Expression and role of long non-coding RNA *H19* in aortic valve development and disease

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

In

Biomedical Engineering

January 31, 2020

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ACKNOWLEDGEMENTS

This body of work was only achieved with the generous help of many individuals, groups, and funding agencies. In particular, I'd like to acknowledge the Vanderbilt University Biomedical Engineering department, Cardiology department and Graduate School, the National Heart Lung and Blood Institute, and the Leducq Foundation for the funding and institutional support that enabled my research. Additionally, I'd like to thank the members of my dissertation committee for their scientific guidance and support.

My deepest gratitude goes out to the countless individuals and groups who helped me in this endeavor and who bore with me through the joys and trials of graduate school. Firstly, I would like to thank my adviser and mentor, Dave Merryman, for bringing me into his research group and fostering my scientific growth for the past several years. You made this work possible and brought an enthusiasm to it that I could rarely muster for myself. You also assembled a research group that made the work light and without whom I would have been lost. To the many members of the Merryman lab and Baldwin lab – Larisa, Cyndi, Erin, Matt, Lance, MK, Joe, Steve, Nathan, Meghan, Cami, Caleb, Ethan, Natalie, Tessa, Michael, Dr. Baldwin, Kevin, Paige, Leshana, Paige, and many others – I am especially indebted. Thank you for your scientific help and more importantly for your friendship and moral support for the past several years. My sincerest thanks as well to my family – you sparked my interest in science, set me on this journey, and supported me the whole way. Finally, my deepest gratitude to my lab mate, friend, and wife, Alison. You paved the way before me and provided me with every motivation and strength I needed. I could not have done this without you.

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Abbreviations and Key Terms	Definition and relevance to thesis work
αSMA	alpha smooth muscle actin
Actb	beta actin: loading control for RT-PCR
AVC	atrioventricular canal
BAV	bicuspid aortic valve
BMP	bone morphogenetic protein
CAVD	calcific aortic valve disease
CD31	endothelial cell marker (also PECAM-1)
CDH11	cadherin-11, OB-cadherin
CHD	congenital heart disease
CTCF	CCCTC-binding factor
ECM	extracellular matrix
EFVR	ejection fraction to velocity ratio
EMT	endothelial to mesenchymal transformation
GAGs	glycosaminoglycans
Gapdh	loading control for RT-PCR
H19	lncRNA involved in development and disease
$H19^{DMD\text{-}9CG}$ mice	mice strain with mutant *H19/Igf2* ICR
HUVEC	human umbilical vein endothelial cells
ICR	imprinting control region
Igf2	insulin-like growth factor 2: epigenetic regulation linked to *H19*
iPSC	induced pluripotent stem cells
lncRNA	long non-coding RNA
MACS	magnetic assisted cell sorting
NFATC1	nuclear factor of activated T-cells: endocardial marker
NOTCH1	transmembrane receptor involved in valve development
OFT	outflow tract
PSV	peak systolic velocity
RT-PCR	reverse transcription polymerase chain reaction
RUNX2	Runt-related transcription factor
TEHV	tissue engineered heart valve
TGFβ	transforming growth factor beta
Tuba1b	tubulin alpha 1 beta chain: loading control for RT-PCR
VEC	valve endothelial cell
VE-cadherin	vascular endothelial cadherin (also CDH5 or CD144)
VEGFR2	vascular endothelial growth factor receptor 2
VIC	valve interstitial cell

Chapter 1

Introduction and motivation

1.1 Valvular heart disease – burden and current therapeutic needs

Valvular heart disease, including developmental complications and late-stage disease, is a major source of illness in the developed world. Congenital heart disease (**CHD**) is the most common type of birth defect and is the leading cause of infant morbidity in the Western world [1], [2]. Valve defects are the most common form of CHD, and include a variety of pathologies such as bicuspid aortic valve (**BAV**), mitral valve prolapse, and congenital aortic stenosis [3]–[7]. These pathologies are often life-threatening, and in the case of less immediately dangerous diseases such as BAV, hemodynamic changes resulting from the altered valve often lead to development of additional valvular pathologies later in life [6], [8]. Age-related valve pathologies are also quite common. Because valve disease is typically progressive, incidence and severity increase with age [8]. In the United States, over 25% of those over the age of 65 show signs of aortic sclerosis, a thickening of the aortic valve leaflets indicative of a diseased state. By age 75, roughly half of those affected progress to potentially life-threatening stenosis, which is characterized by significant aortic valve tissue remodeling, narrowing of the aortic opening, and hemodynamic changes [9], [10]. One of the most common forms of age-related valve disease is calcific aortic valve disease (**CAVD**), which is especially characterized by fibrocalcific remodeling of the aortic valve. The high prevalence and cost of valve disease necessitate new approaches to treat the many people suffering from valvular heart disease.

In the case of congenital valve defects, surgical replacement is the only viable treatment strategy. In the majority of these cases, the replacement valves – either mechanical or tissue-

based prostheses – are unable to grow along with the recipient, necessitating one or more subsequent replacements in later life. Furthermore, these inert valves are incapable of reparative remodeling, leading to the potential for mechanical failure. The Ross procedure, in which the aortic valve is replaced with a pulmonary autograft, is available for pediatric patients and does allow for limited growth, though it is less frequently recommended due to its complexity, high mortality in those under 1 year of age, and availability of alternatives in older patients [11]. In any case, follow-up surgeries are required in up to 40% of patients within the first 10 years following the initial operation, with additional cardiovascular complications persisting throughout the life of the individual [12].

Therapeutic options at the other end of the age spectrum are slightly more diverse, but are still insufficient to address the high degree of disease complexity. Surgical replacement is still the only method to address the diseased valve directly, though the complications of the available replacement options limit the number of patients for whom replacement is appropriate. Mechanical replacements require anticoagulant treatment, which may be counter-indicated in certain patients. Tissue-based prostheses, which are the more common choice for adult valve replacement, do not require anticoagulants but do have a lifespan of less than 20 years. Recently, transcatheter aortic valve replacement has been introduced as a means to lower surgical morbidity, though it also uses bioprosthetic valves which have a limited lifespan. Pharmaceutical means to address adult valve disease mainly focus on preventing downstream complications that result from the diseased valve. Anti-arrhythmics and anticoagulants prevent development of clots, which are more likely to form in certain instances of disease with highly altered hemodynamics, and angiotensin converting enzymes (ACE) inhibitors and other vasodilators may help in case of aortic regurgitation, though long-term effects on morbidity and mortality are still questionable [13].

Clearly, treatment options for valve disease are far from ideal. For both pediatric patients and those with age-related disease, identification of novel approaches to valve replacement, particularly novel source material for replacement valves, would represent a major clinical milestone with immense potential benefit. Tissue engineered heart valves (**TEHVs**) are an area of active research, but to date no suitable cell source has been identified that can create a viable valve capable of long-term growth. One potential source of cells would be patient-derived induced pluripotent stem cells (**iPSCs**) driven down a developmental pathway which recapitulates valve development. However, given the complexity of such a system, additional research into the basic factors regulating differentiation of the endothelial/endocardial cells which give rise to the cardiac valves is a crucial step towards creating a viable cell source. Additionally, findings applicable to a broader variety of endothelial cells would also have wider implications for other tissue engineering applications. Finally, identification of novel drug targets to prevent or treat adult valve disease would be a massive benefit for this patient population.

1.2 Shared signaling creates a therapeutic window

While a cursory glance at tissue-level pathologies observed in patients with congenital valve defects and age-related valve disease make them seem quite different, there are numerous similarities between the two. One of the unique hallmarks of later stage valve disease is a re-activation of developmental protein signaling pathways leading to activated cellular phenotypes commonly observed during development, as shown in Figure 1.1. These activated states are characterized by increased cellular motility, including endothelial to mesenchymal transformation (**EMT**), and a high degree of extracellular matrix (**ECM**) remodeling, leading to alterations in the mechanical structure of the cellular microenvironment. Alterations in major signaling pathways such as NOTCH1 and members of the transforming growth factor beta (**TGFβ**) family are also common. Explanations for the re-emergence of this developmental state

in age-related instances of disease are unclear, but the similarities create an attractive target for research which would have potential benefit in both instances of disease.

