 6: Layout of all the experiment plates; each plate contained nine filled wells as described in Figure 5. I prepared triplicate plates for each combination of inhibitor, MSC type, and oxygen tension to study effects and their interactions.
Note that insufficient BM-MSCs were available to produce the LDN plates in triplicate; for these conditions, I only made a single hypoxic and normoxic plate (as shown).

4

RESULTS

4.1 IPSC PULSATILE GROWTH

Four vessels were successfully grown, two samples each under static and pulsatile conditions.

I measured DNA content using the PicoGreen© dsDNA assay as described in Section 3.1.6, comparing two non-pulsatile vessel samples with two pulsatile vessel samples (4 weeks of growth under 2.5% uniaxial strain at 2.75 Hz). I plated samples in technical triplicate for absorbance measurement, and results for each sample lay within the assay's linear region, as demonstrated in Figure A.15 (Appendix) against a series of standard concentrations. Coefficients of variation between replicate wells averaged 3.5%, indicating reasonable intra-assay consistency. Results are shown in Figure 7 (orange), with ds-DNA expressed as a percentage of the dry weight of the resultant vessel. The non-pulsatile vessel averaged 0.0780 ± 0.002% dsDNA by dry weight, where 0.008% represents the standard error, i.e.,

$$s_{\bar{x}} = \frac{s}{\sqrt{n}}$$

where s is the standard deviation of either the pulsatile vessels or the non-pulsatile vessels, and n is the number of independent biological duplicates under each treatment condition (in this case, 2). Similarly, pulsatile conditions produced vessels comprised of 0.1414 ± 0.008% dsDNA.

Assuming roughly equal variance in both conditions ("homoscedastic")[1], the two-sample t-test is given as [165]

$$t = \frac{(\bar{x}_\alpha - \bar{x}_\beta)}{\sqrt{s_\alpha^2/n_\alpha + s_\beta^2/n_\beta}} = \frac{(\bar{x}_\alpha - \bar{x}_\beta)}{\sqrt{s_{\bar{x}_\alpha}^2 + s_{\bar{x}_\beta}^2}}$$

for equal sample sizes (i.e., $n_\alpha = n_\beta$), where $\bar{x}_\alpha$ and $\bar{x}_\beta$ are the means of the two treatment conditions. In this case, the difference in dsDNA fractions has a $t = 80.0$ with an accompanying two-tailed $p = 0.00015 \ll 0.05$, i.e., we would only expect differences of this magnitude 0.015% of the time if the null hypothesis were true, and the iPSCs were *not* actually responding to pulsatile stimulation. The

1 Per Krzywinski et al., the equal-variance t-test is surprisingly robust to slight inequalities in variance, especially when n is equal in both populations (as in our case) [165].

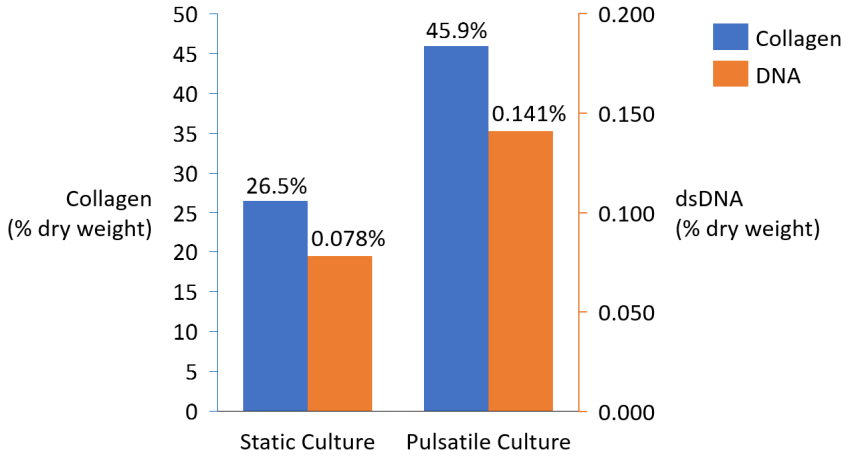


Figure 7: EFFECT OF PULSATILE STIMULATION on collagen and dsDNA production, both expressed as percentages of dry weight of the resultant vessels. Both cases represent the average of two separate vessel samples. dsDNA is measured via a PicoGreen© assay (Section 3.1.6), and increased from 0.0780 ± 0.002% of dry weight to 0.1414 ± 0.008% (p=0.015%). Collagen values reflect the results of a modified Bradford hydroxyproline assay (Section 3.1.5), showing an increase from 25 ± 2% to 46 ± 2% (p = 2.3%) with pulsatile stimulation.

difference thus easily achieves statistical significance even at this low n=2.

I also measured collagen weights, again as a percentage of dry weight, under both growth conditions; results are also shown in Figure 7 (blue) and summarized in Table 8. Pulsatile growth conditions resulted in a statistically-significant doubling of collagen fractions from 25 ± 2% to 46 ± 2% ($p = 0.023 < 0.05$, using again the homoscedastic two-tailed t-test).

Results are based on the colorimetric assay described in Section 3.1.5, which measures absorbance based on hydroxyproline concentrations. Again, samples lay in the linear region of the assay, per Figure A.16 (Appendix). I also compared TEVG samples against a sample of human aorta (Yale Pathology), as indicated, which exceeded both vessel samples in collagen fraction at 55% (one measurement).

4.2 DIFFERENTIATION CONTROL VIA O_2 AND BMP-INHIBITION

I successfully cultured BM-MSCs (passage 7) over the two-week experimental period with the chosen concentrations of BMP inhibitors, both in hypoxic and normoxic conditions, and analyzed the resulting lysates with RT-qPCR. BM-MSCs formed robust, confluent sheets of spindled, plastic-adherent cells visible under an optical microscope.

Conditions	Sample	Dry Weight (mg)	Estimated Collagen (mg)	(%)
Static	*# 1*	2.42	0.54	22.3%
	# 2	1.67	0.45	26.9%
			Average:	**25 ± 2%**
Pulsatile	*# 1*	2.86	1.38	48.1%
	# 2	2.61	1.14	43.6%
			Average:	**46 ± 2%**
Aorta		15.21	8.35	**54.9%**

Figure 8: Collagen produced in pulsatile and non-pulsatile iPSC-derived vessels, as measured by a colorimetric hydroxyproline assay and expressed as a fraction of vessel dry weight samples. Error terms represent standard error based on the two vessel samples measured at each condition.
Values for both TEVGs are compared with a sample of human aorta analyzed with the same hydroxyproline assay; pulsatile growth conditions moved collagen production significantly closer to physiological conditions.

Unfortunately, none of the iPSC-derived MSCs survived to the experiment's completion; cells grew thin and web-like, ultimately losing adherence and perishing. I thus discarded all iPSC-derived wells before they could produce lysates useful for analysis. There were no obvious signs of fungal or bacterial contamination under the optical microscope. With this setback, the new layout of the surviving plates is shown in Figure 9.

Some subsets of the BM-MSC population were also lost to analysis. Two plates of MesenCult controls (well "A") did not produce enough RNA to conduct PCR, as did one entire plate in what was likely a technical error in RNA extraction. Notably, no wells exposed to 10 μM LDN193189 produced measurable amounts of nucleic acid.

Fortunately, nearly all DMH-1 plates and conditions produced two or more usable results, and LDN193189 wells remained intact. The complete set of recorded ΔC_q values and their standard errors are supplied in the Appendix as Figures 17 and Figure 18. Where enough raw material persisted, I repeated PCR on samples with $\geqslant 1$ cycle discrepancy between technical PCR duplicates. I discarded technical duplicates with $\geqslant 2$ cycle discrepancies that I was unable to re-plate. In each remaining case, values represent the mean of two duplicates.

4.2.1 *Differentiation into smooth muscle phenotypes*

For all surviving wells, I quantified smooth muscle 22-α gene expression (SM22-α; see Table 2 in Section 3.1.3) as a marker of smooth muscle differentiation, alongside pro-α1 (I) collagen (Col1a1), the chon-

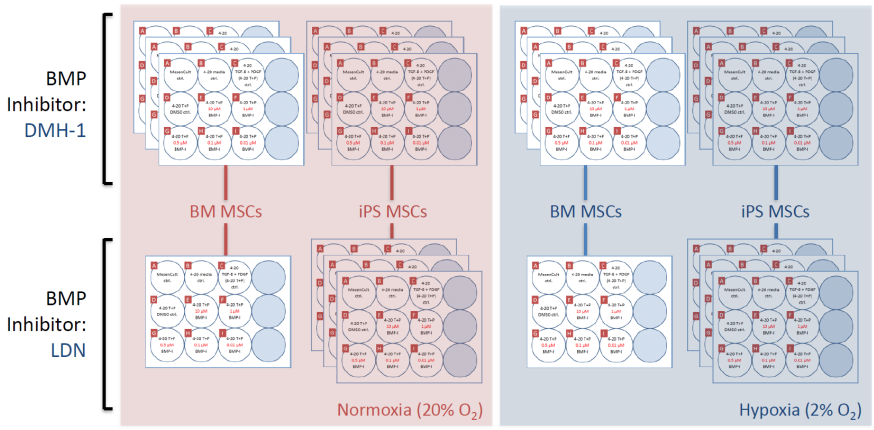


Figure 9: FINAL LAYOUT OF THE EXPERIMENTAL PLATES; compare with original experimental design in Figure 6. Shaded plates indicate cell death; all iPSC-derived MSC lines died during the course of the experiment, restricting inhibition studies to the BM-MSC cell lines.

drogenic marker pro-α (II) collagen (Col2a), and the osteogenic marker osteocalcin (OCN).

Figure 10 compares fold change values relative to the housekeeping gene GAPDH in both culture media and wells containing the added differentiation factors $PDFG_{BB}$ and TGF-β. SM22-α is significantly unregulated 2.3-fold by the factors ($\Delta\Delta C_q$ = -1.19, paired two-tailed t-test p = 0.37%), suggesting successful movement towards a smooth muscle phenotype, while OCN and Col2a are both significantly downregulated ($\Delta\Delta C_q$ = 1.38, p = 0.31% and $\Delta\Delta C_q$ = 2.37, p = 0.18, respectively). Col1a1 is not significantly affected.

The BMP inhibitor DMH-1 is dissolved in DMSO; to test the impact of the solvent itself, I similarly compared differentiation factor wells with (well "C") and without (well "D") a DMSO concentration equal to the highest concentration of DMSO present in the treatment groups—in this case, preparing a 10 µM solution from 50mM stock DMSO, 0.02% v/v DMSO in the 4-20 medium (DMH-1) and 0.1% v/v DMSO (LDN193189). The impact of the solvent was not statistically significant for any of the markers tested, as shown in Figure 11. Furthermore, overall GAPDH C_q values with and without the solvent did not change significantly, as seen in Figures A.17 and A.18 (Appendix).

4.2.2 *Effects of hypoxia and BMP-inhibitors*

Figure 12 shows fold-change impact on each gene marker of each combination of DMH-1 concentration and oxygen tension, with Figure 13 showing similar data for LDN193189.

Multiple linear regression on all surviving well ΔC_q values, controlling for plate-to-plate variation and presence or absence of hy-

poxic growth conditions, produces the factors summarized in Table 14 (STATA/IC Version 14.2; see Appendix Figure 19 for raw inputs and outputs).

The resultant regression takes the standard form

$$y_i = \beta_0 + \beta_1 x_{i1} + \cdots + \beta_p x_{ip} + \epsilon_i$$

for each individual well ($i = 1, 2, \ldots n$), where y_i represents the ΔC_q value of the measured gene (i.e., either SM22-α, OCN, Col2a, or Col1a1), β_0 is a constant associated with each gene's regression, and β_1 through β_p are the regression constants calculated for each independent variable (x_1 through x_p) included in the regression. This includes variables indicating BMP-inhibitor concentrations in each well (DMH-1 and LDN193189) as well as binary ("dummy") variables for the presence of hypoxia and the identity of the physical plate from which each measurement derives, accounting for inter-plate variability.

More specifically, each gene's expression (ΔC_q) is predicted as:

$$\Delta C_{qi} = \beta_0 + h_i \beta_{\text{hypoxia}} + C_{LDN} \beta_{LDN193189} + C_{DMH} \beta_{\text{DMH-1}} + p_{1i} \beta_{\text{plate 1}} + \cdots + p_{5i} \beta_{\text{plate 5}} + \epsilon_i$$

where:

- h_i represents the presence or absence of hypoxia, "1" for 2% growth conditions and "0" for normoxic 20% growth.
- C_{LDN} and C_{DMH} represent the concentrations of BMP-inhibitors LDN193189 and DMH-1, respectively, in μM—as these molecules were not tested in combination, only one is non-zero for a given data point.
- p_1 through p_5 are dummy variables for each plate, i.e., for the first plate ("plate 1"), p_1 is coded as "1," and p_2 through p_5 are "0." Note that since plates are either "hypoxic" or "normoxic" in their entirety, and because one plate can be described by a complete string of "$p_{xi} = 0$" values, only $n - 1$ binary variables are required to uniquely code for the full set of plates.

The resultant β coefficients thus represent the strength of the influence of their associated factor on each gene's expression.

I ran multiple separate regressions that also included interaction terms between oxygen levels and inhibitor concentration; these did *not* reveal any coefficients approaching statistical significance, and I therefore assumed independence for the remainder of the analysis.

Hypoxia had a significant negative effect on SM22-α expression ($\Delta\Delta C_q$ = 1.77 ± 0.22 S. E. M., p <0.0%), corresponding to an average 3.4-fold decrease in SM22-α mRNA concentration. On the contrary,

the reduced oxygen percentage tended to strongly *increase* the expression of chondrocyte marker Col2a ($\Delta\Delta C_q$ = -2.85 $\pm$ 0.73, p =0.1%), equivalent to a 7.2-fold upregulation. Though the osteocyte marker OCN showed no significant response to oxygen concentration, type-I collagen expression increased 3.4-fold ($\Delta\Delta C_q$ = -1.75 $\pm$ 0.65, p = 1.2%) in the low-oxygen environment. In net, this suggests that physiological oxygen tensions increased the incidence of chondrogenic differentiation.

DMH-1 had no significant effects on any of the studied markers. In contrast, LDN193189 produced a slight but significant 1.7-fold increase in SM22-α expression per μM of inhibitor across the studied 0.01-1 μM range of concentrations ($\Delta\Delta C_q$ = -0.78 $\pm$ 0.27, p =0.8%) and a substantial 4.5-fold reduction ($\Delta\Delta C_q$ = -2.17 $\pm$ 0.89, p =2.2%) in the expression of the cartilage-associated Col2a gene, in agreement with the stated hypothesis.

All four regressions produced F-statistics that indicated significance at the $p<0.05$ level. Several plates additionally exhibited statistically significant "plate effects," i.e., all the wells on a given physical plate saw correlated up- or down-regulation of the respective gene.

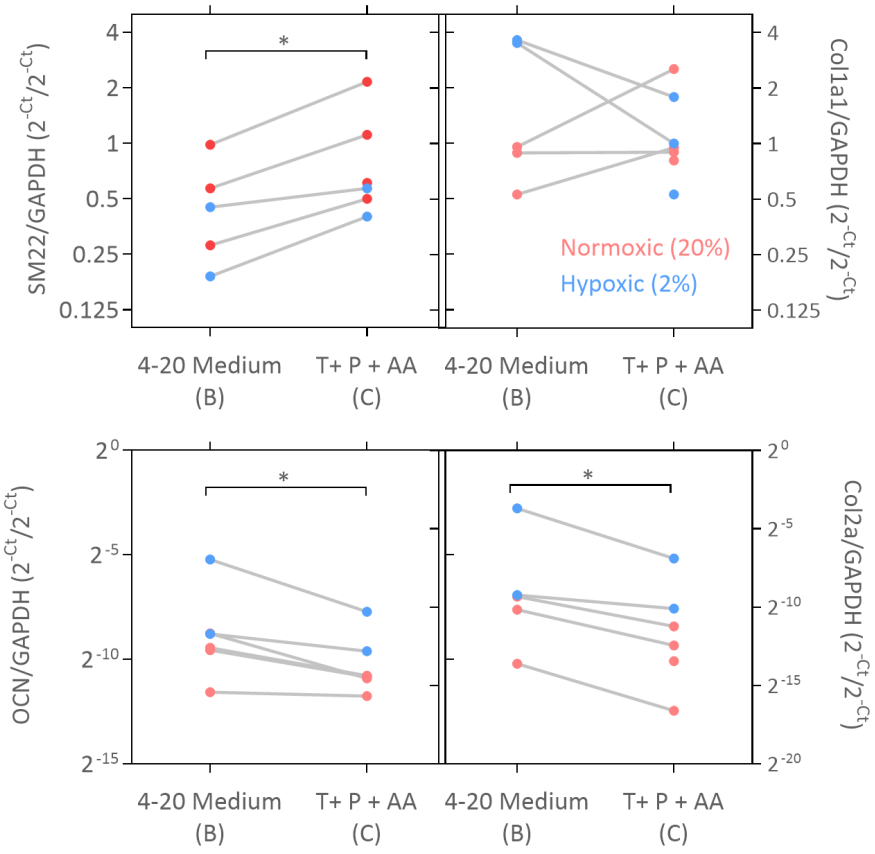


Figure 10: GROWTH FACTOR EFFECTS. Comparison of marker expression levels between 4-20 growth media (well "B") and cells with added growth media ($PDGF_{BB}$ and TGF-β, well "C"); each dot represents a well in normoxic (20% O_2, red) or hypoxic (2% O_2, blue) growth conditions, with gray lines connecting wells grown on the same 12-well plate. All values are measured in fold-change (i.e., $2^{C_q}/2^{C_q}$ from the GAPDH reference gene; asterisks indicate relationships significant at the p=0.01 level. Growth factors induced a significant increase in SMC-marker SM22 ($\Delta\Delta C_q$ = -1.19, paired two-tailed t-test p = 0.37%) and significant decreases in osteochondrogenic markers OCN ($\Delta\Delta C_q$ = 1.38, p = 0.31%) and Col2a ($\Delta\Delta C_q$ = 2.37, p = 0.18%). Col1a1 was not significantly changed.