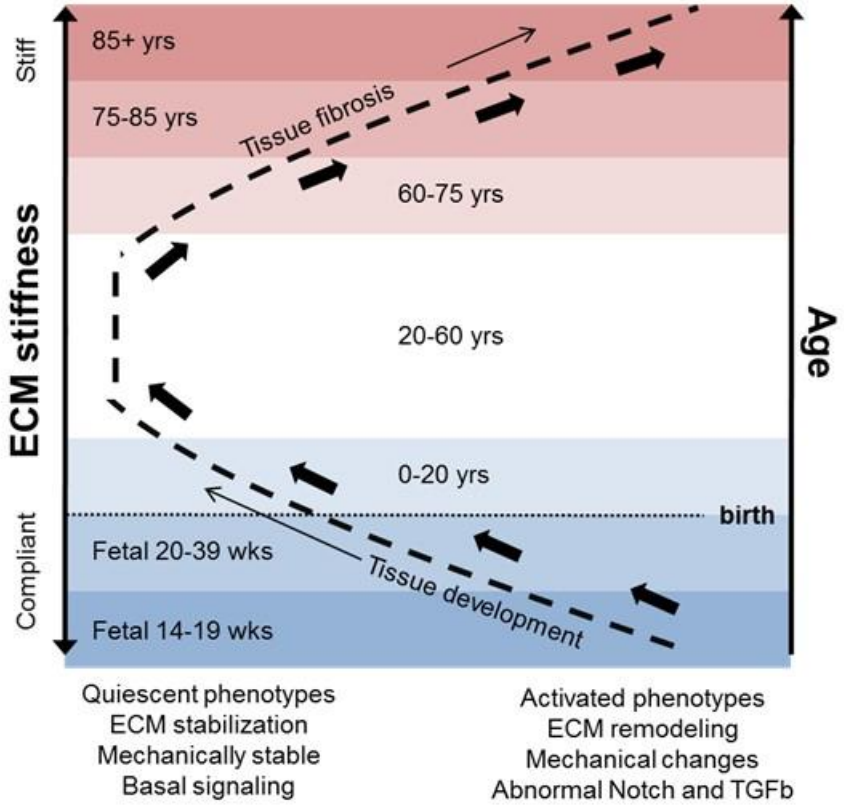

Figure 1.1 Reemergence of developmental features in later stages of valve disease
(Reprinted with permission from [14])

1.3 Non-genetic factors which affect valve health may have therapeutic potential

While certain highly specific mutations – such as the *NOTCH1* loss of function mutation – are known to affect valve health in both pediatric and old patients, countless instances of disease lack any clear genetic explanation. This has motivated research into the roles that non-genetic factors such as epigenetics, mechanobiology, and non-coding RNAs may play in valve disease and development. While these factors have been investigated in isolation and in connection with known mutations and traditional signaling pathways, research into their intersection is sparse. Nevertheless, they constitute a major component of valve development

and disease, and a thorough understanding of their role in cell behavior could either identify novel targets for disease treatment or lead to better approaches for tissue engineering and cell-based therapies.

The dynamic mechanical environment of the cardiovascular system is known to affect cellular activity and gene expression. In development, mechanical forces imparted by hemodynamic pressure, fluid shear stress, mechanical stretch, and ECM stiffness all act in concert to direct proper morphological development of the heart, valves, and vasculature. Prior work in the Merryman lab has identified dynamic mechanical contraction as a regulator of EMT in atrioventricular valve development [15]. Likewise, alterations to tissue mechanics are hallmarks of later stage disease. Increased mechanical strain and hemodynamic shear and pressure play major roles in the progression of aortic valve disease and vascular pathologies such as atherosclerosis [16], [17]. Even in the context of mutation-driven cardiovascular disease, altered mechanotransduction is thought to be a major driver of disease. Collectively, these results demonstrate the crucial role that mechanical forces play in cardiovascular development and disease, though the means by which they do so, especially the interaction of mechanics and non-genetic factors requires further investigation.

Recently, long non-coding RNAs (**lncRNAs**) have emerged as a novel class of signaling molecule which may regulate components of classical signaling pathways, potentially explaining the shared features of cardiovascular development and disease. LncRNAs are typically defined as transcribed RNAs longer than 200 base pairs with no known protein coding sequence. Traditionally, lncRNAs was thought to be largely inactive, though their varied functions and roles are being increasingly identified as a crucial component of cell function. One of the earliest discovered lncRNAs, *H19*, has long been known to be highly active during embryonic development, and recently has been implicated in a variety of cardiovascular diseases, including CAVD.

1.4 Dissertation overview

My doctoral work has sought to address the clinical need for new treatments of valvular heart disease by investigating the role of mechanical signaling and epigenetic factors influencing valve development and health. The first major focus of this work was the mechanical regulation and role of lncRNA *H19* in endothelial differentiation. Next, my work investigated the role of *H19* in the initiation and progression of aortic valve disease. In this thesis, I present first a thorough background of normal valve development, structure, and function. Next, I present a summary of valve disease burden as well as the need for new therapeutic approaches which has motivated my work. Next, I present a thorough background of the field of valvular heart disease and the various known drivers of valve disease, as well as a summary of the role that mechanical forces and lncRNA *H19* are currently known to play in the valve. Following this, I present a summary of my doctoral research into the role of mechanical strain and lncRNA *H19* expression in endothelial development, with implications for TEHVs and other tissue engineering applications. Also presented is my research into the role of *H19* as an initiating factor in aortic valve disease in mice along with the potential for *H19*-based models of mouse cardiovascular disease as well as preliminary work on other transgenic mouse models which I pursued. I conclude with a discussion of the impact of this work and the potential applications it may have on the treatment of valve disease, as well as future directions the research could be taken.

Chapter 2

Background – heart valve development and healthy function

2.1 Heart physiology and valve function

The cardiac valves are critical structures within the heart that are responsible for maintaining unidirectional blood flow (Fig 2.1). During a healthy cardiac cycle, atrial contraction forces blood from the right atria to the right ventricle through the tricuspid valve and from the left atria to the left ventricle through the mitral valve. Contraction of the ventricles increases ventricular pressure and forces the tricuspid and mitral valves – also known as the atrioventricular valves – closed. This allows the aortic and pulmonary valves – also known as the semilunar valves – to open and for the ventricles to send blood through the circulatory system. The right ventricle passes blood through the pulmonary valve to be oxygenated in the lungs, and the left ventricle passes blood through the aortic valve, sending oxygenated blood to the rest of the body. This cycle repeats when oxygen-depleted blood from the body is returned to the right atria and oxygen-rich blood from the lungs is returned to the left atria.

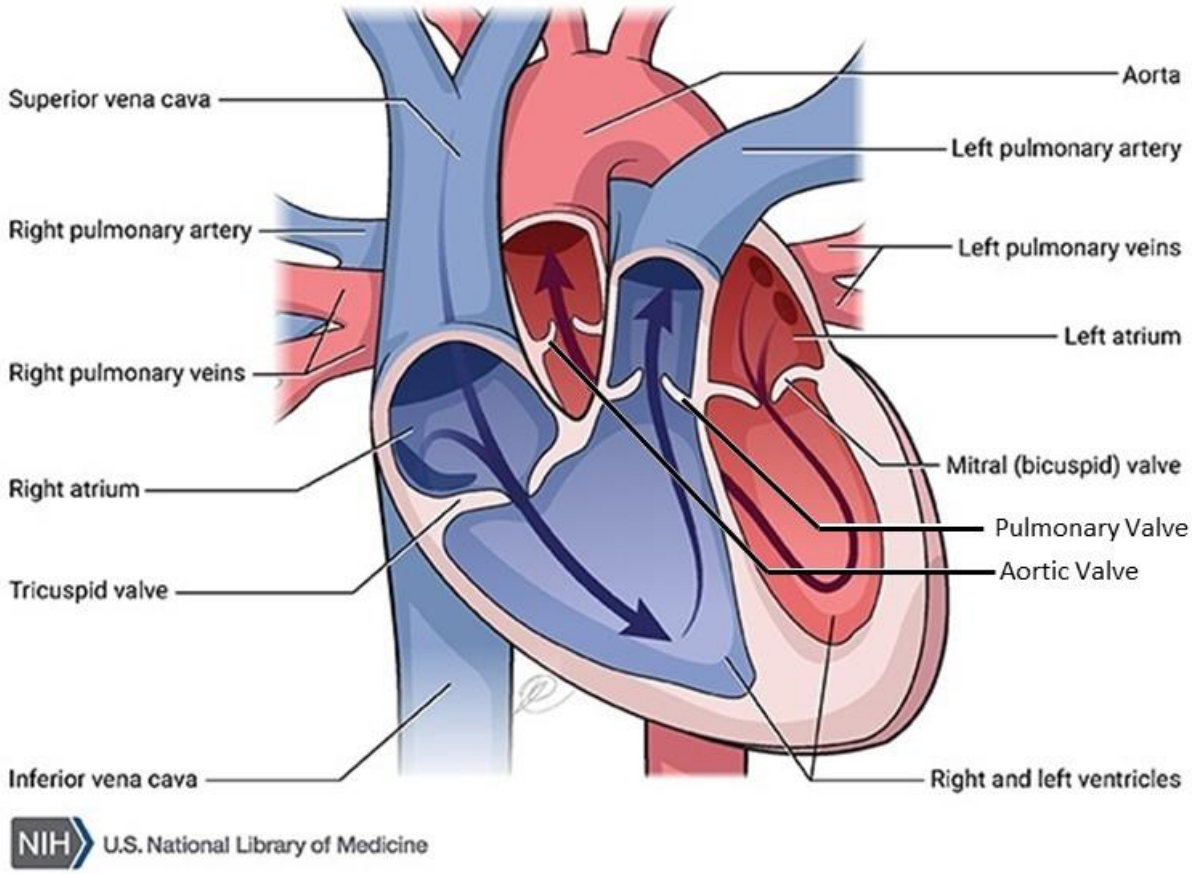

Figure 2.1 Heart anatomy and circulation
(Reprinted with permission from ghr.nlm.nih.gov)

Throughout this process, the cardiac valves must operate in harmony to ensure proper blood flow. Closing of the atrioventricular valves during ventricular contraction, or systole, ensures that blood is forced through the semilunar valves and out to the rest of the body and lungs. Likewise, the semilunar valves must close as the heart relaxes during diastole to fill the ventricles properly and prevent retrograde blood flow. This critical function means that pathologies which affect the heart valves are typically quite dire. In general, valvular heart disease occurs primarily during early development or in age-associated disease. Despite the occurrence of disease at such disparate points in a lifespan, there are many similarities between disease at these points, and a thorough understanding of normal valve function at each stage may be necessary for the development of successful therapeutic strategies for either.

2.2 Heart valve development

The cardiac valves form extremely early in embryonic development. As the heart is required for the circulation of nutrients to the embryo, it is one of the first organs to form a recognizable structure. In humans, primordial valve structures first appear at around 4 weeks of gestation. At this point, the heart is tubular and made of an outer layer of contractile myocardia and an inner layer of specialized endothelial cells, called endocardial cells (Fig 2.2) [18], [19]. The myocardia generates blood flow via peristaltic contraction from one end to the other, squeezing blood through the heart tube [20]. However, as the circulatory demands of the developing embryo grow the need for valve structures which maintain this unidirectional blood flow also increases. Cardiac jelly, which consists mainly of glycosaminoglycans (**GAGs**) and separates the myocardial cells from the endocardial cells, accumulates near the beginning and end of the heart tube, called the atrioventricular canal (**AVC**) and outflow tract (**OFT**) respectively [21]. This accumulation forms the endocardial cushions, or localized swellings on the interior of the heart tube which are covered in endocardial cells [3].

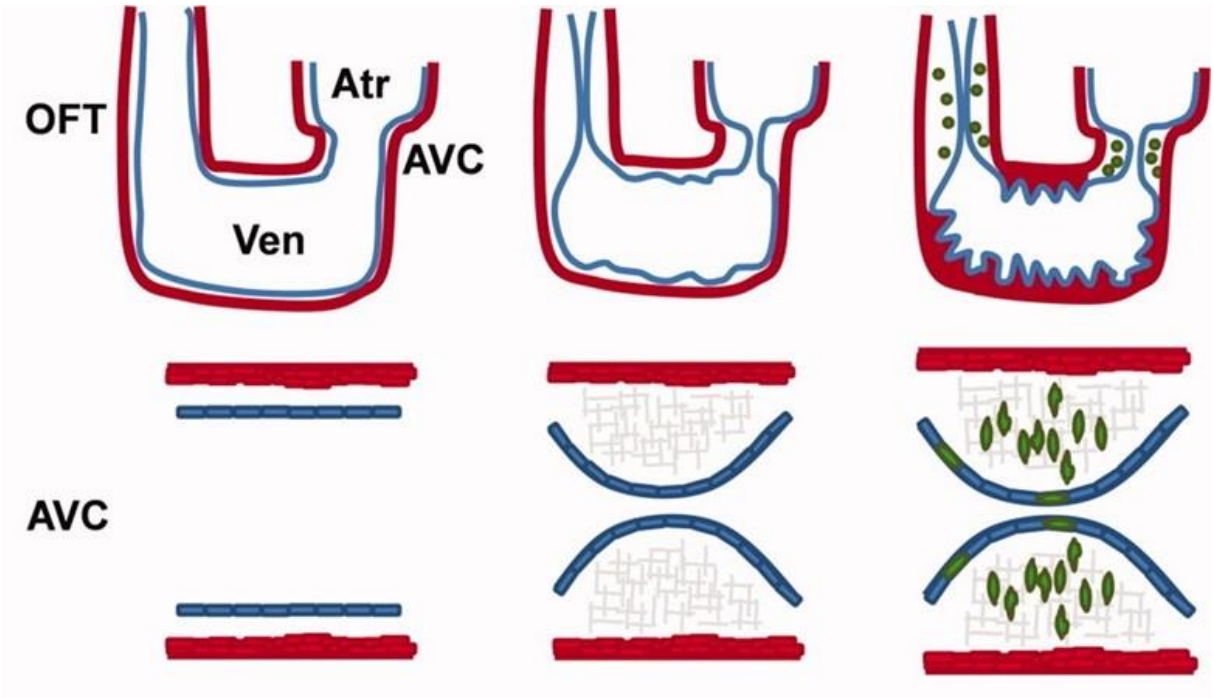

Figure 2.2 Endocardial cushion development and EMT
The heart tube consists of an inner layer of endocardial cells (blue), and an outer layer of myocardial cells (red). At around 4 weeks of human gestation, cardiac jelly accumulates in the AVC and OFT, and endocardial cells subsequently undergo EMT and invade and remodel the endocardial cushions (green). (Reprinted with permission from [22])

The endocardial cushions are the earliest structures which resemble heart valves in both form and function. Like valves, they constrict blood flow to a much narrower opening and act to maintain unidirectional blood flow [23]. Because of this constriction, the endocardial cushions are able to partially block retrograde blood flow, much like the mature valves that they will develop into. Most importantly, the endocardial cushions present a unique mechanical structure within the heart tube, leading to alterations in the hemodynamic profile of the developing heart. These altered hemodynamics, discussed in subsequent sections of this work, are thought to be major players in the progression of valve development.