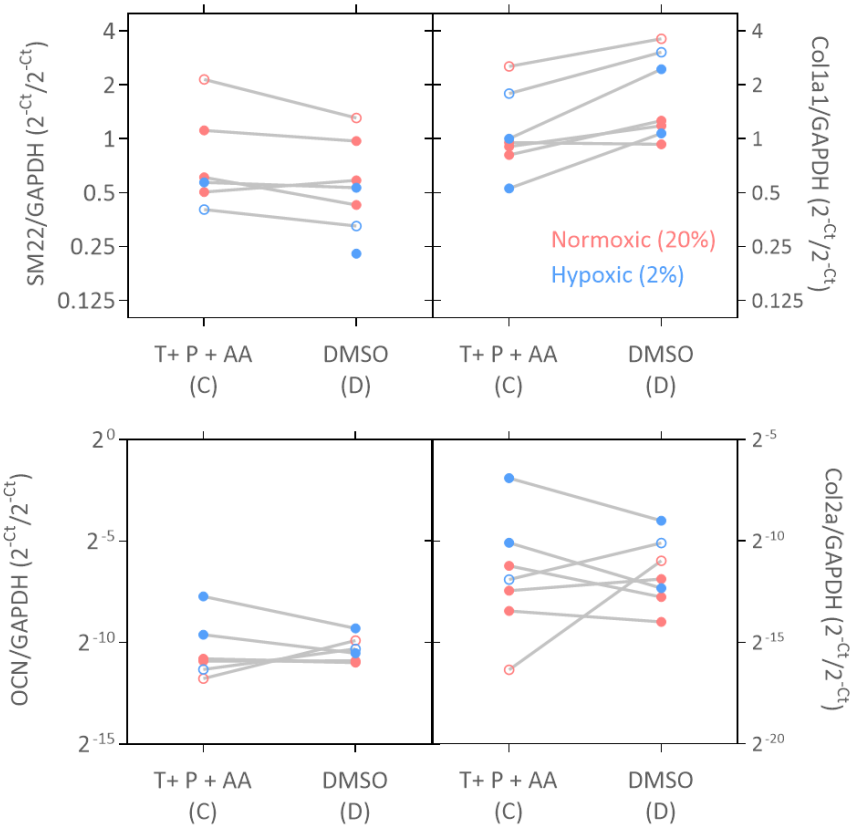


Figure 11: DMSO EFFECTS. Comparison of wells containing growth media and differentiation factors ($PDGF_{BB}$ and TGF-β) with ("C") and without ("D") the addition of 0.02% v/v DMSO, the highest concentration used in the DMH-1 experiment (solid dots), and 0.1%, the control in the LDN193189 experiment (hollow dots). All values are measured in fold-change (i.e., $2^{C_q}/2^{C_q}$ from the GAPDH reference gene. Neither group exhibited statistically significant trends in a paired, two-tailed t-test ($p > 0.05$).

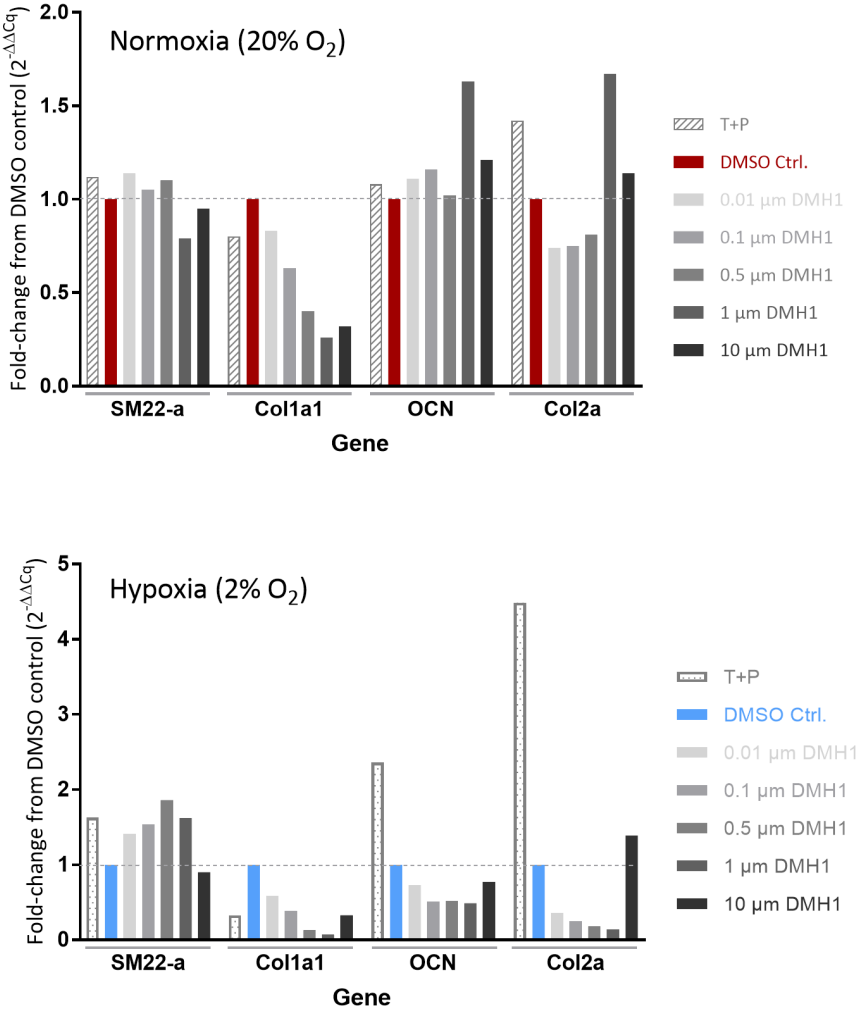


Figure 12: DMH-1 AND OXYGEN TENSION EFFECTS. Plot of fold-change variation between a DMSO control at both 20% & 2% O_2 and wells containing various concentrations of the BMP-1 inhibitor DMH-1. In all cases, fold-change is calculated as $2^{-\Delta\Delta C_q}$, as defined in Section 3.1.3. Interaction significance is not measured between each condition; instead, ΔC_q data across all concentrations is integrated into the multiple linear regression model summarized in Figure 14. The number of contributing measurements in each sample is summarized in Figure A.17 (Appendix).

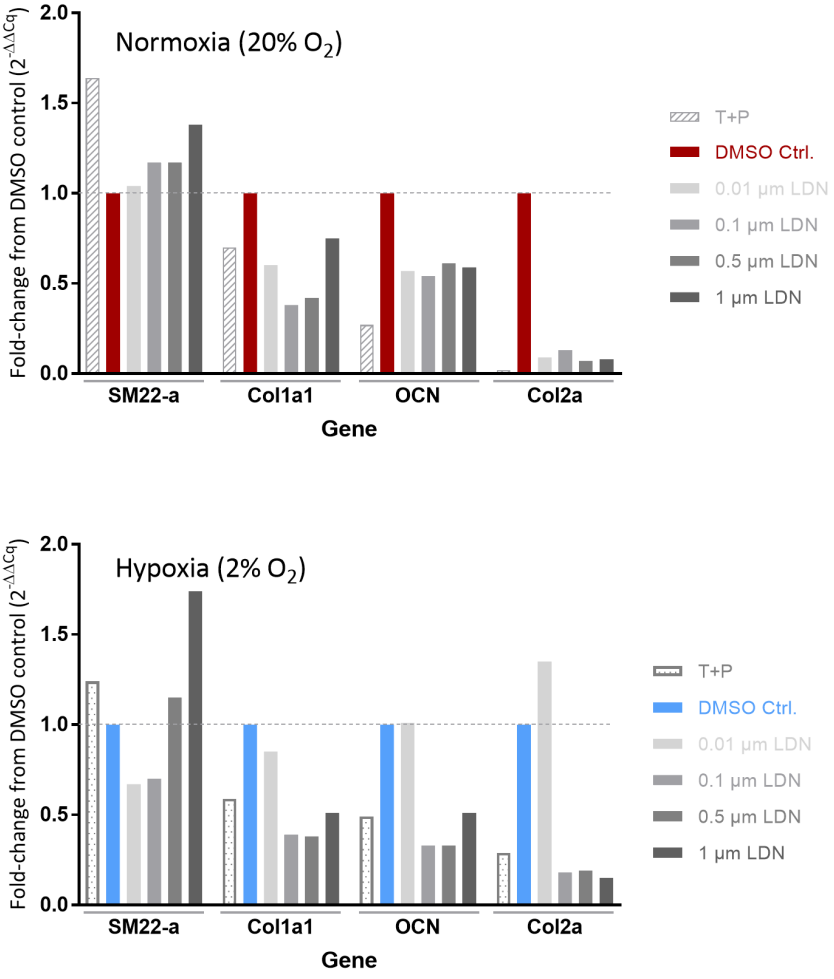


Figure 13: DMH-1 AND OXYGEN TENSION EFFECTS. Plot of fold-change variation between a DMSO control at both 20% & 2% O_2 and wells containing various concentrations of the BMP-1 inhibitor LDN193189. In all cases, fold-change is calculated as $2^{-\Delta\Delta C_q}$, as defined in Section 3.1.3. Interaction significance is not measured between each condition; instead, ΔC_q data across all concentrations is integrated into the multiple linear regression model summarized in Figure 14. The number of contributing measurements in each sample is summarized in Figure A.17 (Appendix).

	OCN	P-value	SM22-α	P-value	Col2a	P-value	Col1a1	P-value
$\beta_{hypoxia}$	0.17 ± 0.29	56.5%	**1.77 ± 0.22**	**0.0%**	**-2.85 ± 0.73**	**0.1%**	**-1.75 ± 0.65**	**1.2%**
β_{DMH-1}	0.00 ± 0.02	93.9%	0.02 ± 0.02	31.4%	-0.06 ± 0.06	29.4%	0.09 ± 0.05	7.2%
$\beta_{LDN193189}$	0.53 ± 0.36	15.5%	**-0.78 ± 0.27**	**0.8%**	**2.17 ± 0.89**	**2.2%**	0.34 ± 0.79	67.6%
β_0	10.79 ± 0.19	0.0%	0.12 ± 0.14	39.9%	13.68 ± 0.46	0.0%	0.83 ± 0.41	5.1%
Plate-specific factors:								
$\beta_{plate\ 1}$	-0.31 ± 0.29	29.5%	**-0.96 ± 0.22**	**0.0%**	**1.62 ± 0.73**	**3.5%**	**1.94 ± 0.65**	**0.6%**
$\beta_{plate\ 2}$	0.08 ± 0.25	74.4%	**0.68 ± 0.19**	**0.1%**	-0.69 ± 0.63	28.5%	-0.39 ± 0.56	49.3%
$\beta_{plate\ 3}$	**-1.18 ± 0.40**	**0.7%**	0.02 ± 0.31	94.6%	-0.97 ± 1.00	34.1%	0.07 ± 0.89	93.9%
$\beta_{plate\ 4}$	-0.30 ± 0.25	25.5%	**0.91 ± 0.19**	**0.0%**	-1.28 ± 0.63	5.1%	-0.20 ± 0.56	72.7%
$\beta_{plate\ 5}$	-0.43 ± 0.29	15.5%	-0.45 ± 0.22	5.3%	-0.63 ± 0.73	39.7%	-2.03 ± 0.65	0.4%
Regression statistics								
F (8,27) =	2.39		22.41		4.86		4.06	
P-value	**4.29%**		**0.00%**		**0.09%**		**0.28%**	

Figure 14: RESULTS OF MULTIPLE LINEAR REGRESSION on the ΔC_q values of four markers (relative to GAPDH) in BM-MSC-derived SMCs, indicating the impact of O_2 and BMP-1 inhibitor concentration on expression. Raw ΔC_q values from surviving wells at each condition diagrammed in Figure 9 are given in Figures A.17 and A.18 (Appendix). STATA (Version 14.2) uses a least-squares approach to generate the various coefficients defined in Section 4.2.2. All errors represent standard error of the mean. Significant effects are in bold. P-values indicate the likelihood that recorded data would be produced if corresponding β values were in fact 0 (i.e., the null hypothesis were true, and the associated variable had no effect).

$\beta_{hypoxia}$ represents the impact of a binary variable representing low oxygen tension, i.e., if grown in 2% O_2, ΔC_q values for the relevant gene would be expected to be $\beta_{hypoxia}$ higher than if grown in 20% O_2. Coefficients are thus given in units of ΔC_q i.e., ng/mg (marker/GAPDH). Similarly, "Plate-specific factors" are the coefficients controlling for each physical plate; $\beta_{plate\ 1}$, for instance, indicates that the ΔC_q of that gene's expression on *all* wells in Plate 1 were are predicted to be $\beta_{plate\ 1}$ higher than Plate 6. These presumably represent effects of plate position in the incubator, pipetting, etc.

$\beta_{LDN193189}$ and β_{DMH-1} represent the coefficients on the concentrations in μM of both studied BMP inhibitors. They represent the expected change in ΔC_q of the specified gene with each μ-molar increase in inhibitor concentration; units are given ng/mg (marker/GAPDH) per μM. Coefficient values of "0" indicate no effect of that inhibitor on the gene's ΔC_q values.

Finally, "Regression statistics" describe the quality of the resultant model in describing each gene's expression data in the form of the F statistic: the mean sum of squares captured by the linear model (MSM) divided by the residual sum of squares (MSR)[2]. The bracketed numbers indicate the degrees of freedom of the model and the residual, respectively. The F test can be viewed as a measure of the joint significance of the group of β variables, i.e., the associated p-value represents the chance that the recorded data would be produced by the null hypothesis that the included variables have no effect on gene expression.

5 DISCUSSION

5.1 IPSC PULSATILE GROWTH

Pulsatile growth conditions produced vessels with significant increases in both dsDNA and total collagen as a fraction of dry weight. The encouraging, statistically significant increase in dsDNA suggests that the iPSC-derived SMCs are indeed proliferating in the vessel scaffold, adding additional support to the viability of iPSC-derived cell lines as a future TEVG source.

The pulsatile conditions caused significantly increased collagen production similar to that of physiological vessel wall, also suggesting that these iPSC-derived cells are behaving as expected from SMCs. Solan et al. performed nearly identical studies on SMCs isolated from directly from porcine arteries, measuring collagen fractions in both a control group and a group pulsed at the same 2.75 Hz frequency used herein: dry weight fractions increased from $24.90 \pm 8.28\%$ to $35.37 \pm 8.28\%$, in close agreement with the 25% to 46% upregulation presented here [166]. Combined with the histological, mechanical, and biomarker testing performed in concert by Sundaram et al. [110], this argues strongly for the viability of iPSC-derived cell lines as an ultimate TEVG source. The potential advantages of such an option are numerous: banks of iPSCs could be used both to provide a well-characterized, readily reproduced cell source for future grafts, or even used to produce a personalized, patient-specific graft derived from the patient's own cells that permanently eliminates any risk of future immunorejection.

5.2 DIFFERENTIATION CONTROL VIA O_2 AND BMP-INHIBITION

Smooth muscle 22 (SM-22, also called transgelin [TAGLN] in fibroblasts [167]) is a specific, actin-binding 22-kDa cytoskeletal protein abundantly found in smooth muscle lineages [168] with three isoelectric isoforms, α, β, and γ. Sharing considerable sequence homology with calponin—which tonically inhibits the ATPase activity of myosin to cause smooth-muscle relaxation—SM22 is currently thought to regulate smooth muscle tone in vivo [169, 170]. Long before its functional role was hypothesized, however, it emerged as a common marker of SMC phenotypes, alongside α-smooth muscle actin (SMA), muscle-myosin heavy chain (MYH11), SM-calponin (CNN1), and smoothelin (SMTH) [171].

The Col1a1 gene codes for pro-α1 (I) collagen protein; two chains of pro-α1 (I) combine with one pro-α2 (I) chain to form the triple-helix of type I collagen. Type I collagen is not specific to a single cell linage, and is found abundantly in bone, ligaments, dermis, and vessel walls. Its importance to vascular integrity is highlighted in some subtypes of the diseases osteogenesis imperfecta and Ehlers-Danlos syndrome, in which Col1a1 and Col1a2 defects can lead to arterial rupture in early adulthood [172] and a plethora of additional connective tissue deficiencies. Alongside type III, type I collagen forms the primary load-bearing component of the vessel's load-bearing extracellular matrix, and its production is thus commonly tracked in TEVG enhancement studies [144, 173–175].

In contrast to type I and III collagen, type II collagen is found predominately in cartilage [176], and is the expression of the associated Col2a gene is used as a marker of chondroprogenitor cells [177]. Osteocalcin (also called bone gamma-carboxyglutamic acid-containing protein) is the primary noncollagenous protein constituent in bone and binds with high affinity to the hydroxyapatite that gives bone and teeth their hardness [178]. In multiple studies of MSC differentiation, the osteocalcin-associated gene OCN (also called BGLAP) is used alongside alkaline phosphatase (ALP), sclerostin (SOST), osterix (SP7), and others [179–181].