2.2.1 Endocardial cells

Endocardial cells are a specialized subtype of endothelial cell that lines the chambers of the heart. They share many hallmarks of other vascular endothelial cells, both in function and

signaling profile. Vascular endothelial and endocardial cells maintain a protective barrier between the blood and tissues underneath, promote the exchange of nutrients and oxygen from the circulatory system to the underlying tissues, and mediate immune cell infiltration. Both cell types express classical endothelial markers platelet and endothelial cell adhesion molecule 1 (PECAM-1, or **CD31**), vascular endothelial cadherin (**VE-cadherin**, CDH5, or CD144), and vascular endothelial growth factor receptor 2 (**VEGFR2**). Endocardial cells are developmentally similar to other vascular endothelial cells as well, though developmental studies in chicken and mice have revealed potential differences in embryonic origin that may explain the unique nature of endocardial cells.

Vascular endothelial and endocardial cells develop in early embryogenesis, in close spatial and temporal proximity to the rest of the heart [24]. Identifying the precise moments of cell-type-specification has been challenging, due to the difficulties inherent to such precise embryonic studies as well as the differences between various model animals [25]. Early evidence in mice and zebrafish showed that tyrosine kinases Tie1 and Tie2 were crucial for development of the endocardium. However, ablation of their function did not prevent formation of vascular endothelium, suggesting that endocardial cells may be a specialized subset of a larger endothelial population [26], [27]. Similarly, work in quail identified an endocardium-forming population of cells which was not capable of myocardial differentiation, suggesting divergent fate commitment of endocardium and myocardium at an early developmental timepoint [28]. Along with other work in chicken and zebrafish, these results suggest pre-specification of the cells which become the endocardium [29], [30].

Other models suggest a different developmental origin for the endocardium. Using genetic mouse models, investigators have more recently argued for the existence of a multipotent progenitor that is capable of differentiation into myocardial, endocardial, and vascular endocardial lineages [25], [31]–[33]. Lineage tracing combined with *in vitro*

differentiation of mouse embryonic stem cells identified a multipotent progenitor which gave rise to both endocardium and myocardial cells [32]. A lineage tree representation of this model of lineage development is shown in Figure 2.3. Additional studies identified other markers for progenitor cells capable of differentiation to myocardial and endocardial lineages, including *Isl1* and *Mef2c* [34], [35]. Similarly, Nkx2.5, a marker for cells of a myocardial lineage, has been shown to be necessary for proper development of the endocardium, and deletions of Nkx2.5 completely abrogates development of the endocardial cushions, demonstrating a close relationship between the myocardial, and endothelial/endocardial lineages [36], [37].

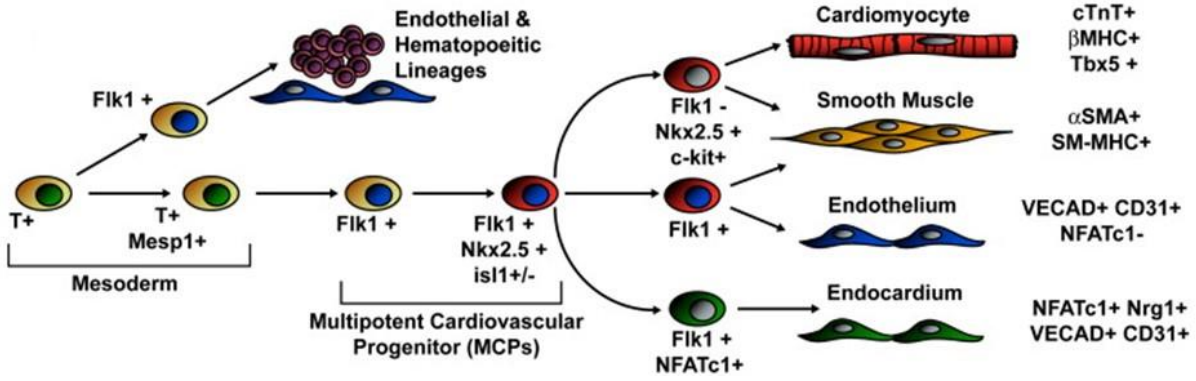

Figure 2.3 Endocardial cell lineage
(Reprinted with permission from [32])

Evidence for both models of the developmental origins of endocardial cells is compelling, and given the phenotypic flexibility that endothelial cells are known to demonstrate, it is conceivable that aspects of both models may be involved depending on the animal model used or context of the study. Regardless of origin, however, mature endocardial cells represent a functionally distinct population of endothelial cells and can be distinguished by their expression of certain endocardial specific markers. Among these, nuclear factor of activated T-cells (**Nfatc1**), is one of the most widely used [38], [39]. Nfatc1 is a transcription factor that is highly expressed in endocardial cells, but not in vascular endothelium. It coordinates the morphological development of the cardiac valves from the endocardial cushions and is highly expressed within

the endocardial cells overlying the cushions [40]–[43]. This specificity, in conjunction with other endothelial markers, has allowed for highly precise identification of endocardial cells for developmental studies, as well as detailed investigation of their role in valve development.

2.2.2 EMT and cushion remodeling

After formation of the endocardial cushions, the endocardial cells overlaying the cushions undergo EMT and invade the cushion matrix. These cells are crucial for the remodeling processes that transforms the loose cushions into compact, mature valve structures. As such, EMT represents a major component of successful formation of a healthy valve. EMT is characterized by the transition of a cell from an endothelial or epithelial monolayer to a migratory, mesenchymal phenotype, and is a major component of many large-scale morphological changes during development. EMT is governed by a plethora of signaling pathways, many of which intersect or overlap. The largest players in endocardial cushion EMT are represented in Figure 2.4.

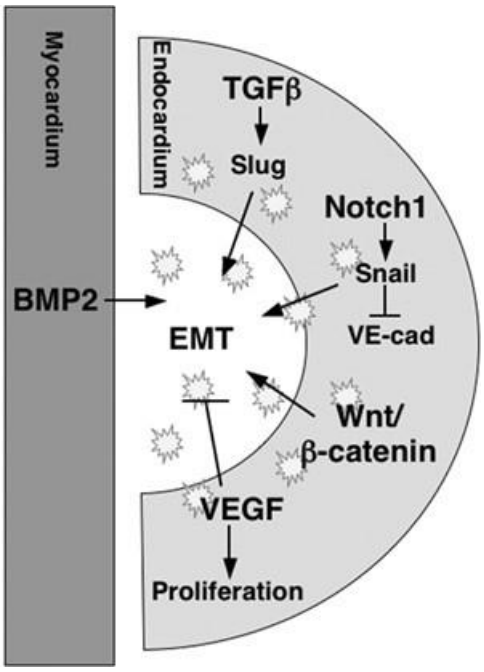

Figure 2.4 Signaling pathways which regulate endocardial EMT
(Reprinted with permission from [3])

In particular, the TGFβ superfamily is a crucial regulator of endocardial EMT. One member of this family, bone morphogenetic protein 2 (**BMP2**) is expressed by the myocardium and signals through endocardial receptors ALK2/3 and BMP-receptor 2 to activate signal transducers SMAD1/5/8 [44], [45]. BMP2 may also promote endocardial cushion development through the deposition of additional ECM components prior to EMT [18], [46]. Specific patterning of TGFβ receptors is thought to limit the extent of EMT to the cushion region, though mechanisms leading to these expression patterns remain unclear [47]. Deletions of certain TGF family members are embryonically lethal around the time of valve development due to insufficient cardiovascular development, but other deletions still produce viable mice, often with slight delays in developmental timepoints. This indicates a degree of redundancy in these signaling pathways, and points to the possible existence of a higher level regulator of this signaling family [48].

Another signaling pathway heavily regulating endocardial EMT and valve development is the NOTCH1 pathway. NOTCH1 is a transmembrane receptor that binds ligands in the Jagged and Delta families presented on neighboring cells. Ligand binding to the extracellular NOTCH1 domain induces proteolytic cleavage of the receptor, and the intracellular domain translocates to the nucleus, where it can act as a transcription factor. In the context of endocardial EMT, NOTCH1 signaling activates transcription of SLUG, a SNAIL family member which downregulates VE-cadherin [49]. NOTCH1 signaling also converges with the TGFβ signaling network in its upregulation of SNAIL, which is synergistically increased in the presence of TGFβ2 signaling. Additional crosstalk between the two pathways is seen in the inhibition of TGFβ-driven EMT through chemical inhibition of the NOTCH1 pathway [50], and in a study showing that TGFβ signaling can drive expression of NOTCH1 ligands [51]. *NOTCH*1 mutation is associated with numerous developmental and age-related diseases as well, discussed further in Chapter 3.

2.3 Aortic valve physiology

While the four cardiac valves vary in their physiology and susceptibility to disease, the aortic valve is by far the most prone to disease. This is no surprise, as it is the gateway from the heart to the systemic circulation, and therefore undergoes the greatest hemodynamic demands [10], [52], [53]. For this reason, the aortic valve is the primary focus of the following background discussion and of my dissertation research.

The aortic valve is a tri-leaflet valve, with all three leaflets of roughly equal size. The leaflets attach at their base to the aortic annulus and their free ends meet at the center of the aortic opening. Openings to coronary arteries are located in the sinuses behind two of these leaflets, while the third leaflet typically has no coronary. During systole, the valve is forced open into the aorta by hemodynamic pressure. Blood flows across the exposed sides of the leaflets at a rapid, unidirectional rate as it is delivered to the body. During diastole, expansion of the ventricles pulls the aortic valve closed, with the cusps of the leaflets coapting to form a seal. Blood flow on the aortic side of the valve is recirculatory as flow is stopped by the closed valve.

2.3.1 Valve structure

The architecture of the valve is well-suited for the dynamic mechanical environment in which it exists. Valve leaflets are trilaminar, with distinct ECM components in each layer. The ventricularis faces the left ventricle and is composed primarily of elastin and collagen. Elastin provides strength and elasticity as the valve is flexed open during systole. The elastin fibers are aligned radially, providing excellent mechanical integrity to the high demands of systolic blood flow [54]. The fibrosa, which faces the aorta, is composed of type I collagen. This strong ECM component is circumferentially aligned and provides the majority of the valves stiffness and mechanical resistance to deformation during diastole [55]. As might be expected from the high mechanical demands placed upon this layer, it is the thickest of the three. Separating either side of the valve is the spongiosa – a shock-absorbing layer composed of GAGs and proteoglycans.

These ECM components act as a cushion and absorb the shear forces imparted by the high flexure of the valve during each open and close [56], [57]. These layers are shown in histological staining and illustration in Figure 2.5.

In addition to the well-ordered ECM components of the aortic valve, cellular components act to maintain valve function. The two major resident cell populations within the aortic valve are valve interstitial cells (**VICs**), which populate the interior of the valve and maintain structural integrity of the ECM components of the valve, and valve endothelial cells (**VECs**), which cover the exterior surfaces of the valve and mediate interactions between the valve and the circulatory system. These cell populations are represented in Figure 2.5B.

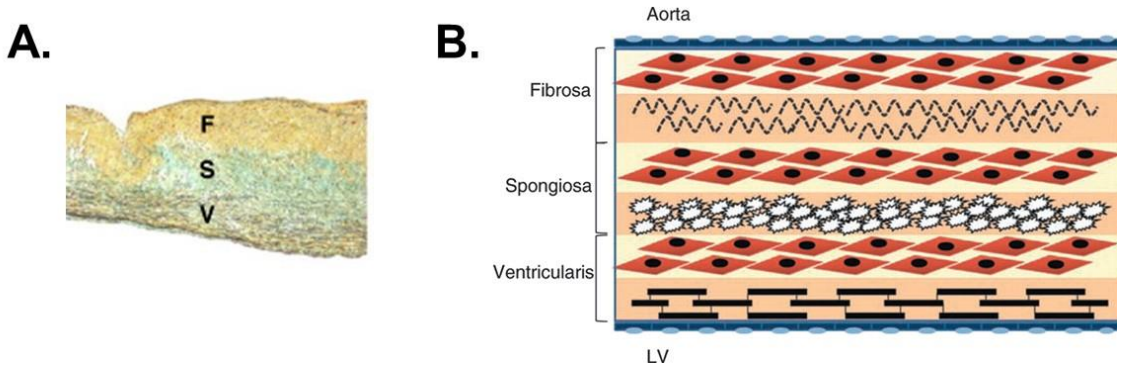

Figure 2.5 Aortic valve composition
The aortic valve is trilaminar, with a largely collagen compose fibrosa (F), spongiosa containing GAGs (S), and ventricularis composed of elastin (V) (A). Cells of the aortic valve include VECs lining the valve surfaces and VICs residing in the valve interior. (Reprinted with permission from [58] and [59])

2.3.2 Valve endothelial cells

VECs are the cellular descendants of endocardial cells which did not undergo EMT and invade the embryonic endocardial cushion. These cells share a similar function to other cardiovascular endothelial cells – that is, they create a barrier between the complex biochemical and hemodynamic environment of pulsatile blood flow and the VICs residing within the interior of the valve [60]. VECs mediate inflammatory signaling from the circulatory system, and they

provide an initial response to other injury stimuli such as oxidized LDL, bacterial or other pathogenic molecules, and altered hemodynamic loads resulting from injury [61]. VECs are capable of recruiting monocytes via upregulation of leukocyte adhesion molecules in response to injury, and they are non-thrombogenic, acting to prevent coagulation [62], [63]. Finally, VECs are in direct contact with VICs, which are a very different cell type than those that other vascular endothelial cells interact with (typically smooth muscle cells).

2.3.3 Valve interstitial cells

VICs populate the interior of the valve and are principally responsible for preserving the structural integrity of the valve by maintaining and remodeling the ECM components within the valve [64]. VICs constitute a unique cell population, though comparisons are often drawn between them and fibroblasts, myofibroblasts, smooth muscle cells, osteoblasts, and stem cells, depending on the experimental methods and disease state of the valve [64]–[68]. It appears that many of these phenotypes can exist simultaneously, and individual cells may change from one state to another in response to external stimuli, so an accurate description of VIC populations and behavior should focus on their various functions.

Under healthy, maintenance-mode conditions, many VICs closely resemble fibroblasts and are thought to play a role in ECM maintenance and angiogenesis inhibition [64], [69]. These cells may be quiescent and have very low metabolic rates. Nevertheless, due to the high mechanical demands placed on the valve, even VICs under healthy conditions are highly secretory, generating collagens, GAGs, and various matrix degrading factors including matrix metalloproteinases and enzymes which degrade GAGs [70], [71]. These cells are characterized by expression of vimentin with low expression of alpha smooth muscle actin (**αSMA**). Upon activation by injury, mechanical stress, or signaling stimuli including cytokines and growth factors such as TGFβ, VICs enter an activated state similar to myofibroblasts. These cells are characterized by high expression of αSMA, increased migration and tissue invasion, and

increased ECM degradation and secretion [64]. Activated VICs are responsible for responding to more acute injury or higher demand for valve maintenance [72]. Unfortunately, overactivity by this VIC subtype is also one of the factors which leads to valve disease, the progression of which is discussed in the following chapter. In cases of advanced calcific disease, VICs can undergo an osteoblast-like differentiation, becoming highly secretory and depositing large amounts of collagen and calcium into the body of the valve [64]. This differentiation process shares many hallmarks with classical osteoblast differentiation and activity, including the prominence of Runt-related transcription factor (**Runx2**) and various BMP family members as well as the expression of osteocalcin and osteopontin [73]. Progenitor VICs, or stem cell-like VICs, maintain the VIC population itself. These cells assist in the injury response process by proliferating and creating more of the other required VIC subtypes [64], [74]. The origin of these cells is unclear, but they may be resident VICs that exist in an undifferentiated state or they may be either circulating cells that migrate into the valve or cells from nearby tissues that migrate to the site of injury. Yet another hypothesis for their origin is that VECs lining the valve may undergo EMT in a mechanism similar to valve development, though recent evidence suggests this mechanism might not normally occur *in vivo* [75], [76].