5.2.1 *Differentiation into smooth muscle phenotypes*

Based on the above, acceptable SMC differentiation should thus result in a substantial increase in SM22 mRNA expression with concomitant down-regulation in the osteochondrogenic markers OCN and Col2a. All of these trends are observed with statistical significance, most crucially the $\approx$ 2.3-fold increase in SM22 expression. Notably, when analyzing normoxic data alone only *paired* t-tests exhibit statistical significance, suggesting the influence of inter-plate differences. These may be the consequences of inconsistent pipetting technique or even plate positioning within the incubators.

Though SM22 is commonly employed as a mid-differentiation marker of smooth muscle development, some groups have reported its presence in multipotent stem cell lineages, suggesting its specificity as an SMC marker might be more limited in differentiation experiments than in measurement of adult tissue [182, 183]. Though the presence of statistically significant up-regulation in response to known promoters of the SMC lineage still argues for successful differentiation, a more convincing case would see the simultaneous measurement of other known biomarkers, e.g., α-smooth muscle actin (α-SMA) and calponin (CNN1). Studies in murine BM-MSCs have shown much more impressive increases in SM22-α 21 days after $PDGF_{BB}$ treatment (1.50 $\pm$ 0.27 vs. 0.005 $\pm$ 0.001 (ng/ng GAPDH) at 0 d), though

there is some indication that related intracellular signaling differs between the species [184].

Some controversy also exists around the use of $PDGF_{BB}$ as a promoter of SMC differentiation. While studies of $PDGF_{BB}$ on embryonic stem cells produced smooth muscle morphologies with induced α-SMA, calponin, and myosin on immunofluorescent staining, other groups have reported $PDGF_{BB}$ as a highly efficacious *negative* regulator of SMC differentiation and the suppression of SM22 in adult SMCs [157, 185]. Studies in our lab have indicated no increase in another SMC marker (CNN1) with $PDGF_{BB}$ alone, but statistically-significant increases in expression after treatment with $PDGF_{BB}$ and TGF-β in combination [110].

Some groups have reported ascorbic acid as inducing SMC phenotypes in MSCs, reporting α-actin positivity on immunofluorescence after two weeks of growth in 0.3 mM L-ascorbic acid [127]. This could compromise the choice of 4-20 media as a negative control for differentiation in this experiment. The concentration of ascorbic acid added to our well—50 µg/mL, or 0.3 µM—is negligible in comparison, and other studies have shown ascorbic acid concentrations up to 500 µM as promoting MSC proliferation without hindering differentiation potential [186].

5.2.2 *DMSO as a confounding factor*

Dimethyl sulfoxide (DMSO) is a widely-utilized, organic, amphipathic solvent, employed here as the carrier solution for the DMH-1 BMP-inhibitor. DMSO is well known to cause a host of phenotypic and morphogenic changes in cultured cells [187]. In embryonic stem cells, DMSO has been shown to induce differentiation at concentrations as low as 0.125% v/v, well below the DMSO's metabolic cytotoxicity IC-50 of 1-3% [188]. The solvent seems to enhance mesodermal differentiation, most powerfully affecting endodermal and hepatic lineages; while 0.1% v/v has been suggested as a safe ceiling concentration, even this may not be without impact [189].

Some groups have specifically reported the effect of DMSO on SMCs—one noting no change in SMC cell count after three days of treatment with 0.01% DMSO [190], and another seeing no significant change in SM22 expression after 2 weeks of DMSO treatment with unreported concentration [191].

Given the uncertainty surrounding the impact of DMSO even at low concentration, I included DMSO control wells on each plate at the highest concentration—0.02% v/v—used in the DMH-1 experiment. The data in Figure 11 suggests that at these concentrations the DMSO factor has no major impact. This is particularly reassuring given that, as this concentration is low enough to be considered negli-

gible, DMSO concentration was not specifically controlled for across DMH-1 treatment wells.

Though LDN193189 is water-soluble, a miscommunication during the course of the experiment led to the addition of 0.1% v/v DMSO to all treatment wells and the DMSO control during two media changes at the experiment's outset. Though again no statistically significant effects emerged, the N=2 dataset is inherently insufficiently-powered to detect minor differences. As concentrations were equal across all treatment wells, however, they should not directly impact existing trends.

5.2.3 *Notes on qPCR analysis*

The statistical analysis of the qPCR data in this experiment is complicated by the presence of missing values, generally due to factors unrelated to the experimental conditions themselves: pipetting errors, incorrect buffer preparation, insufficient initial cellular material, etc. They can thus reasonably be considered Missing Completely at Random (MCAR) in the statistical sense, somewhat simplifying their treatment.

Given the presence of multiple treatment concentrations, the data is not amenable to treatment with simple analysis of variance (ANOVA), and I instead relied on a least-squares multivariate linear regression. Even if the underlying relationship is non-linear—which could reasonably be argued, given a potentially sigmoidal dose-response curve from the inhibitor-receptor interaction, whose results are then examined in logarithmic C_q space—this may provide a first-order Taylor approximation of the real effects. Thus, I rely on a variant of the $\Delta\Delta C_q$ approach of Livak et al., comparing ΔC_q values for each gene marker; the β coefficients produced by the model correspond with $\Delta\Delta C_q$s cross the considered treatment effects.

Multivariate regression does require the assumption of homoscedasticity, or roughly symmetric variances across the dataset; it is not clear that this is fully satisfied in this approach, as equal variances in concentration result in asymmetric variance after exponential conversion. Furthermore, I have assumed equivalent PCR efficiencies between genes of interest and housekeeping genes, which may not be valid across all markers.

5.2.4 *Effects of hypoxia and BMP-inhibitors*

The most striking observed experimental effect surrounded the comparison of normoxia (20% O_2) and hypoxia (2% O_2), with the latter unexpectedly decreasing smooth muscle markers while enhancing expression of both Type I and Type II collagen. This was in contrast to the expectation that hypoxia might hinder chondrocytic differentia-

tion. The motivating examples from bone engineering literature hint at a subtler interplay: while still permitting early chondrocyte differentiation, low oxygen levels hinder subsequent hypertrophic differentiation of these chondrocytes [142, 143, 192]. Hypertrophic differentiation refers to phenotypic changes in adult chondrocytes after completing their proliferative phase, growing into large, round, terminally-differentiated cells that aid endochondral ossification in vivo [193]. These demonstrate decreased Type II collagen production relative to younger, proliferative chondrocytes. If this is indeed the case, hypoxia may yet be valuable to tissue engineers: not to prevent chondrogenesis, but to prevent subsequent hypertrophic differentiation and ossification. Future experiments might directly measure calcium production rather than the OCN marker, which may not capture the incidence of these hypertrophic cells.

Disappointingly, the DMH-1 inhibitor did not significantly alter any of the studied genes, suggesting limited utility in future differentiation control. In contrast, despite its relative paucity of replications, LDN193189 seemed to produce a significant, dose-dependent effect to both up-regulate smooth muscle markers and down-regulate the chondrocyte gene Col2a. Though this result may be impacted by DMSO erroneously added during the course of the experiment, this would not seem to explain the dose-dependence of the result. This suggests LDN193189 as a target for future study into calcification suppression.

It is possible that the genes selected as osteo- and chondrogenesis markers are expressed too late in their differentiation to be fully captured by this study. Osteocalcin is sometimes described as a late marker of osteoblast progression [194], and in one study of osteoblastic MSC differentiation it reached maximum expression only on day 21 (albeit demonstrating substantial up-regulation by day four in culture by Kulterer et al. [195]). A study of rat MSC osteogenesis compared the expression of multiple osteogenic markers, seeing a twofold increase in OCN at 13 days and four-fold at 16 days, at the experiment's conclusion; osteocalcin was among the last markers to demonstrate substantial change [181]. For osteogenesis, alkaline phosphatase and the PTH/PTHrP may be more reliable markers in future studies, appearing both more universally amongst osteoblast subtypes and at an earlier stage or bone production [196].

The use of a single housekeeping gene (GAPDH) may also have confounded qPCR results; many have suggested that unexpected and unmeasured variation from sample to sample can lead to large errors, and that truly accurate normalization of requires geometric averaging of multiple housekeeping genes in each sample [197]. While this may be less of a problem in clonal cultures than with human tissue biopsies, direct study of the stability of GAPDH as compared to other common housekeeping genes in the cell lines may be warranted, as

would be specific validation of GAPDH as a reference gene [198] to achieve compliance with MIQE guidelines [159].

The study did indicate several statistical inter-plate differences, which may be due to inconsistent media changes or pipetting techniques, or even more minor factors: the relative location of the plates in the incubator, time spent at room temperature awaiting manipulation, etc. It must also be noted that this experiment simultaneously estimates the magnitude of multiple presumably independent effects; with qPCR data alone producing 36 separate factor estimates, it is more than likely that the null hypothesis will produce Type 1 errors, i.e., incorrect rejection of the null hypothesis based on p-values less than the 5% threshold. Some have proposed p-value adjustments to account for multiple comparisons, but these have not achieved consensus and tend to simply trade reduction in Type I error for increases in Type II error, i.e., failing to find real effects [199]. In our case, statistically significant conclusions—some of which vastly exceed the relatively weak threshold of p=0.05—argue at the least for further study and corroboration of the presented results.

6

CONCLUSION

The work herein studied both the potential of iPSC-derived MSCs as a viable source for future TEVG construction and the potential use of oxygen tension control or small-molecule BMP-inhibitors to improving differentiation specificity, with a view to reducing vessel calcification.

Regarding iPSC-derived vessels, pulsatile vessel-growth conditions resulted in significant and substantial increases both in dsDNA—indicative of successfully proliferating SMCs—and collagen fraction as a percentage of dry weight, closely mimicking similar work in more typically-derived MSCs. This agrees with Hypothesis 1 in Chapter 2, and adds to the growing body of evidence pointing to iPSCs as an attractive future option for TEVG design.

In the case of oxygen tension, stated hypotheses were incorrect: hypoxia (2% O_2) actually *decreased* SMC marker expression and increased chondrogenic expression. This may reflect the true engineering utility of hypoxia lying not in preventing all chondrogenic differentiation, but rather in preventing hypertrophic differentiation and subsequent ossification of chondrocytes. This suggests more direct measurement of calcification as a target for future work, rather than simply tracking osteoblast-associated gene markers.

Unfortunately, the small molecule DMH-1 produced no statistically-significant results. This may be due to insufficient experimental power, especially given the low baseline presence of osteochondrogenesis in the studied cell populations.

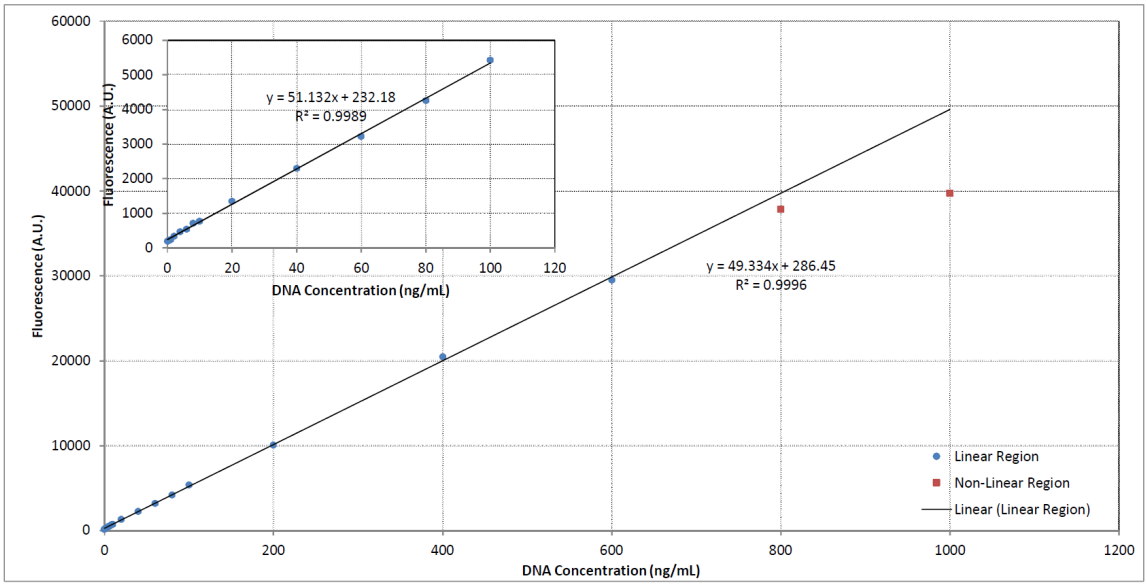
LDN193189, in contrast, was capable of inducing an impressive 4.5-fold reduction in Col2a expression while modestly up-regulating SMC markers. Though this initial study served as a broad survey across a variety of conditions and concentrations, these positive results warrant further study of this BMP-inhibitor as a potential addition to the arsenal of the vascular tissue engineer.

Part III

APPENDIX

a

APPENDIX



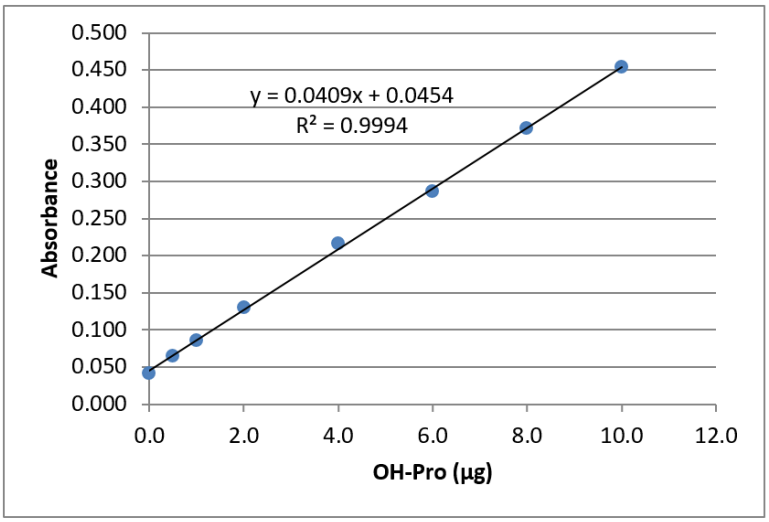
Sample	Dilution		Fluor.	Dil. [DNA] (ng/mL)	Sample [DNA] (ng/mL)	Avg. [DNA] (ng/mL)	Sample Conc. (mg/mL)	DNA Portion
468 1	α	0.075	15683.9	302.19254	4029.23385			
468 1	β	0.025	5605.3	105.08397	4203.35863	4023.465	2.86	0.1407%
468 1	γ	0.0125	2685.1	47.972524	3837.80194			
468 2	α	0.075	15324.8	295.16932	3935.59096			
468 2	β	0.025	5010.9	93.458282	3738.33129	3711.09	2.61	0.1422%
468 2	γ	0.0125	2443.2	43.241849	3459.34792			
471 1	α	0.075	8174.8	155.33517	2071.13556			
471 1	β	0.025	2730.8	48.865638	1954.6255	1892.21	2.42	0.0782%
471 1	γ	0.0125	1287.3	20.635871	1650.86964			
471 2	α	0.075	5616.3	105.2991	1403.98794			
471 2	β	0.025	1898.3	32.585335	1303.41339	1298.559	1.67	0.0778%
471 2	γ	0.0125	991.7	14.853451	1188.2761			

Slope: 51.132
Intercept: 232.18

Figure 15: Standard curve data for PicoGreen© dsDNA assay demonstrating assay linearity up to approximately 600 ng/mL based on serial-dilution controls. Inset magnifies region below 100 ng/mL of DNA concentration, demonstrating comparable linearity.
I diluted each sample (i.e., 468 1 [pulsatile] is one vessel) at three different strengths (i.e., α, β, and γ) as the full extent of the region of linearity was not know in advance. As discussed in Section 4.1, I used 50 measurements to generate an average well absorbance number, and pipetted each sample in technical triplicate; I then scanned the 96-well plate three times. Every different data point is thus the average of nine values: three separate scans for each well, and three replicate wells per sample or standard.
I use the regression from the controls to generate a slope allowing me to convert the fluorescence of each vessel's dilutions into estimates of DNA content; I then combine the three dilutions for each vessel to give a net estimate of DNA content for each sample, demonstrating a near doubling in dsDNA concentration from non-pulsatile to pulsatile growth conditions.

Standard Curve

OH-pro (µg)	Absorbance (mean)	(st.dev.)
0.0	0.042	0.0012
0.5	0.064	0.0015
1.0	0.086	0.0006
2.0	0.131	0.0015
3.0		
4.0	0.216	0.0026
6.0	0.286	0.0084
8.0	0.371	0.0026
10.0	0.454	0.0030



Slope:	0.0409	(µg)^-1
Int.:	0.0454	

Data calculations

Sample	Absorbance (mean)	(st.dev.)	OH-Pro (µg)	Vol. (mL)	Dil. Fact.	Dry Weight (mg)	Collagen (mg)
468 (1)	0.2330	0.0036	4.587	3	30	2.86	48.1%
468 (2)	0.1930	0.0044	3.609	3.15	31.5	2.61	43.6%
471 (1)	0.1083	0.0047	1.539	3.5	35	2.42	22.3%
471 (2)	0.1067	0.0015	1.498	3	30	1.67	26.9%
Hu. Aort	0.2730	0.0035	5.565	15	150	15.21	54.9%

Figure 16: Standard curve data demonstrating hydroxyproline assay linearity up to 10 µg; sample values fell well within the linear range. Conversion from hydroxyproline to collagen mass assumed a 1:10 w/w ratio, as described in [162].

Average ΔCt

	Normoxia				Hypoxia			
	OCN	**Col2a**	**Col1a1**	**SM-22α**	**OCN**	**Col2a**	**Col1a1**	**SM-22α**
Mesencult	9.01	8.26	-0.41	0.75	8.71	8.42	-0.97	0.95
4-20 Media	9.25	9.74	0.54	1.32	7.00	6.45	-0.31	2.33
T+P	10.84	12.37	0.17	0.52	8.67	8.49	0.92	0.81
DMSO Ctrl.	10.94	12.87	-0.16	0.68	9.90	10.65	-0.69	1.52
10 µm DMH1	10.67	12.68	1.47	0.76	10.28	10.18	0.89	1.67
1 µm DMH1	10.24	12.13	1.81	1.03	10.95	13.50	3.12	0.82
0.5 µm DMH1	10.92	13.17	1.17	0.54	10.85	13.15	2.30	0.63
0.1 µm DMH1	10.73	13.28	0.51	0.61	10.89	12.66	0.65	0.89
0.01 µm DMH1	10.80	13.30	0.11	0.49	10.35	12.12	0.06	1.02

= 3 contributing wells
= 2 contributing wells
= 1 contributing wells

Standard Error ΔCt

	Normoxia				Hypoxia			
	OCN	**Col2a**	**Col1a1**	**SM-22α**	**OCN**	**Col2a**	**Col1a1**	**SM-22α**
Mesencult					1.29	2.19	1.08	0.65
4-20 Media	0.25	0.42	0.38	0.52	1.78	2.76	1.50	1.19
T+P	0.03	0.64	0.07	0.34	0.94	1.59		
DMSO Ctrl.	0.02	0.61	0.13	0.35	0.61	1.66	0.60	0.61
10 µm DMH1	0.14	0.35	0.34	0.23	0.04	0.06	0.35	0.19
1 µm DMH1	0.39	1.38	0.90	0.62				
0.5 µm DMH1	0.13	0.05	0.36	0.25				
0.1 µm DMH1	0.05	0.43	0.33	0.25				
0.01 µm DMH1	0.07	0.14	0.13	0.20				

Figure 17: Raw ΔC_q values and their standard error for all conditions and measured genes in the DMH-1 BMP-inhibitor experiment. ΔC_q is calculated by subtracting the C_q of the GAPDH reference gene from the C_q of the gene of interest; as concentration varies as 2^{-C_q}, more strongly positive ΔC_q values indicate lower gene expression levels, and vice versa. The color of the cell indicates the number of separate samples averaged to generate the C_q value in question. Where sufficient results exist, I also supply standard error.

Average ΔCt

	Normoxia				Hypoxia			
	OCN	**Col2a**	**Col1a1**	**SM-22α**	**OCN**	**Col2a**	**Col1a1**	**SM-22α**
Mesencult	10.27	10.51	0.07	1.06	10.18	10.02	-1.88	2.08
4-20 Media	11.55	13.53	0.06	0.03	9.99	10.36	-1.86	2.39
T+P	11.77	16.29	-1.34	-1.10	11.32	11.90	-0.84	1.31
DMSO Ctrl.	9.89	10.97	-1.85	-0.38	10.30	10.09	-1.60	1.62
10 µm LDN								
1 µm LDN	10.66	14.55	-1.44	-0.85	11.27	12.83	-0.64	0.82
0.5 µm LDN	10.62	14.82	-0.59	-0.61	11.89	12.48	-0.19	1.42
0.1 µm LDN	10.77	13.92	-0.47	-0.61	11.91	12.58	-0.26	2.14
0.01 µm LDN	10.71	14.50	-1.11	-0.45	10.29	9.66	-1.36	2.18

Figure 18: Raw ΔC_q values and their standard error for all conditions and measured genes in the LDN193189 BMP-inhibitor experiment. As in Figure 17, ΔC_q is calculated by subtracting the C_q of the GAPDH reference gene from the C_q of the gene of interest; as concentration varies as 2^{-C_q}, more strongly positive ΔC_q values indicate lower gene expression levels, and vice versa. As only one cell is measured at each location, standard error calculations are not possible.

```
. reg col2a plated1n plated1h plated2n plated2h plated3n plateldn hypoxic dmh1 ldn
note: plated1n omitted because of collinearity
```

Source	SS	df	MS		
				Number of obs	= 36
				F(8, 27)	= 4.86
Model	46.131324	8	5.7664155	Prob > F	= 0.0009
Residual	32.0501422	27	1.1870423	R-squared	= 0.5901
				Adj R-squared	= 0.4686
Total	78.1814662	35	2.23375618	Root MSE	= 1.0895

col2a	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
plated1n	0	(omitted)				
plated1h	1.621227	.72828	2.23	0.035	.1269201	3.115534
plated2n	-.686337	.6290316	-1.09	0.285	-1.977003	.6043293
plated2h	-.9660756	.9975801	-0.97	0.341	-3.012941	1.08079
plated3n	-1.282701	.6290316	-2.04	0.051	-2.573367	.0079652
plateldn	-.6268449	.72828	-0.86	0.397	-2.121152	.8674621
hypoxic	-2.847655	.72828	-3.91	0.001	-4.341962	-1.353348
dmh1	-.0610106	.0570138	-1.07	0.294	-.1779933	.0559721
ldn	2.171647	.8945607	2.43	0.022	.3361597	4.007134
_cons	13.67751	.4582699	29.85	0.000	12.73722	14.6178

```
. reg col1a1 plated1n plated1h plated2n plated2h plated3n plateldn hypoxic dmh1 ldn
note: plated1n omitted because of collinearity
```

Source	SS	df	MS		
				Number of obs	= 36
				F(8, 27)	= 4.06
Model	30.4693672	8	3.8086709	Prob > F	= 0.0028
Residual	25.2986443	27	.936986825	R-squared	= 0.5464
				Adj R-squared	= 0.4119
Total	55.7680115	35	1.59337176	Root MSE	= .96798

col1a1	Coef.	Std. Err.	t	P>|t|	[95% Conf.	Interval]
plated1n	0	(omitted)				
plated1h	1.944056	.6470413	3.00	0.006	.6164366	3.271675
plated2n	-.3888381	.558864	-0.70	0.493	-1.535532	.757856
plated2h	.068011	.8863013	0.08	0.939	-1.750529	1.886551
plated3n	-.1973611	.558864	-0.35	0.727	-1.344055	.9493331
plateldn	-2.031222	.6470413	-3.14	0.004	-3.358841	-.703603
hypoxic	-1.749215	.6470413	-2.70	0.012	-3.076834	-.4215958
dmh1	.0949263	.050654	1.87	0.072	-.0090072	.1988597
ldn	.3356011	.7947737	0.42	0.676	-1.29514	1.966342
_cons	.8314064	.4071505	2.04	0.051	-.0039974	1.66681

Figure 19: Sample STATA inputs and outputs for linear regression on two markers, Col2a (top) and Col1a1 (bottom). Variables involved in the regression include a binary or "dummy" variable for each plate ("`plated_`"), a binary variable indicating whether the well was grown under hypoxic or normoxic conditions, and variables giving μM concentration of the BMP inhibitors. Variables are assumed to be independent in the above; I also tested interaction effects by including multiplicative terms (i.e., `ldn*hypoxia`) in the regression equation. STATA provides statistical treatment of each variable as shown.

[1] L R Kaiser. "The future of multihospital systems." In: *Topics in health care financing* 18.4 (1992), pp. 32–45. ISSN: 0095-3814. URL: http://www.ncbi.nlm.nih.gov/pubmed/1631884.

[2] Tissue Engineering Part A. *Origins*. July 2009. DOI: 10.1089/ten.tea.2007.0412. URL: http://www.liebertonline.com/doi/abs/10.1089/ten.tea.2007.0412.

[3] Jessica Viola, Bhavya Lal, and Oren Grad. "The Emergence of Tissue Engineering as a Research Field." In: *The National Science Foundation* A-07 (2003).

[4] R Langer and JP Vacanti. "Tissue engineering." In: *Science* 260.5110 (1993).

[5] Richard. Skalak and C. Fred. Fox. *Tissue engineering : proceedings of a workshop held at Granlibakken, Lake Tahoe, California, February 26-29, 1988*. Liss, 1988, p. 343. ISBN: 0845147064.

[6] Sumati Sundaram, Joshua Siewert, Jenna Balestrini, Ashley Gard, Kevin Boehm, Elise Wilcox, and Laura Niklason. "Tissue engineering and regenerative medicine." In: *Rossi's Principles of Transfusion Medicine*. Chichester, WestSussex: John Wiley & Sons, Ltd., Apr. 2016, pp. 488–504. ISBN: 9781119013020. DOI: 10.1002/9781119013020.ch42. URL: http://doi.wiley.com/10.1002/9781119013020.ch42.

[7] Alexis Carrel. "Suture of Blood-Vessels and Transplantation of Organs: Nobel Prize Lecture." In: (1912).

[8] Alexis Carrel. "La technique des anastomoses vasculaires et la transplantation des viscères." In: *Lyon Médical* 98 (1902), pp. 859–864.

[9] Alexis Carrel and Robert R. Hyman. "The Operative Technique of Vascular Anastomoses and the Transplantation of Viscera." In: *Clinical Orthopaedics & Related Research* 29.1 (1963), pp. 3–6. URL: http://journals.lww.com/corr/citation/1963/00290/1%7B%5C_%7Dthe%7B%5C_%7Doperative%7B%5C_%7Dtechnique%7B%5C_%7Dof%7B%5C_%7Dvascular%7B%5C_%7Danastomoses.1.aspx.

[10] J Kunlin. "Le traitement de l'ischemie arteritique par la greffe veineuse longue." In: *Revue de Chirurgie* 70 (1951), pp. 206–235.

[11] Jesse E. Thompson. "History of vascular surgery." In: *Surgery: Basic Science and Clinical Evidence: Second Edition* (2008), pp. 1299–1315. ISSN: 0098-7484. DOI: 10.1007/978-0-387-68113-9_61.

[12] Jordan D Haller and Andrew S Olearchyk. "Cardiology's 10 greatest discoveries." In: *Texas Heart Institute journal* 29.4 (2002), pp. 342–4. ISSN: 0730-2347. URL: http://www.ncbi.nlm.nih.gov/pubmed/12484626.

[13] R G Favaloro, D B Effler, C Cheanvechai, R A Quint, and F M Sones. "Acute coronary insufficiency (impending myocardial infarction and myocardial infarction): surgical treatment by the saphenous vein graft technique." In: *The American journal of cardiology* 28.5 (Nov. 1971), pp. 598–607. ISSN: 0002-9149. URL: http://www.ncbi.nlm.nih.gov/pubmed/5116978.

[14] Nirav J Mehta and Ijaz A Khan. "Cardiology's 10 greatest discoveries of the 20th century." In: *Texas Heart Institute journal* 29.3 (2002), pp. 164–71. ISSN: 0730-2347. URL: http://www.ncbi.nlm.nih.gov/pubmed/12224718.

[15] Ruben Y. Kannan, Henryk J. Salacinski, Peter E. Butler, George Hamilton, and Alexander M. Seifalian. "Current status of prosthetic bypass grafts: A review." In: *Journal of Biomedical Materials Research Part B: Applied Biomaterials* 74B.1 (July 2005), pp. 570–581. ISSN: 1552-4973. DOI: 10.1002/jbm.b.30247. URL: http://doi.wiley.com/10.1002/jbm.b.30247.

[16] Ion C. Ţintoiu, Malcolm John Underwood, Stephane Pierre Cook, Hironori Kitabata, and Aamer Abbas. *Coronary graft failure: State of the art*. 2016, pp. 1–758. ISBN: 9783319265155. DOI: 10.1007/978-3-319-26515-5.

[17] Ehsan Benrashid, Christopher C. McCoy, Linda M. Youngwirth, Jina Kim, Roberto J. Manson, James C. Otto, and Jeffrey H. Lawson. "Tissue engineered vascular grafts: Origins, development, and current strategies for clinical application." In: *Methods* 99 (2016), pp. 13–19. ISSN: 10959130. DOI: 10.1016/j.ymeth.2015.07.014. URL: http://dx.doi.org/10.1016/j.ymeth.2015.07.014.

[18] Michale E. Debakey, Denton A. Cooley, E. Stanley Crawford, and George C. Jr. Morris. "Clinical application of a new flexible knitted Dacron arterial substitute." In: *The American Surgeon* 24 (1958), pp. 862–869. ISSN: 00031348. DOI: 10.1001/archsurg.1958.01290040061008.

[19] Belding H. Scribner, J E Caner, R Buri, and W Quinton. "The technique of continuous hemodialysis." In: *Transactions - American Society for Artificial Internal Organs* 6 (1960), pp. 88–103. ISSN: 0066-0078. URL: http://www.ncbi.nlm.nih.gov/pubmed/13749430.

[20] Wayne Quinton, David Dillard, and Belding H. Scribner. "Cannulation of blood vessels for prolonged hemodialysis." In: *Trans Am Soc Artif Intern Organs* 6 (1960), pp. 104–107.

[21] William Drukker. "Haemodialysis : a Historical Review." In: *Replacement of Renal Function by Dialysis: A Text Book of Dialysis*. Ed. by J. F Maher. Vol. 60. 1861. Springer Science & Business Media, 1989, p. 1188. ISBN: 0898384141. DOI: 10.1016/S0025-6196(12)60309-7.

[22] Michael J. Brescia, James E. Cimino, Kenneth Appel, and Baruch J. Hurwich. "Chronic Hemodialysis Using Venipuncture and a Surgically Created Arteriovenous Fistula." In: *New England Journal of Medicine* 275.20 (Nov. 1966), pp. 1089–1092. ISSN: 0028-4793. DOI: 10.1056/NEJM196611172752002. URL: http://www.nejm.org/doi/abs/10.1056/NEJM196611172752002.

[23] Sherene Shalhub. "Historical Perspectives on Hemodialysis Access." In: *Hemodialysis Access*. Cham: Springer International Publishing, 2017, pp. 3–12. DOI: 10.1007/978-3-319-40061-7_1. URL: http://link.springer.com/10.1007/978-3-319-40061-7%7B%5C_%7D1.

[24] Beat H Walpoth and Gary L Bowlin. "The daunting quest for a small diameter vascular graft." In: *Expert Review of Medical Devices* 2.6 (Nov. 2005), pp. 647–651. ISSN: 1743-4440. DOI: 10.1586/17434440.2.6.647. URL: http://www.ncbi.nlm.nih.gov/pubmed/16293089.

[25] C B Weinberg and E Bell. "A blood vessel model constructed from collagen and cultured vascular cells." In: *Science (New York, N.Y.)* 231.4736 (Jan. 1986), pp. 397–400. ISSN: 0036-8075. URL: http://www.ncbi.nlm.nih.gov/pubmed/2934816.

[26] N L'Heureux, L Germain, R Labbé, and F A Auger. "In vitro construction of a human blood vessel from cultured vascular cells: a morphologic study." In: *Journal of vascular surgery* 17.3 (Mar. 1993), pp. 499–509. ISSN: 0741-5214. URL: http://www.ncbi.nlm.nih.gov/pubmed/8445745.

[27] L. E. Niklason, J. Gao, W. M. Abbott, K. K. Hirschi, S. Houser, R. Marini, and R. Langer. "Functional Arteries Grown in Vitro." In: *Science* 284.5413 (1999).

[28] Toshiharu Shin'oka, Yasuharu Imai, and Yoshito Ikada. "Transplantation of a Tissue-Engineered Pulmonary Artery." In: *New England Journal of Medicine* 344.7 (Feb. 2001), pp. 532–533. ISSN: 0028-4793. DOI: 10.1056/NEJM200102153440717. URL: http://www.nejm.org/doi/abs/10.1056/NEJM200102153440717.

[29] Shannon L. M. Dahl, Jennifer Koh, Vikas Prabhakar, and Laura E. Niklason. "Decellularized Native and Engineered Arterial Scaffolds for Transplantation." In: *Cell Transplantation* 12.6 (Jan. 2003), pp. 659–666. ISSN: 09636897. DOI: 10.3727/000000003108747136. URL: http://openurl.ingenta.com/content/xref?genre=article%7B%5C&%7Dissn=0963-6897%7B%5C&%7Dvolume=12%7B%5C&%7Dissue=6%7B%5C&%7Dspage=659.

[30] Todd N McAllister et al. "Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: a multicentre cohort study." In: *The Lancet* 373.9673 (Apr. 2009), pp. 1440–1446. ISSN: 01406736. DOI: 10.1016/S0140-6736(09)60248-8. URL: http://www.ncbi.nlm.nih.gov/pubmed/19394535.

[31] Wei Wu, Robert A Allen, and Yadong Wang. "Fast-degrading elastomer enables rapid remodeling of a cell-free synthetic graft into a neoartery." In: *Nature medicine* 18.7 (July 2012), pp. 1148–53. ISSN: 1546-170X. DOI: 10.1038/nm.2821. URL: http://www.ncbi.nlm.nih.gov/pubmed/22729285.

[32] C. E. Lok and R. Foley. "Vascular Access Morbidity and Mortality: Trends of the Last Decade." In: *Clinical Journal of the American Society of Nephrology* 8.7 (July 2013), pp. 1213–1219. ISSN: 1555-9041. DOI: 10.2215/CJN.01690213. URL: http://www.ncbi.nlm.nih.gov/pubmed/23824198.