VECs and VICs act in concert to maintain homeostasis and respond to the unique mechanical environment of the aortic valve. Communication between the two cell types is largely paracrine, and includes nitric oxide synthesis by the VECs, which acts to prevent myofibroblast differentiation and calcification by the VICs [77], [78]. Conversely, injury to the endothelium leads to a pronounced activation of underlying VICs, with increased calcium deposition and ECM remodeling [79], [80]. *In vitro* coculture experimentation shows that VECs act to promote a quiescent phenotype in VICs, marked by lower expression of αSMA, lower calcific nodule formation, and lower collagen secretion [81], [82].

2.4 Mechanical control of valve development and function

The developing valve experiences mechanical stimuli from the flow of blood over the surface of the endocardial cushions, blood pressure, and the contraction of the myocardial layer beneath the cushions. Hemodynamic flow over the endocardial cushions has been shown to be responsible for side specific remodeling of the cushions into mature valve structures, and alterations to blood flow by surgical intervention in chick embryos revealed that blood pressure rather than flow rate may mediate morphological changes in the valve [83], [84]. In an *ex vivo* explant model of endocardial EMT, contraction from the myocardium was found to promote EMT and successful valve development [15]. Finally, numerous *in vitro* experiments have demonstrated the crucial role for mechanical forces in stimulating developmental functions such as EMT and ECM remodeling [85], [86].

The adult aortic valve is similarly subject to a variety of mechanical forces. Flow of blood across the ventricularis during systole or on the fibrosa during diastole imparts fluid shear stress, with distinct flow patterns imparting different shear patterns and magnitudes to either side of the valve [87], [88]. During diastole, the retrograde push of blood against the fibrosa side of the valve creates a large mechanical strain on the valve, stretching the leaflets radially and circumferentially [89]. Finally, movement from open to closed creates a large flexural strain, primarily around the annulus of the valve where the leaflets must bend. This is distinct from in-plane stresses, which affect the whole thickness of the valve equally, in that each layer of the valve experiences different flexural tension [90].

At a cellular level, VECs and VICs must contend with particularly extreme mechanical environments compared with the rest of the circulatory system [61], [77]. Estimates of the hemodynamic shear across the ventricularis are as high as 1,500 dyne/cm^2 at peak flow, though other studies suggest cycle average values around 20-30 dyne/cm^2 [87], [91]. These values far exceed estimates for other parts of the circulatory system, which are below 10 dyne/cm^2 for

larger vessels and below 15 dyne/cm^2 for sprouting capillaries [92], [93]. Interestingly, when exposed to shear stress, VECs respond by aligning perpendicular to flow, whereas vascular endothelial cells align parallel [82], [94], [95]. Furthermore, VECs exhibit side-specific mechanical properties, depending on their origin from the ventricle or aortic side of the valve [96]. VICs are also subject to mechanical stresses, primarily in the form of strain as the valve is stretched. These forces place strain on the VICs directly, but also damage ECM components within the valve, necessitating VIC-mediated repair.

2.5 Non-coding RNAs are novel regulators of valve health

Non-coding RNAs (ncRNAs) are a relatively recently discovered class of molecule that are increasingly being implicated in the regulation of cellular processes. As their name would imply, ncRNAs are RNA molecules that are not known to code for any protein product, yet they still exert signaling function as an RNA. Two major classes of ncRNAs which are known to affect cardiovascular development and disease are microRNAs (miRNAs), which are roughly 22 base pairs long and regulate gene expression through RNA silencing, and lncRNAs, which are over 200 base pairs and function through a variety of mechanisms.

MiRNAs are known to be involved in many aspects of embryonic development, including cardiac development and valve formation [97], [98]. One of the strongest pieces of evidence for their importance comes not from manipulation of miRNA species directly, but rather alterations in Dicer, an RNase involved in miRNA-mediated RNA interference. Disruption of Dicer impairs proper cardiac development, including ventricular-septal defects and lack of valve development [99]. Several specific miRNA species responsible for these defects have been identified [100], [101]. In the valve specifically, *miR-126* is expressed in endocardial cells and has been shown to interact with VEGF and NFAT signaling to promote leaflet extension following EMT [102]. Additionally, *miR-23* inhibits synthesis of hyaluronic acid, a major component of cardiac jelly in the endocardial cushions, to restrict cushion formation to the proper spatial location [103].

Likewise, numerous miRNA species have been implicated in mature valve disease, discussed in the following chapter.

Less has been definitively shown about the role of lncRNAs in valve development and healthy function. Still, specific associations between lncRNAs and valve development have been observed. For example, lncRNA *uc.4* has been associated with mitral valve formation, and lncRNA *LL33* is active in the AVC region during valve development [104], [105]. One lncRNA in particular, *H19*, has been shown to be relevant in adult valve disease, and observations suggest it plays a role in valve development as well. *H19* is a highly-conserved mammalian gene and was one of the first lncRNAs discovered. It is currently thought to act as a transcriptional regulator of several key developmental genes through competitive binding of promoter sites and by acting as a molecular decoy for miR-141 and miR-22, among others [106], [107]. *H19* also harbors a highly conserved miRNA sequence within its first exon, miR-675, which likely accounts for some of the translational inhibitory action of *H19* [108].

The developmental relevance of *H19* stems from the unique manner in which its expression is regulated. During development, *H19* is highly expressed in the majority of embryonic tissues, including the developing heart [109]. Following birth, *H19* becomes strongly imprinted, meaning only one copy of the two genomic copies is expressed. In the case of *H19*, the maternal copy is expressed while the paternal copy is epigenetically silenced. This imprint is regulated by a differentially methylated imprinting control region (**ICR**), which is found a few kilobases upstream of the *H19* promoter. On the other side of the ICR is insulin-like growth factor 2 (**IGF2**), which is co-regulated by the ICR. Enhancer elements downstream of *H19* promote expression of both *H19* and *IGF2* through interaction with their promoter sites. In most adult tissues, the maternal ICR is bound by CCCTC-binding factor (**CTCF**), a transcriptional regulator which promotes interaction of the distal enhancer elements with the *H19* promoter. On the paternal allele, hypermethylation of the ICR prevents CTCF binding, allowing the enhancer

elements to activate *IGF2* transcription. A schematic of this unique regulatory mechanism is shown in Figure 2.6. This methylation pattern is a key indicator of *H19* expression and potentially pathologic dysregulation. *H19* is hypothesized to effect many pathways involved in embryonic development, and has a definite role in regulating embryonic growth [110]–[112]. It has also been shown to impact EMT, endothelial function, and many other aspects of cardiovascular disease, which are discussed in Chapter 3 [113]–[115]. Given these known effects and its high expression in the developing heart, it's highly likely that *H19* plays an active role in valve development.