[33] Jean Ethier, David C Mendelssohn, Stacey J Elder, Takeshi Hasegawa, Tadao Akizawa, Takashi Akiba, Bernard J Canaud, and Ronald L Pisoni. "Vascular access use and outcomes: an international perspective from the dialysis outcomes and practice patterns study." In: *Nephrol Dial Transplant* 23 (2008), pp. 3219–3226. DOI: 10.1093/ndt/gfn261.

[34] David A. Rose, Emmanuel Sonaike, and Kakra Hughes. "Hemodialysis Access." In: *Surgical Clinics of North America* 93.4 (2013), pp. 997–1012. ISSN: 00396109. DOI: 10.1016/j.suc.2013.05.002.

[35] Prabir Roy-Chaudhury, Burnett S. Kelly, Mary Ann Miller, Anita Reaves, Janice Armstrong, Nuwan Nanayakkara, and Sue C. Heffelfinger. "Venous neointimal hyperplasia in polytetrafluoroethylene dialysis grafts." In: *Kidney International* 59.6 (2001), pp. 2325–2334. ISSN: 00852538. DOI: 10.1046/j.1523-1755.2001.00750.x.

[36] Ziv J. Haskal et al. "Stent Graft versus Balloon Angioplasty for Failing Dialysis-Access Grafts." In: *New England Journal of Medicine* 362.6 (Feb. 2010), pp. 494–503. ISSN: 0028-4793. DOI: 10.1056/NEJMoa0902045. URL: http://www.nejm.org/doi/abs/10.1056/NEJMoa0902045.

[37] Peter T. Kennealey et al. "A prospective, randomized comparison of bovine carotid artery and expanded polytetrafluoroethylene for permanent hemodialysis vascular access." In: *Journal of Vascular Surgery* 53.6 (June 2011), pp. 1640–1648. ISSN: 07415214. DOI: 10.1016/j.jvs.2011.02.008. URL: http://linkinghub.elsevier.com/retrieve/pii/S0741521411003016.

[38] M. Harlander-Locke, J. C. Jimenez, P. F. Lawrence, H. A. Gelabert, B. G. Derubertis, D. A. Rigberg, and S. M. Farley. "Bovine Carotid Artery (Artegraft) as a Hemodialysis Access Conduit

in Patients Who Are Poor Candidates for Native Arteriovenous Fistulae." In: *Vascular and Endovascular Surgery* 48.7-8 (Oct. 2014), pp. 497–502. ISSN: 1538-5744. DOI: 10.1177/1538574414561231. URL: http://ves.sagepub.com/cgi/doi/10.1177/1538574414561231.

[39] Neelan Das, Mark J. Bratby, Vivek Shrivastava, Alison J. Cornall, Christopher R. Darby, Philip Boardman, Susan Anthony, and Raman Uberoi. "Results of a Seven-Year, Single-Centre Experience of the Long-Term Outcomes of Bovine Ureter Grafts Used as Novel Conduits for Haemodialysis Fistulas." In: *CardioVascular and Interventional Radiology* 34.5 (Oct. 2011), pp. 958–963. ISSN: 0174-1551. DOI: 10.1007/s00270-011-0096-z. URL: http://link.springer.com/10.1007/s00270-011-0096-z.

[40] Matthew P Welberry-Smith, James Ian Spark, Jai V. Patel, David J. Border, Dilip Oswal, and Andrew J P Lewington. "Massive aneurysmal dilatation of a depopulated ureteric hemodialysis xenograft." In: *Hemodialysis International* 13.1 (Jan. 2009), pp. 3–5. ISSN: 14927535. DOI: 10.1111/j.1542-4758.2009.00349.x. URL: http://doi.wiley.com/10.1111/j.1542-4758.2009.00349.x.

[41] Gearoid T Kingston, Christopher R Darby, and Ian S D Roberts. "The pathology of depopulated bovine ureter xenografts utilized for vascular access in haemodialysis patients." In: *Histopathology* 55.2 (Aug. 2009), pp. 154–160. ISSN: 03090167. DOI: 10.1111/j.1365-2559.2009.03360.x. URL: http://doi.wiley.com/10.1111/j.1365-2559.2009.03360.x.

[42] A Hatzibaloglou, Yiannis Velissaris, D Kaitzis, D Grekas, A Avdelidou, and D Kiskinis. "ProCol vascular bioprosthesis for vascular access: Midterm results." In: *Journal of Vascular Access* 5.1 (2004), pp. 16–18. ISSN: 11297298. URL: http://www.ncbi.nlm.nih.gov/pubmed/16596534.

[43] Sinasi Manduz, Nurkay Katrancioglu, Emre Ozker, and Kasim Dogan. "Early thrombosis in bovine mesenteric vein grafts after infrainguinal reconstruction." In: *International Journal of Angiology* 17.01 (Mar. 2008), pp. 37–39. ISSN: 1061-1711. DOI: 10.1055/s-0031-1278278. URL: http://www.thieme-connect.de/DOI/DOI?10.1055/s-0031-1278278.

[44] J Schmidli et al. "Bovine Mesenteric Vein Graft (ProCol) in Critical Limb Ischaemia with Tissue Loss and Infection." In: *European Journal of Vascular and Endovascular Surgery* 27.3 (Mar. 2004), pp. 251–253. ISSN: 10785884. DOI: 10.1016/j.ejvs.2003.12.001. URL: http://linkinghub.elsevier.com/retrieve/pii/S107858840300563X.

[45] Robert Madden et al. "Decellularized cadaver vein allografts used for hemodialysis access do not cause allosensitization or preclude kidney transplantation." In: *American journal of kidney diseases : the official journal of the National Kidney Foundation* 40.6 (Dec. 2002), pp. 1240–3. ISSN: 1523-6838. DOI: 10.1053/ajkd.

2002.36892. URL: http://www.ncbi.nlm.nih.gov/pubmed/12460043.

[46] Wojciech Wystrychowski, Todd N. McAllister, Krzysztof Zagalski, Nathalie Dusserre, Lech Cierpka, and Nicolas L'Heureux. *First human use of an allogeneic tissue-engineered vascular graft for hemodialysis access*. Tech. rep. 5. 2014, pp. 1353–1357. DOI: 10.1016/j.jvs.2013.08.018.

[47] Jeffrey H. Lawson et al. "Bioengineered human acellular vessels for dialysis access in patients with end-stage renal disease: Two phase 2 single-arm trials." In: *The Lancet* 387.10032 (2016), pp. 2026–2034. ISSN: 1474547X. DOI: 10.1016/S0140-6736(16)00557-2.

[48] Julien I.E Hoffman and Samuel Kaplan. "The incidence of congenital heart disease." In: *Journal of the American College of Cardiology* 39.12 (2002), pp. 1890–1900. ISSN: 07351097. DOI: 10.1016/S0735-1097(02)01886-7.

[49] Matthew E. Oster, Kyung A. Lee, Margaret A. Honein, Tiffany Riehle-Colarusso, Mikyong Shin, and Adolfo Correa. "Temporal Trends in Survival Among Infants With Critical Congenital Heart Defects." In: *Pediatrics* 131.5 (2013).

[50] Yushane Shih. "Effects Of Degradation On Mechanical Properties Of Tissue-Engineering Poly(glycolic Acid) Scaffolds." In: *Yale Medicine Thesis Digital Library* (2015). URL: http://elischolar.library.yale.edu/ymtdl/2011.

[51] Joseph T Patterson, Thomas Gilliland, Mark W Maxfield, Spencer Church, Yuji Naito, Toshiharu Shinoka, and Christopher K Breuer. "Tissue-engineered vascular grafts for use in the treatment of congenital heart disease: from the bench to the clinic and back again." In: *Regenerative medicine* 7.3 (May 2012), pp. 409–19. ISSN: 1746-076X. DOI: 10.2217/rme.12.12. URL: http://www.ncbi.nlm.nih.gov/pubmed/22594331.

[52] Jonathan Johnson and Heidi M Connolly. "Management of patients post-Fontan procedure - UpToDate." In: *UpToDate*. Ed. by Ted W. Post. Waltham, MA: UpToDate, 2017. URL: https://www.uptodate.com/contents/management-of-patients-post-fontan-procedure?source=search%7B%5C_%7Dresult%7B%5C&%7Dsearch=fontan%7B%5C&%7DselectedTitle=1%7B~%7D24.

[53] F Fontan and E Baudet. "Surgical repair of tricuspid atresia." In: *Thorax* 26.3 (May 1971), pp. 240–8. ISSN: 0040-6376. DOI: 10.1136/THX.26.3.240. URL: http://www.ncbi.nlm.nih.gov/pubmed/5089489.

[54] Toshiharu Shinoka and Christopher Breuer. "Tissue-Engineered Blood Vessels in Pediatric Cardiac Surgery CONGENITAL HEART DISEASE: A SIGNIFICANT MEDICAL PROBLEM." In: *YALE*

JOURNAL OF BIOLOGY AND MEDICINE 81 (2008), pp. 161–166.

[55] Salvatore Giannico, Fatma Hammad, Antonio Amodeo, Guido Michielon, Fabrizio Drago, Attilio Turchetta, Roberto Di Donato, and Stephen P. Sanders. "Clinical Outcome of 193 Extracardiac Fontan Patients." In: *Journal of the American College of Cardiology* 47.10 (May 2006), pp. 2065–2073. ISSN: 07351097. DOI: 10.1016/j.jacc.2005.12.065. URL: http://www.ncbi.nlm.nih.gov/pubmed/16697327.

[56] Joseph A Dearani et al. "Late follow-up of 1095 patients undergoing operation for complex congenital heart disease utilizing pulmonary ventricle to pulmonary artery conduits." In: *The Annals of Thoracic Surgery* 75.2 (Feb. 2003), pp. 399–411. ISSN: 00034975. DOI: 10.1016/S0003-4975(02)04547-2. URL: http://linkinghub.elsevier.com/retrieve/pii/S0003497502045472.

[57] Yuji Naito et al. "Successful clinical application of tissue-engineered graft for extracardiac Fontan operation." In: *The Journal of Thoracic and Cardiovascular Surgery* 125.2 (2003), pp. 419–420. ISSN: 00225223. DOI: 10.1067/mtc.2003.134.

[58] Narutoshi Hibino, Edward McGillicuddy, Goki Matsumura, Yuki Ichihara, Yuji Naito, Christopher Breuer, and Toshiharu Shinoka. "Late-term results of tissue-engineered vascular grafts in humans." In: *The Journal of Thoracic and Cardiovascular Surgery* 139.2 (Feb. 2010), 431–436.e2. ISSN: 00225223. DOI: 10.1016/j.jtcvs.2009.09.057. URL: http://www.ncbi.nlm.nih.gov/pubmed/20106404.

[59] Brooks V Udelsman, Mark W Maxfield, and Christopher K Breuer. "Tissue engineering of blood vessels in cardiovascular disease: moving towards clinical translation." In: *Heart (British Cardiac Society)* 99.7 (Apr. 2013), pp. 454–60. ISSN: 1468-201X. DOI: 10.1136/heartjnl-2012-302984. URL: http://www.ncbi.nlm.nih.gov/pubmed/23363931.

[60] Toshihide Nakano, Hideaki Kado, Tsuyoshi Tachibana, Kazuhiro Hinokiyama, Akira Shiose, Masaki Kajimoto, and Yusuke Ando. "Excellent Midterm Outcome of Extracardiac Conduit Total Cavopulmonary Connection: Results of 126 Cases." In: *The Annals of Thoracic Surgery* 84.5 (2007), pp. 1619–1626. ISSN: 00034975. DOI: 10.1016/j.athoracsur.2007.05.074.

[61] Hirotsugu Kurobe, Mark W Maxfield, Christopher K Breuer, and Toshiharu Shinoka. "Concise review: tissue-engineered vascular grafts for cardiac surgery: past, present, and future." In: *Stem cells translational medicine* 1.7 (July 2012), pp. 566–71. ISSN: 2157-6564. DOI: 10.5966/sctm.2012-0044. URL: http://www.ncbi.nlm.nih.gov/pubmed/23197861.

[62] Cameron Best, Ekene Onwuka, Victoria Pepper, Malik Sams, Jake Breuer, and Christopher Breuer. "Cardiovascular Tissue Engineering: Preclinical Validation to Bedside Application." In: *Physiology* 31.1 (2015).

[63] WHO. *WHO | Cardiovascular diseases (CVDs)*. 2015. DOI: FactsheetNÂř317. URL: www.who.int/mediacenter/factsheets/fs317en/. (visited on 01/24/2017).

[64] Dariush Mozaffarian et al. "Heart Disease and Stroke Statistics—2016 Update." In: *Circulation* 133.4 (Jan. 2016), e38–e360. ISSN: 0009-7322. DOI: 10.1161/CIR.0000000000000350. URL: http://www.ncbi.nlm.nih.gov/pubmed/26673558.

[65] Yoshiki Matsuo et al. "Plaque characteristics and arterial remodeling in coronary and peripheral arterial systems." In: *Atherosclerosis* 223.2 (Aug. 2012), pp. 365–71. ISSN: 1879-1484. DOI: 10.1016/j.atherosclerosis.2012.05.023. URL: http://www.ncbi.nlm.nih.gov/pubmed/22721702.

[66] Dawit G. Seifu, Agung Purnama, Kibret Mequanint, and Diego Mantovani. "Small-diameter vascular tissue engineering." In: *Nature Reviews Cardiology* 10.7 (2013), pp. 410–421. ISSN: 1759-5002. DOI: 10.1038/nrcardio.2013.77. URL: http://www.nature.com/doifinder/10.1038/nrcardio.2013.77.

[67] Joseph F. Sabik. "Understanding Saphenous Vein Graft Patency." In: *Circulation* 124.3 (2011).

[68] Ninos Samano, Håkan Geijer, Mats Liden, Stephen Fremes, Lennart Bodin, and Domingos Souza. "The no-touch saphenous vein for coronary artery bypass grafting maintains a patency, after 16 years, comparable to the left internal thoracic artery: A randomized trial." In: *The Journal of Thoracic and Cardiovascular Surgery* 150.4 (2015), pp. 880–888. ISSN: 00225223. DOI: 10.1016/j.jtcvs.2015.07.027.

[69] Nicolas L'Heureux, Nathalie Dusserre, Alicia Marini, Sergio Garrido, Luis de la Fuente, and Todd McAllister. "Technology Insight: the evolution of tissue-engineered vascular grafts—from research to clinical practice." In: *Nature Clinical Practice Cardiovascular Medicine* 4.7 (July 2007), pp. 389–395. ISSN: 1743-4297. DOI: 10.1038/ncpcardio0930. URL: http://www.ncbi.nlm.nih.gov/pubmed/17589429.

[70] Brett C. Isenberg, Chrysanthi Williams, and Robert T. Tranquillo. "Small-Diameter Artificial Arteries Engineered In Vitro." In: *Circulation Research* 98.1 (2006).

[71] Samand Pashneh-Tala, Sheila MacNeil, and Frederik Claeyssens. "The Tissue-Engineered Vascular Graft-Past, Present, and Future." In: *Tissue engineering. Part B, Reviews* 22.1 (Oct. 2016), p. 68. ISSN: 1937-3376. DOI: 10.1089/ten.teb.2015.0100. URL: http://www.ncbi.nlm.nih.gov/pubmed/26447530.

[72] Toshiharu Shin'oka, Goki Matsumura, Narutoshi Hibino, Yuji Naito, Manabu Watanabe, Takeshi Konuma, Takahiko Sakamoto, Masayoshi Nagatsu, and Hiromi Kurosawa. "Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells." In: *The Journal of Thoracic and Cardiovascular Surgery* 129.6 (June 2005), pp. 1330–1338. ISSN: 00225223. DOI: 10.1016/j.jtcvs.2004.12.047. URL: http://www.ncbi.nlm.nih.gov/pubmed/15942574.

[73] Goki Matsumura, Noriko Isayama, Shojiro Matsuda, Kensuke Taki, Yuki Sakamoto, Yoshito Ikada, and Kenji Yamazaki. "Long-term results of cell-free biodegradable scaffolds for in situ tissue engineering of pulmonary artery in a canine model." In: *Biomaterials* 34.27 (2013), pp. 6422–6428. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2013.05.037.

[74] Stacey C Schutte, Zhenzhen Chen, Kelvin G M Brockbank, and Robert M Nerem. "Cyclic strain improves strength and function of a collagen-based tissue-engineered vascular media." In: *Tissue engineering. Part A* 16.10 (Oct. 2010), pp. 3149–57. ISSN: 1937-335X. DOI: 10.1089/ten.TEA.2010.0009. URL: http://www.ncbi.nlm.nih.gov/pubmed/20504073.

[75] Zeeshan H Syedain, Lee A Meier, Jason W Bjork, Ann Lee, and Robert T Tranquillo. "Implantable arterial grafts from human fibroblasts and fibrin using a multi-graft pulsed flow-stretch bioreactor with noninvasive strength monitoring." In: *Biomaterials* 32.3 (Jan. 2011), pp. 714–22. ISSN: 1878-5905. DOI: 10.1016/j.biomaterials.2010.09.019. URL: http://www.ncbi.nlm.nih.gov/pubmed/20934214.

[76] Hak-Joon Sung, Carson Meredith, Chad Johnson, and Zorina S. Galis. "The effect of scaffold degradation rate on three-dimensional cell growth and angiogenesis." In: *Biomaterials* 25.26 (2004), pp. 5735–5742. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2004.01.066.