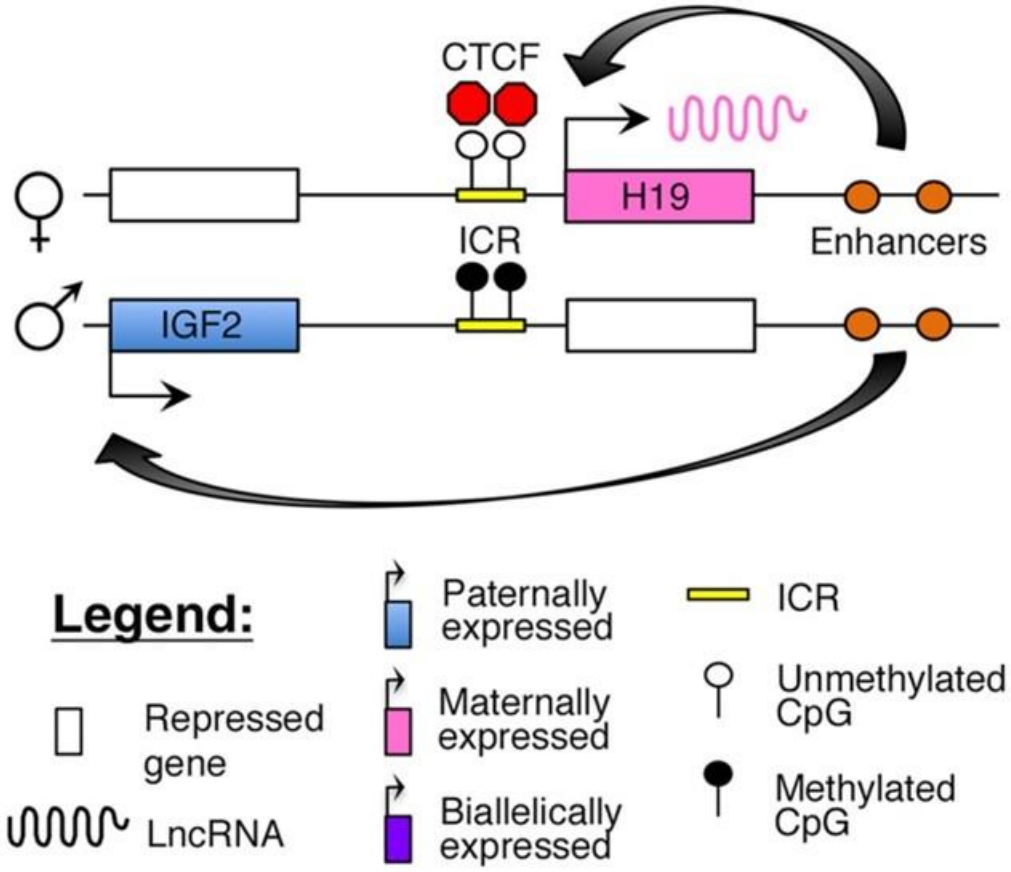

Figure 2.6 The *H19/IGF2* locus imprinting mechanism
(Reprinted with permission from [116])

Chapter 3

Valve disease and treatment

Text for Chapter 3 was adapted in part from **Vander Roest MJ** and Merryman WD. A developmental approach to induced pluripotent stem cells-based tissue engineered heart valves. *Future Cardiology*, 13(1)1-4 10.2217/fca-2016-0071. [117]

In both development and disease, genetic mutation, dysregulation of healthy signaling, injury, or other yet-unknown factors can lead to pathological disease. Given the critical function of the cardiac valves, these diseases can have a profound effect on the affected individual and on the greater cost of healthcare. This chapter provides background information on developmental and age-related valve disease, known factors which drive disease, current treatment strategies, and areas of open research which are covered later in this dissertation.

3.1 Developmental valve disease

CHD is the most common type of birth defect, accounting for roughly a third of the major developmental anomalies in live births worldwide. CHD is the leading cause of infant morbidity in the Western world, and a leading cause of pediatric or *in utero* death worldwide [1], [6]. Valvular defects account for 20-30% of the cases of CHD, making them a major source of developmental and pediatric disease [3], [118]. Estimates of the prevalence of various congenital valve pathologies range from 1-3% of live births, though these estimates may be lower than actuality, given that many valvular pathologies are classified under different pathologies or are not evident at birth (though may become problematic in later life) [1], [119].

3.1.1 Bicuspid aortic valve

BAV is a malformation of the aortic valve in which the typically tri-leaflet valve forms with only two leaflets (Fig 3.1A). It is the most common congenital cardiac defect, and is found in 1-2% of live births, with a 3 to 1 predominance in males [4], [120]. BAV can occur in isolation, but it often appears in conjunction with other forms of CHD. Many genetic links to BAV have been identified and are discussed in section 3.3, though many instances occur with no clear explanation. Despite the striking structural differences between BAV and a tricuspid aortic valve, symptoms are not always immediately consequential. Numerous individuals grow and develop with a fully functioning bicuspid valve which leads to little decrease in the life expectancy of younger affected populations [121], [122]. However, overall valve-related morbidity is increased, and the altered hemodynamics resulting from the bicuspid valve lead to CAVD and aortic stenosis in later life (Fig 3.1B) [123].

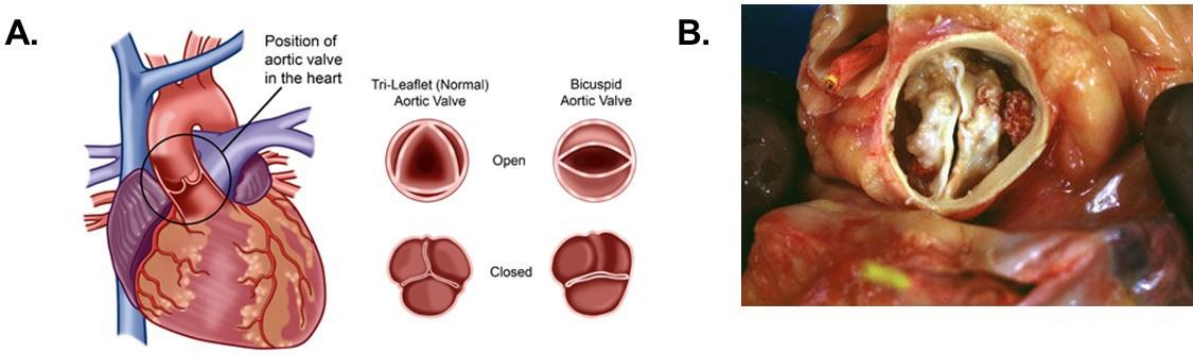

Figure 3.1 Bicuspid aortic valve
In BAV, the normally trileaflet aortic valve forms with only two leaflets (A). This can cause hemodynamic changes which lead to calcification (B). (reprinted from valleyheart.com and clevelandclinic.org)

3.1.2 Congenital aortic stenosis

Congenital aortic stenosis is a common hallmark of pathologic BAV, but it can also occur in tricuspid aortic valves [6]. Congenital stenosis is characterized by a narrowed aortic opening, which results in hemodynamic obstruction. Given the early presentation,

congenital aortic stenosis can progress quickly to congestive heart failure [7]. However, because stenosis exists on a spectrum, disease manifestation may be mild in early development with significant ramifications in later life, including a risk for sudden death in adolescence and early adulthood [124]. Infants and children with congenital aortic stenosis require surgery in over 40% of cases, with more needing further surgical intervention or valve replacement in later life [124], [125].

3.1.3 Other congenital defects

Other, less common, congenital valve defects are also major sources of childhood morbidity. The mitral valve is prone to congenital prolapse or regurgitation, Ebstein's anomaly, isolated cleft, and stenosis [5], [126]–[129]. Similar to the pulmonary valve may become stenosed, though typically to a lesser degree [130]. Finally, many other cardiovascular complications are exacerbated by accompanying valve malformations, many of which are not counted among valve statistics due to the presence of more severe pathologies. Collectively, the burden of congenital valve disease is great, and both the development of new therapeutic approaches for treatment and a better understanding of disease progression would be of immense impact to a large number of affected patients.

3.2 Age-related valve disease – Calcific aortic valve disease

CAVD is the predominant form of aortic valve disease in adults. It is characterized by progressive fibrocalcific remodeling of the aortic valve tissue (Fig 3.2). In the early stages of CAVD, often called sclerosis, remodeling stiffens the valve and reduces the flexibility of the leaflets without significant impact on hemodynamic function. As the disease progresses into stenosis, valve remodeling is so extensive as to obstruct aortic outflow. Due to the progressive nature of CAVD, it is most commonly found in older patient populations. More than 1 in 4 people over the age of 65 have signs of mild sclerosis, while 12% have more severe stenosis [9], [131].

In the more elderly, disease prevalence is even higher, with nearly 50% of those over 75 estimated to have some degree of valve calcification [132].