[77] Daniel D. Swartz, James A. Russell, and Stelios T. Andreadis. "Engineering of fibrin-based functional and implantable small-diameter blood vessels." In: *American Journal of Physiology - Heart and Circulatory Physiology* 288.3 (2005).

[78] Benedetto Marelli, Matteo Achilli, Antonio Alessandrino, Giuliano Freddi, Maria Cristina Tanzi, Silvia Farè, and Diego Mantovani. "Collagen-Reinforced Electrospun Silk Fibroin Tubular Construct as Small Calibre Vascular Graft." In: *Macromolecular Bioscience* 12.11 (Nov. 2012), pp. 1566–1574. ISSN: 16165187. DOI: 10.1002/mabi.201200195. URL: http://www.ncbi.nlm.nih.gov/pubmed/23060093.

[79] Chao Deng, Pingchuan Zhang, Branka Vulesevic, Drew Kuraitis, Fengfu Li, Ann Fook Yang, May Griffith, Marc Ruel, and Erik J. Suuronen. "A Collagen–Chitosan Hydrogel for Endothelial Differentiation and Angiogenesis." In: *Tissue Engi-*

neering Part A 16.10 (Oct. 2010), pp. 3099–3109. ISSN: 1937-3341. DOI: 10.1089/ten.tea.2009.0504. URL: http://www.ncbi.nlm.nih.gov/pubmed/20586613.

[80] Salma Amensag and Peter S McFetridge. "Rolling the human amnion to engineer laminated vascular tissues." In: *Tissue engineering. Part C, Methods* 18.11 (Nov. 2012), pp. 903–12. ISSN: 1937-3392. DOI: 10.1089/ten.TEC.2012.0119. URL: http://www.ncbi.nlm.nih.gov/pubmed/22616610.

[81] Po-Han Lee, Shu-Huai Tsai, Lih Kuo, Chih-Yung Hwang, Chan-Yen Kuo, Vivian C. Yang, and Jan-Kan Chen. "A prototype tissue engineered blood vessel using amniotic membrane as scaffold." In: *Acta Biomaterialia* 8.9 (Sept. 2012), pp. 3342–3348. ISSN: 17427061. DOI: 10.1016/j.actbio.2012.05.012. URL: http://www.ncbi.nlm.nih.gov/pubmed/22609451.

[82] A.V. Piterina, A. Callanan, L. Davis, C. Meaney, M. Walsh, and T.M. McGloughlin. "Extracellular matrices as advanced scaffolds for vascular tissue engineering." In: *Bio-Medical Materials and Engineering* 19.4-5 (2009), pp. 333–348. ISSN: 0959-2989. DOI: 10.3233/BME-2009-0598.

[83] Hao-Fan Peng, Jin Yu Liu, Stelios T. Andreadis, and Daniel D. Swartz. "Hair Follicle-Derived Smooth Muscle Cells and Small Intestinal Submucosa for Engineering Mechanically Robust and Vasoreactive Vascular Media." In: *Tissue Engineering Part A* 17.7-8 (Apr. 2011), pp. 981–990. ISSN: 1937-3341. DOI: 10.1089/ten.tea.2010.0109. URL: http://www.ncbi.nlm.nih.gov/pubmed/21083418.

[84] R A Roeder, G C Lantz, and L A Geddes. "Mechanical remodeling of small-intestine submucosa small-diameter vascular grafts–a preliminary report." In: *Biomedical instrumentation & technology* 35.2 (2001), pp. 110–20. URL: http://www.ncbi.nlm.nih.gov/pubmed/11383308.

[85] Timothy J. Keane, Ricardo Londono, Neill J. Turner, and Stephen F. Badylak. "Consequences of ineffective decellularization of biologic scaffolds on the host response." In: *Biomaterials* 33.6 (Feb. 2012), pp. 1771–1781. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2011.10.054. URL: http://www.ncbi.nlm.nih.gov/pubmed/22137126.

[86] J. I. Spark, S. Yeluri, C. Derham, Y. T. Wong, and D. Leitch. "Incomplete cellular depopulation may explain the high failure rate of bovine ureteric grafts." In: *British Journal of Surgery* 95.5 (May 2008), pp. 582–585. ISSN: 00071323. DOI: 10.1002/bjs.6052. URL: http://www.ncbi.nlm.nih.gov/pubmed/18344206.

[87] Marissa Peck, David Gebhart, Nathalie Dusserre, Todd N. McAllister, and Nicolas L'Heureux. "The Evolution of Vascular Tissue Engineering and Current State of the Art." In: *Cells Tissues Organs* 195.1-2 (2012), pp. 144–158. ISSN: 1422-6421. DOI:

10.1159/000331406. URL: http://www.ncbi.nlm.nih.gov/pubmed/21996786.

[88] Hideyuki Okano, Masaya Nakamura, Kenji Yoshida, Yohei Okada, Osahiko Tsuji, Satoshi Nori, Eiji Ikeda, Shinya Yamanaka, and Kyoko Miura. "Steps Toward Safe Cell Therapy Using Induced Pluripotent Stem Cells." In: *Circulation Research* 112.3 (2013).

[89] Marissa Peck, Nathalie Dusserre, Todd N. McAllister, and Nicolas L'Heureux. "Tissue engineering by self-assembly." In: *Materials Today* 14.5 (2011), pp. 218–224. ISSN: 13697021. DOI: 10.1016/S1369-7021(11)70117-1.

[90] Jens M. Kelm, Volker Lorber, Jess G. Snedeker, Dörthe Schmidt, Angela Broggini-Tenzer, Martin Weisstanner, Bernhard Odermatt, Anita Mol, Gregor Zünd, and Simon P. Hoerstrup. "A novel concept for scaffold-free vessel tissue engineering: Self-assembly of microtissue building blocks." In: *Journal of Biotechnology* 148.1 (July 2010), pp. 46–55. ISSN: 01681656. DOI: 10.1016/j.jbiotec.2010.03.002. URL: http://www.ncbi.nlm.nih.gov/pubmed/20223267.

[91] Francoise Marga, Karoly Jakab, Chirag Khatiwala, Benjamin Shepherd, Scott Dorfman, Bradley Hubbard, Stephen Colbert, and Forgacs Gabor. "Toward engineering functional organ modules by additive manufacturing." In: *Biofabrication* 4.2 (June 2012), p. 022001. ISSN: 1758-5082. DOI: 10.1088/1758-5082/4/2/022001. URL: http://www.ncbi.nlm.nih.gov/pubmed/22406433.

[92] Ira S. Cohen and Glenn R. Gaudette. *Regenerating the heart : stem cells and the cardiovascular system*. Humana Press, 2011, p. 556. ISBN: 1617790214.

[93] Cay M Kielty, Michael J Sherratt, and C Adrian Shuttleworth. "Elastic fibres." In: *Journal of cell science* 115.Pt 14 (July 2002), pp. 2817–28. ISSN: 0021-9533. URL: http://www.ncbi.nlm.nih.gov/pubmed/12082143.

[94] Angela H. Huang and Laura E. Niklason. "Engineering of arteries in vitro." In: *Cellular and Molecular Life Sciences* 71.11 (2014), pp. 2103–2118. ISSN: 14209071. DOI: 10.1007/s00018-013-1546-3. arXiv: NIHMS150003.

[95] George Bou-Gharios, Markella Ponticos, Vineeth Rajkumar, and David Abraham. "Extra-cellular matrix in vascular networks." In: *Cell Proliferation* 37.3 (June 2004), pp. 207–220. ISSN: 0960-7722. DOI: 10.1111/j.1365-2184.2004.00306.x. URL: http://www.ncbi.nlm.nih.gov/pubmed/15144498.

[96] Liqiong Gui and Laura E. Niklason. "Vascular tissue engineering: Building perfusable vasculature for implantation." In: *Current Opinion in Chemical Engineering* 3 (2014), pp. 68–74. ISSN: 22113398. DOI: 10.1016/j.coche.2013.11.004. arXiv:

NIHMS150003. URL: http://dx.doi.org/10.1016/j.coche.2013.11.004.

[97] A S Narayanan, L B Sandberg, R Ross, and D L Layman. "The smooth muscle cell. III. Elastin synthesis in arterial smooth muscle cell culture." In: *The Journal of cell biology* 68.3 (Mar. 1976), pp. 411–9. ISSN: 0021-9525. URL: http://www.ncbi.nlm.nih.gov/pubmed/1030702.

[98] Thomas Petersen and Laura Niklason. "Cellular lifespan and regenerative medicine." In: *Biomaterials* 28.26 (Sept. 2007), pp. 3751–3756. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2007.05.012. URL: http://linkinghub.elsevier.com/retrieve/pii/S0142961207004103.

[99] Tohru Minamino, Hideyuki Miyauchi, Toshihiko Yoshida, Kaoru Tateno, Takeshige Kunieda, and Issei Komuro. "Vascular cell senescence and vascular aging." In: *Journal of Molecular and Cellular Cardiology* 36.2 (2004), pp. 175–183. ISSN: 00222828. DOI: 10.1016/j.yjmcc.2003.11.010.

[100] Giselle Chamberlain, James Fox, Brian Ashton, and Jim Middleton. "Concise Review: Mesenchymal Stem Cells: Their Phenotype, Differentiation Capacity, Immunological Features, and Potential for Homing." In: *Stem Cells* 25.11 (Nov. 2007), pp. 2739–2749. ISSN: 10665099. DOI: 10.1634/stemcells.2007-0197. URL: http://doi.wiley.com/10.1634/stemcells.2007-0197.

[101] Ulrich Lindner, Jan Kramer, Jürgen Rohwedel, and Peter Schlenke. "Mesenchymal Stem or Stromal Cells: Toward a Better Understanding of Their Biology?" In: *Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie* 37.2 (Apr. 2010), pp. 75–83. ISSN: 1660-3796. DOI: 10.1159/000290897. URL: http://www.ncbi.nlm.nih.gov/pubmed/20737049.

[102] Peter E. Westerweel and Marianne C. Verhaar. "Directing Myogenic Mesenchymal Stem Cell Differentiation." In: *Circulation Research* 103.6 (2008).

[103] M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F.C Marini, D.S. Krause, R.J. Deans, A. Keating, D.J. Prockop, and E.M. Horwitz. "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." In: *Cytotherapy* 8.4 (2006), pp. 315–317. ISSN: 14653249. DOI: 10.1080/14653240600855905. URL: http://linkinghub.elsevier.com/retrieve/pii/S1465324906708817.

[104] Guojun Sheng. "The developmental basis of mesenchymal stem/stromal cells (MSCs)." In: *BMC Developmental Biology* 15.1 (Dec. 2015), p. 44. ISSN: 1471-213X. DOI: 10.1186/s12861-015-0094-5. URL: http://www.biomedcentral.com/1471-213X/15/44.

[105] Patricia A Zuk, Min Zhu, Peter Ashjian, Daniel A De Ugarte, Jerry I Huang, Hiroshi Mizuno, Zeni C Alfonso, John K Fraser, Prosper Benhaim, and Marc H Hedrick. "Human adipose tissue is a source of multipotent stem cells." In: *Molecular biology of the cell* 13.12 (Dec. 2002), pp. 4279–95. ISSN: 1059-1524. DOI: 10.1091/mbc.E02-02-0105. URL: http://www.ncbi.nlm.nih.gov/pubmed/12475952.

[106] C Campagnoli, I A Roberts, S Kumar, P R Bennett, I Bellantuono, and N M Fisk. "Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow." In: *Blood* 98.8 (Oct. 2001), pp. 2396–402. ISSN: 0006-4971. URL: http://www.ncbi.nlm.nih.gov/pubmed/11588036.

[107] J. Y. Liu, H. F. Peng, and S. T. Andreadis. "Contractile smooth muscle cells derived from hair-follicle stem cells." In: *Cardiovascular Research* 79.1 (Mar. 2008), pp. 24–33. ISSN: 0008-6363. DOI: 10.1093/cvr/cvn059. URL: https://academic.oup.com/cardiovascres/article-lookup/doi/10.1093/cvr/cvn059.

[108] C. K. Hashi, Y. Zhu, G.-Y. Yang, W. L. Young, B. S. Hsiao, K. Wang, B. Chu, and S. Li. "Antithrombogenic property of bone marrow mesenchymal stem cells in nanofibrous vascular grafts." In: *Proceedings of the National Academy of Sciences* 104.29 (July 2007), pp. 11915–11920. ISSN: 0027-8424. DOI: 10.1073/pnas.0704581104. URL: http://www.ncbi.nlm.nih.gov/pubmed/17615237.

[109] Alain Mirza, Jean-Marc Hyvelin, Gael Y. Rochefort, Patrick Lermusiaux, Daniel Antier, Bonaventure Awede, Pierre Bonnet, Jorge Domenech, and Veronique Eder. "Undifferentiated mesenchymal stem cells seeded on a vascular prosthesis contribute to the restoration of a physiologic vascular wall." In: *Journal of Vascular Surgery* 47.6 (June 2008), pp. 1313–1321. ISSN: 07415214. DOI: 10.1016/j.jvs.2007.12.038. URL: http://www.ncbi.nlm.nih.gov/pubmed/18329228.

[110] Sumati Sundaram, Jennifer One, Joshua Siewert, Stephan Teodosescu, Liping Zhao, Sashka Dimitrievska, Hong Qian, Angela H Huang, and Laura Niklason. "Tissue-engineered vascular grafts created from human induced pluripotent stem cells." In: *Stem cells translational medicine* 3.12 (Dec. 2014), pp. 1535–43. ISSN: 2157-6564. DOI: 10.5966/sctm.2014-0065. URL: http://www.ncbi.nlm.nih.gov/pubmed/25378654.

[111] Liang Zhang, Jianye Zhou, Qingping Lu, Yingjie Wei, and Shengshou Hu. "A novel small-diameter vascular graft: In vivo behavior of biodegradable three-layered tubular scaffolds." In: *Biotechnology and Bioengineering* 99.4 (Mar. 2008), pp. 1007–1015. ISSN: 00063592. DOI: 10.1002/bit.21629. URL: http://doi.wiley.com/10.1002/bit.21629.

[112] Jin Yu Liu, Daniel D. Swartz, Hao Fan Peng, Sylvia F. Gugino, James A. Russell, and Stelios T. Andreadis. "Functional tissue-engineered blood vessels from bone marrow progenitor cells." In: *Cardiovascular Research* 75.3 (Aug. 2007), pp. 618–628. ISSN: 00086363. DOI: 10.1016/j.cardiores.2007.04.018. URL: https://academic.oup.com/cardiovascres/article-lookup/doi/10.1016/j.cardiores.2007.04.018.

[113] Youngmee Jung et al. "Scaffold-free, Human Mesenchymal Stem Cell-Based Tissue Engineered Blood Vessels." In: *Scientific Reports* 5 (Oct. 2015), p. 15116. ISSN: 2045-2322. DOI: 10.1038/srep15116. URL: http://www.nature.com/articles/srep15116.

[114] Yilin Zhao, Su Zhang, Jianyin Zhou, Jinling Wang, Maochuan Zhen, Yun Liu, Jibing Chen, and Zhongquan Qi. "The development of a tissue-engineered artery using decellularized scaffold and autologous ovine mesenchymal stem cells." In: *Biomaterials* 31.2 (2010), pp. 296–307. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2009.09.049.

[115] Kazutoshi Takahashi and Shinya Yamanaka. "Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors." In: *Cell* 126.4 (Aug. 2006), pp. 663–676. ISSN: 00928674. DOI: 10.1016/j.cell.2006.07.024. URL: http://linkinghub.elsevier.com/retrieve/pii/S0092867406009767.

[116] Christine Cheung, Andreia S Bernardo, Matthew W B Trotter, Roger A Pedersen, and Sanjay Sinha. "Generation of human vascular smooth muscle subtypes provides insight into embryological origin–dependent disease susceptibility." In: *Nature Biotechnology* 30.2 (Jan. 2012), pp. 165–173. ISSN: 1087-0156. DOI: 10.1038/nbt.2107. URL: http://www.ncbi.nlm.nih.gov/pubmed/22252507.

[117] Narutoshi Hibino, Daniel R. Duncan, Ani Nalbandian, Tai Yi, Yibing Qyang, Toshiharu Shinoka, and Christopher K. Breuer. "Evaluation of the use of an induced puripotent stem cell sheet for the construction of tissue-engineered vascular grafts." In: *The Journal of Thoracic and Cardiovascular Surgery* 143.3 (Mar. 2012), pp. 696–703. ISSN: 00225223. DOI: 10.1016/j.jtcvs.2011.06.046. URL: http://www.ncbi.nlm.nih.gov/pubmed/22244569.

[118] Yongyu Wang, Jiang Hu, Jiao Jiao, Zhongning Liu, Zhou Zhou, Chao Zhao, Lung-Ji Chang, Y. Eugene Chen, Peter X. Ma, and Bo Yang. "Engineering vascular tissue with functional smooth muscle cells derived from human iPS cells and nanofibrous scaffolds." In: *Biomaterials* 35.32 (Oct. 2014), pp. 8960–8969. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2014.07.011. URL: http://www.ncbi.nlm.nih.gov/pubmed/25085858.

[119] Sarra de Valence, Jean-Christophe Tille, Damiano Mugnai, Wojciech Mrowczynski, Robert Gurny, Michael Möller, and Beat H. Walpoth. "Long term performance of polycaprolactone vascular grafts in a rat abdominal aorta replacement model." In: *Biomaterials* 33.1 (Jan. 2012), pp. 38–47. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2011.09.024. URL: http://www.ncbi.nlm.nih.gov/pubmed/21940044.

[120] Tadahisa Sugiura, Avione Y. Lee, and Toshiharu Shinoka. "Tissue engineering in vascular medicine." In: *Frontiers in Stem Cell and Regenerative Medicine Research*. Ed. by Atta Ur-Rahman and Shazia Anjum. Vol. 4. Bentham Science Publishers, 2017, pp. 3–35.

[121] M. Y. Speer, H.-Y. Yang, T. Brabb, E. Leaf, A. Look, W.-L. Lin, A. Frutkin, D. Dichek, and C. M. Giachelli. "Smooth Muscle Cells Give Rise to Osteochondrogenic Precursors and Chondrocytes in Calcifying Arteries." In: *Circulation Research* 104.6 (Mar. 2009), pp. 733–741. ISSN: 0009-7330. DOI: 10.1161/CIRCRESAHA.108.183053. URL: http://www.ncbi.nlm.nih.gov/pubmed/19197075.

[122] Y. Sun, C. H. Byon, K. Yuan, J. Chen, X. Mao, J. M. Heath, A. Javed, K. Zhang, P. G. Anderson, and Y. Chen. "Smooth Muscle Cell-Specific Runx2 Deficiency Inhibits Vascular Calcification." In: *Circulation Research* 111.5 (Aug. 2012), pp. 543–552. ISSN: 0009-7330. DOI: 10.1161/CIRCRESAHA.112.267237. URL: http://www.ncbi.nlm.nih.gov/pubmed/22773442.

[123] Young-Sup Yoon, Jong-Seon Park, Tengiz Tkebuchava, Corinne Luedeman, and Douglas W. Losordo. "Unexpected Severe Calcification After Transplantation of Bone Marrow Cells in Acute Myocardial Infarction." In: *Circulation* 109.25 (2004).

[124] Martin Breitbach et al. "Potential risks of bone marrow cell transplantation into infarcted hearts." In: *Blood* 110.4 (2007).

[125] Corin Williams, Angela W. Xie, Sitaram M. Sirisha Emani, Masayuki Yamato, Teruo Okano, Sitaram M. Sirisha Emani, and Joyce Y. Wong. "A Comparison of Human Smooth Muscle and Mesenchymal Stem Cells as Potential Cell Sources for Tissue-Engineered Vascular Patches." In: *Tissue Engineering Part A* 18.9-10 (May 2012), pp. 986–998. ISSN: 1937-3341. DOI: 10.1089/ten.tea.2011.0172. URL: http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2011.0172.

[126] Zhaodi Gong and Laura E. Niklason. "Use of Human Mesenchymal Stem Cells as Alternative Source of Smooth Muscle Cells in Vessel Engineering." In: 2011, pp. 279–294. DOI: 10.1007/978-1-60761-999-4_21. URL: http://link.springer.com/10.1007/978-1-60761-999-4%7B%5C_%7D21.

[127] Emi Arakawa, Kazuhide Hasegawa, Nobuaki Yanai, Masuo Obinata, and Yuzuru Matsuda. "A mouse bone marrow stromal cell line, TBR-B, shows inducible expression of smooth muscle-specific genes." In: *FEBS Letters* 481.2 (Sept. 2000), pp. 193–196. ISSN: 00145793. DOI: 10.1016/S0014-5793(00)01995-5. URL: http://doi.wiley.com/10.1016/S0014-5793%7B%5C%%7D2800%7B%5C%%7D2901995-5.

[128] A. Bobik. "Transforming Growth Factor- s and Vascular Disorders." In: *Arteriosclerosis, Thrombosis, and Vascular Biology* 26.8 (Aug. 2006), pp. 1712–1720. ISSN: 1079-5642. DOI: 10.1161/01.ATV.0000225287.20034.2c. URL: http://atvb.ahajournals.org/cgi/doi/10.1161/01.ATV.0000225287.20034.2c.

[129] Longmei Zhao and Basil M. Hantash. "TGF-β1 Regulates Differentiation of Bone Marrow Mesenchymal Stem Cells." In: 2011, pp. 127–141. DOI: 10.1016/B978-0-12-386015-6.00042-1. URL: http://linkinghub.elsevier.com/retrieve/pii/B9780123860156000421.

[130] Katrien Janssens, Peter ten Dijke, Sophie Janssens, and Wim Van Hul. "Transforming Growth Factor-β1 to the Bone." In: *Endocrine Reviews* 26.6 (Oct. 2005), pp. 743–774. ISSN: 0163-769X. DOI: 10.1210/er.2004-0001. URL: http://press.endocrine.org/doi/10.1210/er.2004-0001.

[131] Hua Chang, Chester W. Brown, and Martin M. Matzuk. "Genetic Analysis of the Mammalian Transforming Growth Factor-β Superfamily." In: *Endocrine Reviews* 23.6 (Dec. 2002), pp. 787–823. ISSN: 0163-769X. DOI: 10.1210/er.2002-0003. URL: http://press.endocrine.org/doi/10.1210/er.2002-0003.

[132] Richard K Assoian, Akira Komoriya, C A Meyers, Dorothea M Miller, and Michael B Sporn. "Transforming growth factor-beta in human platelets. Identification of a major storage site, purification, and characterization." In: *The Journal of biological chemistry* 258.11 (June 1983), pp. 7155–60. ISSN: 0021-9258. URL: http://www.ncbi.nlm.nih.gov/pubmed/6602130.

[133] Stephen G Ball, C Adrian Shuttleworth, and Cay M Kielty. "Platelet-derived growth factor receptors regulate mesenchymal stem cell fate: implications for neovascularization." In: *Expert Opinion on Biological Therapy* 10.1 (Jan. 2010), pp. 57–71. ISSN: 1471-2598. DOI: 10.1517/14712590903379510. URL: http://www.tandfonline.com/doi/full/10.1517/14712590903379510.

[134] L E Niklason and R S Langer. "Advances in tissue engineering of blood vessels and other tissues." In: *Transplant immunology* 5.4 (Dec. 1997), pp. 303–6. ISSN: 0966-3274. URL: http://www.ncbi.nlm.nih.gov/pubmed/9504152.

[135] Allen S. Bulick, Dany J. Muñoz-Pinto, Xin Qu, Mousami Mani, Deissy Cristancho, Matthew Urban, and Mariah S. Hahn. "Impact of Endothelial Cells and Mechanical Conditioning on Smooth

Muscle Cell Extracellular Matrix Production and Differentiation." In: *Tissue Engineering Part A* 15.4 (Apr. 2009), pp. 815–825. ISSN: 1937-3341. DOI: 10.1089/ten.tea.2008.0179. URL: http://www.ncbi.nlm.nih.gov/pubmed/19108675.

[136] Dror Seliktar, Robert M. Nerem, and Zorina S. Galis. "Mechanical Strain-Stimulated Remodeling of Tissue-Engineered Blood Vessel Constructs." In: *Tissue Engineering* 9.4 (Aug. 2003), pp. 657–666. ISSN: 1076-3279. DOI: 10.1089/107632703768247359. URL: http://www.ncbi.nlm.nih.gov/pubmed/13678444.

[137] Jennifer S. Park, Julia S.F. Chu, Catherine Cheng, Fanqing Chen, David Chen, and Song Li. "Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells." In: *Biotechnology and Bioengineering* 88.3 (Nov. 2004), pp. 359–368. ISSN: 0006-3592. DOI: 10.1002/bit.20250. URL: http://doi.wiley.com/10.1002/bit.20250.

[138] Stephen G Ball, Adrian C Shuttleworth, and Cay M Kielty. "Direct cell contact influences bone marrow mesenchymal stem cell fate." In: *The International Journal of Biochemistry & Cell Biology* 36.4 (Apr. 2004), pp. 714–727. ISSN: 13572725. DOI: 10.1016/j.biocel.2003.10.015. URL: http://linkinghub.elsevier.com/retrieve/pii/S1357272503003558.

[139] Karen K. Hirschi, Stephanie A. Rohovsky, and Patricia A. D'Amore. "PDGF, TGF-β, and Heterotypic Cell–Cell Interactions Mediate Endothelial Cell–induced Recruitment of 10T1/2 Cells and Their Differentiation to a Smooth Muscle Fate." In: *The Journal of Cell Biology* 141.3 (May 1998), pp. 805–814. ISSN: 0021-9525. DOI: 10.1083/jcb.141.3.805. URL: http://www.jcb.org/lookup/doi/10.1083/jcb.141.3.805.

[140] Thomas P. Lozito, Juan M. Taboas, Catherine K. Kuo, and Rocky S. Tuan. "Mesenchymal stem cell modification of endothelial matrix regulates their vascular differentiation." In: *Journal of Cellular Biochemistry* 107.4 (2009), pp. 706–713. ISSN: 07302312. DOI: 10.1002/jcb.22166.

[141] Julie Basu Ray, Sara Arab, Yupu Deng, Peter Liu, Linda Penn, David W. Courtman, and Michael E. Ward. "Oxygen regulation of arterial smooth muscle cell proliferation and survival." In: *American Journal of Physiology - Heart and Circulatory Physiology* 294.2 (2008).

[142] Yong-Can Huang, Hong-Ming Zhu, Jia-Qin Cai, Yi-Zhou Huang, Jin Xu, Yi Zhou, Xiao-He Chen, Xiu-Qun Li, Zhi-Ming Yang, and Li Deng. "Hypoxia inhibits the spontaneous calcification of bone marrow-derived mesenchymal stem cells." In: *Journal of Cellular Biochemistry* 113.4 (Apr. 2012), pp. 1407–1415. ISSN: 07302312. DOI: 10.1002/jcb.24014. URL: http://www.ncbi.nlm.nih.gov/pubmed/22135004%20http://doi.wiley.com/10.1002/jcb.24014.

[143] Debby Gawlitta, Mattie H.P. van Rijen, Edmée J.M. Schrijver, Jacqueline Alblas, and Wouter J.A. Dhert. "Hypoxia Impedes Hypertrophic Chondrogenesis of Human Multipotent Stromal Cells." In: *Tissue Engineering Part A* 18.19-20 (Oct. 2012), pp. 1957–66. ISSN: 1937-3341. DOI: 10.1089/ten.tea.2011.0657. URL: http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2011.0657.

[144] Jason W. Bjork, Lee A. Meier, Sandra L. Johnson, Zeeshan H. Syedain, and Robert T. Tranquillo. "Hypoxic culture and insulin yield improvements to fibrin-based engineered tissue." In: *Tissue engineering. Part A* 18.7-8 (Apr. 2012), pp. 785–95. ISSN: 1937-335X. DOI: 10.1089/ten.TEA.2011.0017. URL: http://online.liebertpub.com/doi/abs/10.1089/ten.tea.2011.0017.

[145] Z. Gong and L. E. Niklason. "Small-diameter human vessel wall engineered from bone marrow-derived mesenchymal stem cells (hMSCs)." In: *The FASEB Journal* 22.6 (June 2008), pp. 1635–1648. ISSN: 0892-6638. DOI: 10.1096/fj.07-087924. URL: http://www.fasebj.org/cgi/doi/10.1096/fj.07-087924.

[146] Lan Yao, Daniel D. Swartz, Sylvia F. Gugino, James A. Russell, and Stelios T. Andreadis. "Fibrin-Based Tissue-Engineered Blood Vessels: Differential Effects of Biomaterial and Culture Parameters on Mechanical Strength and Vascular Reactivity." In: *Tissue Engineering* 11.7-8 (July 2005), pp. 991–1003. ISSN: 1076-3279. DOI: 10.1089/ten.2005.11.991. URL: http://www.liebertonline.com/doi/abs/10.1089/ten.2005.11.991.

[147] Jennifer S. Park, Julia S. Chu, Anchi D. Tsou, Rokhaya Diop, Zhenyu Tang, Aijun Wang, and Song Li. "The effect of matrix stiffness on the differentiation of mesenchymal stem cells in response to TGF-β." In: *Biomaterials* 32.16 (June 2011), pp. 3921–3930. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2011.02.019. URL: http://linkinghub.elsevier.com/retrieve/pii/S0142961211001712.

[148] I. Stefani, M. A. Asnaghi, J.J. Cooper-White, and S. Mantero. "A double chamber rotating bioreactor for enhanced tubular tissue generation from human mesenchymal stem cells." In: *Journal of Tissue Engineering and Regenerative Medicine* (2016). ISSN: 19326254. DOI: 10.1002/term.2341. URL: http://doi.wiley.com/10.1002/term.2341.

[149] Mao-Shih Liang and Stelios T. Andreadis. "Engineering fibrin-binding TGF-β1 for sustained signaling and contractile function of MSC based vascular constructs." In: *Biomaterials* 32.33 (Nov. 2011), pp. 8684–8693. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2011.07.079. URL: http://linkinghub.elsevier.com/retrieve/pii/S0142961211008763.

[150] W E Fibbe. "Mesenchymal stem cells. A potential source for skeletal repair." In: *Annals of the rheumatic diseases* 61 Suppl 2.suppl 2 (Nov. 2002), pp. ii29–31. ISSN: 0003-4967. DOI: 10.1136/ARD.61.SUPPL_2.II29. URL: http://www.ncbi.nlm.nih.gov/pubmed/12379617.

[151] C. Goepfert, A. Slobodianski, A. F. Schilling, P. Adamietz, and R. Pörtner. "Cartilage Engineering from Mesenchymal Stem Cells." In: *Bioreactor Systems for Tissue Engineering II*. Berlin, Heidelberg: Springer Berlin Heidelberg, 2010, pp. 163–200. DOI: 10.1007/10_2010_67. URL: http://link.springer.com/10.1007/10%7B%5C_%7D2010%7B%5C_%7D67.

[152] Brian Johnstone, Thomas M. Hering, Arnold I. Caplan, Victor M. Goldberg, and Jung U. Yoo. "In VitroChondrogenesis of Bone Marrow-Derived Mesenchymal Progenitor Cells." In: *Experimental Cell Research* 238.1 (Jan. 1998), pp. 265–272. ISSN: 00144827. DOI: 10.1006/excr.1997.3858. URL: http://www.ncbi.nlm.nih.gov/pubmed/9457080.

[153] Koichiro Kawamura, Constance R Chu, Satoshi Sobajima, Paul D Robbins, Freddie H Fu, Nicholas J Izzo, and Christopher Niyibizi. "Adenoviral-mediated transfer of TGF-β1 but not IGF-1 induces chondrogenic differentiation of human mesenchymal stem cells in pellet cultures." In: *Experimental Hematology* 33 (2005), pp. 865–872.

[154] Paul B Yu et al. "BMP type I receptor inhibition reduces heterotopic [corrected] ossification." In: *Nature medicine* 14.12 (Dec. 2008), pp. 1363–9. ISSN: 1546-170X. DOI: 10.1038/nm.1888. URL: http://www.ncbi.nlm.nih.gov/pubmed/19029982.

[155] Dirk Hockemeyer et al. "Genetic engineering of human pluripotent cells using TALE nucleases." In: *Nature Biotechnology* 29.8 (July 2011), pp. 731–734. ISSN: 1087-0156. DOI: 10.1038/nbt.1927. URL: http://www.nature.com/doifinder/10.1038/nbt.1927.

[156] J. Solway, J. Seltzer, F. F. Samaha, S. Kim, L. E. Alger, Q. Niu, E. E. Morrisey, H. S. Ip, and M. S. Parmacek. *Structure and expression of a smooth muscle cell-specific gene, SM22α*. 1995. DOI: 10.1074/jbc.270.22.13460.

[157] G. K. Owens. "Molecular Regulation of Vascular Smooth Muscle Cell Differentiation in Development and Disease." In: *Physiological Reviews* 84.3 (July 2004), pp. 767–801. ISSN: 0031-9333. DOI: 10.1152/physrev.00041.2003. URL: http://physrev.physiology.org/cgi/doi/10.1152/physrev.00041.2003.

[158] Clay Quint, Melissa Arief, Akihito Muto, Alan Dardik, and Laura E. Niklason. "Allogeneic human tissue-engineered blood vessel." In: *Journal of Vascular Surgery* 55.3 (Mar. 2012), pp. 790–798. ISSN: 07415214. DOI: 10.1016/j.jvs.2011.07.098. URL: http://linkinghub.elsevier.com/retrieve/pii/S0741521411019471.

[159] Stephen A Bustin et al. "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments." In: *Clinical Chemistry* 55.4 (Apr. 2009), pp. 611–622. ISSN: 0009-9147. DOI: 10.1373/clinchem.2008.112797. URL: http://www.rdml.org/clinchem.2008.112797v1.pdf.

[160] Kenneth J. Livak and Thomas D. Schmittgen. "Analysis of relative gene expression data using real-time quantitative PCR." In: *Methods* 25.4 (Dec. 2001), pp. 402–408. ISSN: 1046-2023 (Print)1046-2023. DOI: 10.1006/meth.2001.1262. arXiv: 1003.3921v1. URL: http://linkinghub.elsevier.com/retrieve/pii/S1046202301912629.

[161] H Stegemann and K Stalder. "Determination of hydroxyproline." In: *Clinica chimica acta; international journal of clinical chemistry* 18.2 (Nov. 1967), pp. 267–73. ISSN: 0009-8981. URL: http://www.ncbi.nlm.nih.gov/pubmed/4864804.

[162] Thomas H Petersen, Elizabeth A Calle, Maegen B Colehour, and Laura E Niklason. "Matrix composition and mechanics of decellularized lung scaffolds." In: *Cells, tissues, organs* 195.3 (2012), pp. 222–31. ISSN: 1422-6421. DOI: 10.1159/000324896. URL: http://www.ncbi.nlm.nih.gov/pubmed/21502745.

[163] Chunhui Xu, Margaret S. Inokuma, Jerrod Denham, Kathaleen Golds, Pratima Kundu, Joseph D. Gold, and Melissa K. Carpenter. "Feeder-free growth of undifferentiated human embryonic stem cells." In: *Nature Biotechnology* 19.10 (Oct. 2001), pp. 971–974. ISSN: 10870156. DOI: 10.1038/nbt1001-971. URL: http://www.nature.com/doifinder/10.1038/nbt1001-971.

[164] Laura Menendez, Tatiana A Yatskievych, Parker B Antin, and Stephen Dalton. "Wnt signaling and a Smad pathway blockade direct the differentiation of human pluripotent stem cells to multipotent neural crest cells." In: *Proceedings of the National Academy of Sciences of the United States of America* 108.48 (Nov. 2011), pp. 19240–5. ISSN: 1091-6490. DOI: 10.1073/pnas.1113746108. URL: http://www.ncbi.nlm.nih.gov/pubmed/22084120.

[165] Martin Krzywinski and Naomi Altman. "Points of significance: Comparing samples—part I." In: *Nature Methods* 11.3 (Feb. 2014), pp. 215–216. ISSN: 1548-7091. DOI: 10.1038/nmeth.2858. URL: http://www.nature.com/doifinder/10.1038/nmeth.2858.

[166] Amy Solan, Shannon Mitchell, Marsha Moses, and Laura Niklason. "Effect of Pulse Rate on Collagen Deposition in the Tissue-Engineered Blood Vessel." In: *Tissue Engineering* 9.4 (Aug. 2003), pp. 579–586. ISSN: 1076-3279. DOI: 10.1089/107632703768247287. URL: http://www.liebertonline.com/doi/abs/10.1089/107632703768247287.

[167] Durward Lawson, Maureen Harrison, and Claire Shapland. "Fibroblast transgelin and smooth muscle SM22α are the same protein, the expression of which is down-regulated in many cell lines." In: *Cell Motility and the Cytoskeleton* 38.3 (1997), pp. 250–257. ISSN: 0886-1544. DOI: 10.1002/(SICI)1097-0169(1997)38:3<250::AID-CM3>3.0.CO;2-9.

[168] J P Lees-Miller, D H Heeley, and L B Smillie. "An abundant and novel protein of 22 kDa (SM22) is widely distributed in smooth muscles. Purification from bovine aorta." In: *The Biochemical journal* 244.3 (June 1987), pp. 705–9. ISSN: 0264-6021. DOI: 10.1042/BJ2440705. URL: http://www.ncbi.nlm.nih.gov/pubmed/3446186.

[169] Satish Rattan and Mehboob Ali. "Role of SM22 in the differential regulation of phasic vs. tonic smooth muscle." In: *American Journal of Physiology - Gastrointestinal and Liver Physiology* 308.7 (Apr. 2015), G605–G612. ISSN: 0193-1857. DOI: 10.1152/ajpgi.00360.2014. URL: http://ajpgi.physiology.org/lookup/doi/10.1152/ajpgi.00360.2014.

[170] J C Zhang, S Kim, B P Helmke, W W Yu, K L Du, M M Lu, M Strobeck, Q Yu, and M S Parmacek. "Analysis of SM22alpha-deficient mice reveals unanticipated insights into smooth muscle cell differentiation and function." In: *Molecular and cellular biology* 21.4 (Feb. 2001), pp. 1336–44. ISSN: 0270-7306. DOI: 10.1128/MCB.2001.21.4.1336-1344.2001. URL: http://www.ncbi.nlm.nih.gov/pubmed/11158319.

[171] S S M Rensen, P A F M Doevendans, and G J J M van Eys. "Regulation and characteristics of vascular smooth muscle cell phenotypic diversity." In: *Netherlands heart journal : monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation* 15.3 (2007), pp. 100–8. ISSN: 1568-5888. URL: http://www.ncbi.nlm.nih.gov/pubmed/17612668.

[172] Fransiska Malfait, Sofie Symoens, Julie De Backer, Trinh Hermanns-Lê, Natzi Sakalihasan, Charles M. Lapière, Paul Coucke, and Anne De Paepe. "Three arginine to cysteine substitutions in the pro-alpha (I)-collagen chain cause Ehlers-Danlos syndrome with a propensity to arterial rupture in early adulthood." In: *Human Mutation* 28.4 (Apr. 2007), pp. 387–395. ISSN: 10597794. DOI: 10.1002/humu.20455. URL: http://doi.wiley.com/10.1002/humu.20455.

[173] Paula Engbers-Buijtenhuijs, Laura Buttafoco, Andre A. Poot, Piet J. Dijkstra, Rob A.I. de Vos, Lotus M.Th. Sterk, Rob H. Geelkerken, Istvan Vermes, and Jan Feijen. "Biological characterisation of vascular grafts cultured in a bioreactor." In: *Biomaterials* 27.11 (Apr. 2006), pp. 2390–2397. ISSN: 01429612. DOI: 10.1016/j.biomaterials.2005.10.016. URL: http://linkinghub.elsevier.com/retrieve/pii/S0142961205009208.

[174] Vincent Falanga, Linda Zhou, and Tatyana Yufit. "Low oxygen tension stimulates collagen synthesis and COL1A1 transcription through the action of TGF-B1." In: *Journal of Cellular Physiology* 191.1 (Apr. 2002), pp. 42–50. ISSN: 0021-9541. DOI: 10.1002/jcp.10065. URL: http://www.ncbi.nlm.nih.gov/pubmed/11920680.

[175] Jillian B. Schmidt, Kelley Chen, and Robert T. Tranquillo. "Effects of Intermittent and Incremental Cyclic Stretch on ERK Signaling and Collagen Production in Engineered Tissue." In: *Cellular and Molecular Bioengineering* 9.1 (Mar. 2016), pp. 55–64. ISSN: 1865-5025. DOI: 10.1007/s12195-015-0415-6. URL: http://link.springer.com/10.1007/s12195-015-0415-6.

[176] Matthew D Shoulders and Ronald T Raines. "Collagen structure and stability." In: *Annual review of biochemistry* 78 (2009), pp. 929–58. ISSN: 1545-4509. DOI: 10.1146/annurev.biochem.77.032207.120833. URL: http://www.ncbi.nlm.nih.gov/pubmed/19344236.

[177] Jung-Sun Cho, Joo-Hoo Park, Ju-Hyung Kang, Sung Eun Kim, Il-Ho Park, and Heung-Man Lee. "Isolation and characterization of multipotent mesenchymal stem cells in nasal polyps." In: *Experimental Biology and Medicine* 240 (2015), pp. 185–193. DOI: 10.1177/1535370214553898. URL: http://journals.sagepub.com/doi/pdf/10.1177/1535370214553898.

[178] Quyen Q. Hoang, Frank Sicheri, Andrew J. Howard, and Daniel S. C. Yang. "Bone recognition mechanism of porcine osteocalcin from crystal structure." In: *Nature* 425.6961 (Oct. 2003), pp. 977–980. ISSN: 0028-0836. DOI: 10.1038/nature02079. URL: http://www.nature.com/doifinder/10.1038/nature02079.

[179] Marita Westhrin, Minli Xie, Magnus Ø Olderøy, Pawel Sikorski, Berit L Strand, and Therese Standal. "Osteogenic differentiation of human mesenchymal stem cells in mineralized alginate matrices." In: *PloS one* 10.3 (2015), e0120374. ISSN: 1932-6203. DOI: 10.1371/journal.pone.0120374. URL: http://www.ncbi.nlm.nih.gov/pubmed/25769043.

[180] Cecilia Granéli, Anna Thorfve, Ulla Ruetschi, Helena Brisby, Peter Thomsen, Anders Lindahl, and Camilla Karlsson. "Novel markers of osteogenic and adipogenic differentiation of human bone marrow stromal cells identified using a quantitative proteomics approach." In: *Stem Cell Research* 12.1 (Jan. 2014), pp. 153–165. ISSN: 18735061. DOI: 10.1016/j.scr.2013.09.009. URL: http://linkinghub.elsevier.com/retrieve/pii/S1873506113001359.

[181] Akifumi Nakamura, Yoshiko Dohi, Manabu Akahane, Hajime Ohgushi, Hiroshi Nakajima, Hiroyuki Funaoka, and Yoshinori Takakura. "Osteocalcin Secretion as an Early Marker of In Vitro Osteogenic Differentiation of Rat Mesenchymal Stem Cells." In: *Tissue Engineering Part C: Methods* 15.2 (June 2009), pp. 169–

180. ISSN: 1937-3384. DOI: 10.1089/ten.tec.2007.0334. URL: http://www.liebertonline.com/doi/abs/10.1089/ten.tec.2007.0334.

[182] Lisa J Harris, Hamid Abdollahi, Ping Zhang, Stephen McIlhenny, Thomas N Tulenko, and Paul J DiMuzio. "Differentiation of adult stem cells into smooth muscle for vascular tissue engineering." In: *The Journal of surgical research* 168.2 (June 2011), pp. 306–14. ISSN: 1095-8673. DOI: 10.1016/j.jss.2009.08.001. URL: http://www.ncbi.nlm.nih.gov/pubmed/19959190.

[183] L. Li, J. M. Miano, P. Cserjesi, and E. N. Olson. "SM22 , a Marker of Adult Smooth Muscle, Is Expressed in Multiple Myogenic Lineages During Embryogenesis." In: *Circulation Research* 78.2 (Feb. 1996), pp. 188–195. ISSN: 0009-7330. DOI: 10.1161/01.RES.78.2.188. URL: http://circres.ahajournals.org/cgi/doi/10.1161/01.RES.78.2.188.

[184] Clifford Lin, Yifan Yuan, David W. Courtman, HI Chang, ML Sung, and HC Kuo. "Differentiation of Murine Bone Marrow-Derived Smooth Muscle Progenitor Cells Is Regulated by PDGF-BB and Collagen." In: *PLOS ONE* 11.6 (June 2016). Ed. by Alain-Pierre Gadeau, e0156935. ISSN: 1932-6203. DOI: 10.1371/journal.pone.0156935. URL: http://dx.plos.org/10.1371/journal.pone.0156935.

[185] Kenichi Tamama, Chandan K. Sen, and Alan Wells. "Differentiation of Bone Marrow Mesenchymal Stem Cells into the Smooth Muscle Lineage by Blocking ERK/MAPK Signaling Pathway." In: *Stem Cells and Development* 17.5 (Oct. 2008), pp. 897–908. ISSN: 1547-3287. DOI: 10.1089/scd.2007.0155. URL: http://www.liebertonline.com/doi/abs/10.1089/scd.2007.0155.

[186] Kyung-Min Choi, Young-Kwon Seo, Hee-Hoon Yoon, Kye-Yong Song, Soon-Yong Kwon, Hwa-Sung Lee, and Jung-Keug Park. "Effect of Ascorbic Acid on Bone Marrow-Derived Mesenchymal Stem Cell Proliferation and Differentiation." In: 105.6 (2008), pp. 586–594. DOI: 10.1263/jbb.105.586. URL: http://ac.els-cdn.com/S1389172308701154/1-s2.0-S1389172308701154-main.pdf?%7B%5C_%7Dtid=1af2d14e-1598-11e7-b8af-00000aacb35d%7B%5C&%7Dacdnat=1490913049%7B%5C_%7D.

[187] Nuno C. Santos, J. Figueira-Coelho, J. Martins-Silva, and Carlota Saldanha. *Multidisciplinary utilization of dimethyl sulfoxide: pharmacological, cellular, and molecular aspects*. 2003. DOI: 10.1016/S0006-2952(03)00002-9. URL: http://www.sciencedirect.com/science/article/pii/S0006295203000029.

[188] S. Adler, C. Pellizzer, M. Paparella, T. Hartung, and S. Bremer. "The effects of solvents on embryonic stem cell differentiation." In: *Toxicology in Vitro* 20.3 (Apr. 2006), pp. 265–271. ISSN: 08872333. DOI: 10.1016/j.tiv.2005.06.043. URL: http://www.ncbi.nlm.nih.gov/pubmed/16112835.

[189] Rajarshi Pal, Murali Krishna Mamidi, Anjan Kumar Das, and Ramesh Bhonde. "Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells." In: *Archives of Toxicology* 86.4 (Apr. 2012), pp. 651–661. ISSN: 0340-5761. DOI: 10.1007/s00204-011-0782-2. URL: http://link.springer.com/10.1007/s00204-011-0782-2.

[190] Bertrand Poussier, Alfredo C. Cordova, Jean-Pierre Becquemin, and Bauer E. Sumpio. "Resveratrol inhibits vascular smooth muscle cell proliferation and induces apoptosis." In: *Journal of Vascular Surgery* 42.6 (2005), 1190–1190.e14. ISSN: 07415214. DOI: 10.1016/j.jvs.2005.08.014. URL: http://www.sciencedirect.com/science/article/pii/S0741521405012759.

[191] Changqing Xie, Jiang Hu, Haiyun Ma, Jifeng Zhang, Lung-Ji Chang, Y Eugene Chen, and Peter X Ma. "Three-dimensional growth of iPS cell-derived smooth muscle cells on nanofibrous scaffolds." In: *Biomaterials* 32.19 (July 2011), pp. 4369–75. ISSN: 1878-5905. DOI: 10.1016/j.biomaterials.2011.02.049. URL: http://www.ncbi.nlm.nih.gov/pubmed/21439638.

[192] Makoto Hirao, Noriyuki Tamai, Noriyuki Tsumaki, Hideki Yoshikawa, and Akira Myoui. "Oxygen tension regulates chondrocyte differentiation and function during endochondral ossification." In: *The Journal of biological chemistry* 281.41 (Oct. 2006), pp. 31079–92. ISSN: 0021-9258. DOI: 10.1074/jbc.M602296200. URL: http://www.ncbi.nlm.nih.gov/pubmed/16905540.

[193] M Pacifici, E B Golden, O Oshima, I M Shapiro, P S Leboy, and S L Adams. "Hypertrophic chondrocytes. The terminal stage of differentiation in the chondrogenic cell lineage?" In: *Annals of the New York Academy of Sciences* 599 (1990), pp. 45–57. ISSN: 0077-8923. URL: http://www.ncbi.nlm.nih.gov/pubmed/2221676.

[194] J.P. Bilezikian, L.G. Raisz, and T. J. Martin. *Principles of Bone Biology*. Vol. 1. 9. Academic Press, 2008, p. 2008. ISBN: 0120986523. DOI: 10.3174/ajnr.A1712.

[195] Birgit Kulterer, Gerald Friedl, Anita Jandrositz, Fatima Sanchez-Cabo, Andreas Prokesch, Christine Paar, Marcel Scheideler, Reinhard Windhager, Karl-Heinz Preisegger, and Zlatko Trajanoski. "Gene expression profiling of human mesenchymal stem cells derived from bone marrow during expansion and osteoblast differentiation." In: *BMC Genomics* 8.1 (Mar. 2007), p. 70. ISSN: 14712164. DOI: 10.1186/1471-2164-8-70. URL: http://www.ncbi.nlm.nih.gov/pubmed/17352823.

[196] Jane E. Aubin. "Bone stem cells." In: *Journal of Cellular Biochemistry* 72.S30-31 (1998), pp. 73–82. ISSN: 07302312. DOI: 10.1002/(SICI)1097-4644(1998)72:30/31+<73::AID-JCB11>3.0.CO;2-L.

[197] Jo Vandesompele, Katleen De Preter, Ilip Pattyn, Bruce Poppe, Nadine Van Roy, Anne De Paepe, and Rank Speleman. "Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes." In: *Genome Biology* 3.711 (2002), pp. 34–1. ISSN: 1465-6914. URL: http://genomebiology.com/2002/3/7/research/0034.

[198] Laurent Gutierrez et al. "The lack of a systematic validation of reference genes: a serious pitfall undervalued in reverse transcription-polymerase chain reaction (RT-PCR) analysis in plants." In: *Plant Biotechnology Journal* 6.6 (Aug. 2008), pp. 609–618. ISSN: 14677644. DOI: 10.1111/j.1467-7652.2008.00346.x. URL: http://www.ncbi.nlm.nih.gov/pubmed/18433420.

[199] Ronald J Feise. "Do multiple outcome measures require p-value adjustment?" In: *BMC Medical Research Methodology* 2.1 (Dec. 2002), p. 8. ISSN: 1471-2288. DOI: 10.1186/1471-2288-2-8. URL: http://bmcmedresmethodol.biomedcentral.com/articles/10.1186/1471-2288-2-8.

COLOPHON

This document was typeset using the typographical look-and-feel `classicthesis` developed by André Miede. The style was inspired by Robert Bringhurst's seminal book on typography "*The Elements of Typographic Style*". `classicthesis` is available for both LaTeX and LyX:

https://bitbucket.org/amiede/classicthesis/

Happy users of `classicthesis` usually send a real postcard to the author, a collection of postcards received so far is featured here:

http://postcards.miede.de/

Final Version as of May 11, 2017 (`classicthesis` version 0.2).

Hermann Zapf's *Palatino* and *Euler* type faces (Type 1 PostScript fonts *URW Palladio L* and *FPL*) are used. The "typewriter" text is typeset in *Bera Mono*, originally developed by Bitstream, Inc. as "Bitstream Vera". (Type 1 PostScript fonts were made available by Malte Rosenau and Ulrich Dirr).