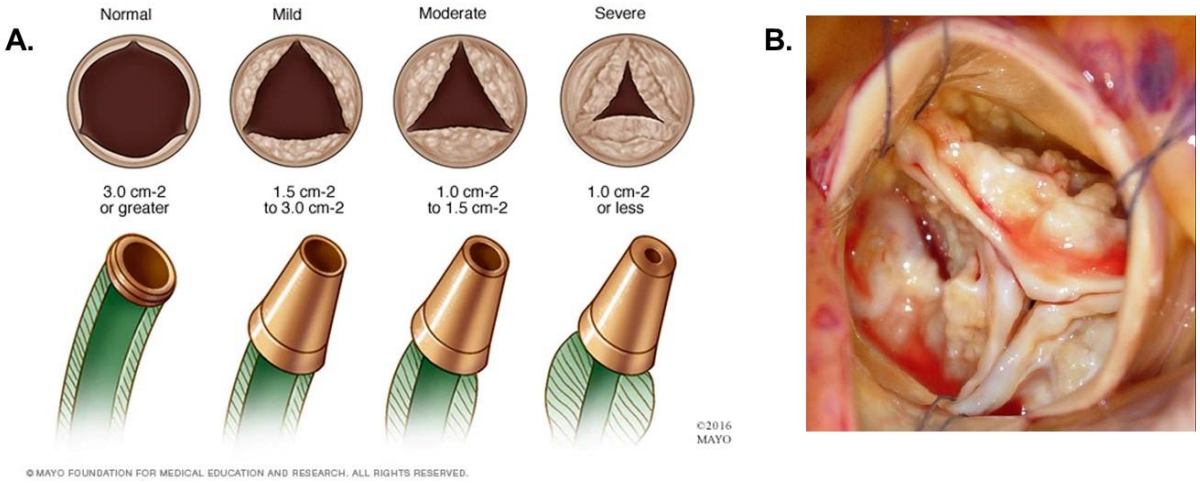

Figure 3.2 Calcific aortic valve disease
CAVD is characterized by progressive remodeling and calcification of the aortic valve with significant narrowing of the aortic opening in severe stenosis (A). Advanced CAVD shows large calcific deposits on valve leaflets (B). (Reprinted with permission from mayoclinic.org and [133])

CAVD is understood to be an active process of remodeling, with VIC-mediated remodeling of the valve ECM as the driving force behind the structural changes in the valved. In this respect, the myofibroblast and osteoblast-like VIC phenotypes are responsible for the majority of these changes. Myofibroblastic VICs are normally a small, beneficial population of cells within the valve, as discussed in Chapter 2. Their response to injury can help maintain valve structure in challenging environments, but overactivity or persistence of the myofibroblastic VIC leads to undesirable remodeling of the valve. Activation of quiescent VICs to a pathologic, myofibroblast state is thought to occur *in vivo* as a result of immune cell infiltration in response to injury or in response to age-related degradation of the valve. This process has been studied *in vitro* using a variety of cytokines to activate VICs, including TGFβ, fibroblast growth factor 2, or serum starvation and mechanical stimuli [58], [64], [65], [73], [134]. Myofibroblast VICs are thought to be the main force behind pathologic remodeling in early

stages of disease, when there is predominantly fibrotic remodeling. As disease progresses into later stages of calcification, myofibroblastic VICs are still active, but are also assisted in their remodeling by osteoblastic VICs.

Osteoblast-like VICs are a pathogenic manifestation of VICs characterized by deposition of mineralized ECM and bone formation. These cells are not found in every instance of CAVD, meaning they may either represent a very advanced stage of CAVD or are part of a distinct pathologic pathway that is not necessary for CAVD [135]. In addition to their functional similarities, osteoblastic VICs share many key signaling components with osteoblasts, including expression of alkaline phosphatase, osteopontin, osteocalcin, and BMP family members [135], [136]. *In vitro* studies have replicated the osteogenic differentiation of VICs through supplementation with organic phosphate and ascorbic acid, demonstrating that such a dramatic phenotypic shift of VICs is certainly possible *in vivo* [137], [138].

3.3 Causes of valve disease

A unified explanation for the initiation and progression of valve disease has not been developed, and given the heterogenous nature of valve disease, it likely doesn't exist. Still, many genetic conditions, signaling pathways, and external stimuli are known to play significant roles in CAVD, and are discussed at present. The high degree of overlap between factors involved in valve development and disease are of special importance as we consider research into open questions at the end of this chapter.

3.3.1 Genetic Factors

Various genetic syndromes have been linked to developmental abnormalities in the valves and are therefore also associated with late-stage valve disease [139]. These include DiGeorge syndrome, Down syndrome, and Noonan syndrome, among others. Many genetic syndromes linked to a mutation in or deletion of ECM components also affect the valves, in p. These include Williams, Marfan, and Ehlers-Danlos syndrome. As previously mentioned, these

developmental changes in valve shape and structure often manifest eithr as CAVD or a similar stenotic disease later in life. Specific mutations in genes involved in CAVD are also linked to both congenital valve disease and CAVD, including mutations in αSMA, NOTCH1, and Jagged1, which is a member of the Notch signaling family [140]–[142]. Mutations in NOTCH1 are also known to lead to BAV and CAVD [143]. Importantly, *NOTCH1* loss of function mutation leads to 100% penetrance of CAVD even in subjects without developmental defects, suggesting an important role for disruption of the Notch pathway in many cases of CAVD. However, *NOTCH1* mutation is relatively rare, and does not explain the majority of cases of CAVD, leading researchers to look in more depth at the NOTCH1 pathway to determine non-genetic causes of CAVD.

3.3.2 Signaling pathways

The NOTCH1 signaling pathway is highly involved in many developmental processes, including cell-fate specification, proliferation, and apoptosis [144]. NOTCH1 and other Notch receptors are single pass transmembrane receptors. NOTCH1 signaling is initiated by the presentation of one of the Notch ligands (Jagged 1 and 2, Delta 1, 3 and 5) from a neighboring cell to the extracellular portion of NOTCH1, a process which may be mechanosensitive. This binding induces proteolytic cleavage of the Notch receptor, at which point the Notch intracellular domain can translocate to the nucleus, where it joins with additional transcription factors to effect transcriptional changes in the cell [144]–[146]. Downstream effects of altered NOTCH1 signaling are varied, and multiple mechanisms for the relationship between NOTCH1 loss of function and CAVD have been proposed. NOTCH1 has been shown to promote expression of Sox9, an osteogenic repressor [147], [148]. Loss of NOTCH1 signaling also leads to increased expression of TGFβ and BMP family members, and this effect seems to be mediated through transcription factors that act downstream of Notch [149]. Finally, decreased NOTCH1 signaling

is associated with an increase in cadherin 11 (**CDH11** or OB-cadherin), another signaling molecule implicated in CAVD.

CDH11 is a cell adhesion molecule which forms strong homotypic bonds with neighboring cells. CDH11 expression is highly elevated in calcified human aortic valves, suggesting a role in disease progression (Fig 3.3A) [150]. CDH11 may play a role in mediating strain driven myofibroblastic activation of VICs, leading to a process of dystrophic valve calcification [151]. Indeed, genetic overexpression of CDH11 has been shown to promote valve calcification [152]. Additional investigation of CDH11 signaling in a *Notch1* haploinsufficiency model of valve calcification showed that decreased CDH11 activity through genetic loss of Cdh11 (*Cdh11*$^{+/-}$) alleviated calcific stenosis in mice (Fig 3.3B). Even more encouraging was the fact that treatment with a CDH11 functional blocking antibody, SYN0012, also prevented calcific stenosis (Fig 3.3C) Interestingly, blocking antibody treatment increased Sox9 nuclear activity over that of control mice (Fig 3.3D), indicating that CDH11 signaling may be an integral part of the signaling network downstream of NOTCH1 that promotes valve calcification. Finally, CDH11 forms intercellular bonds which are significantly stronger than those between other cadherins, pointing to a potential mechanobiological role for upregulation of CDH11 [153].

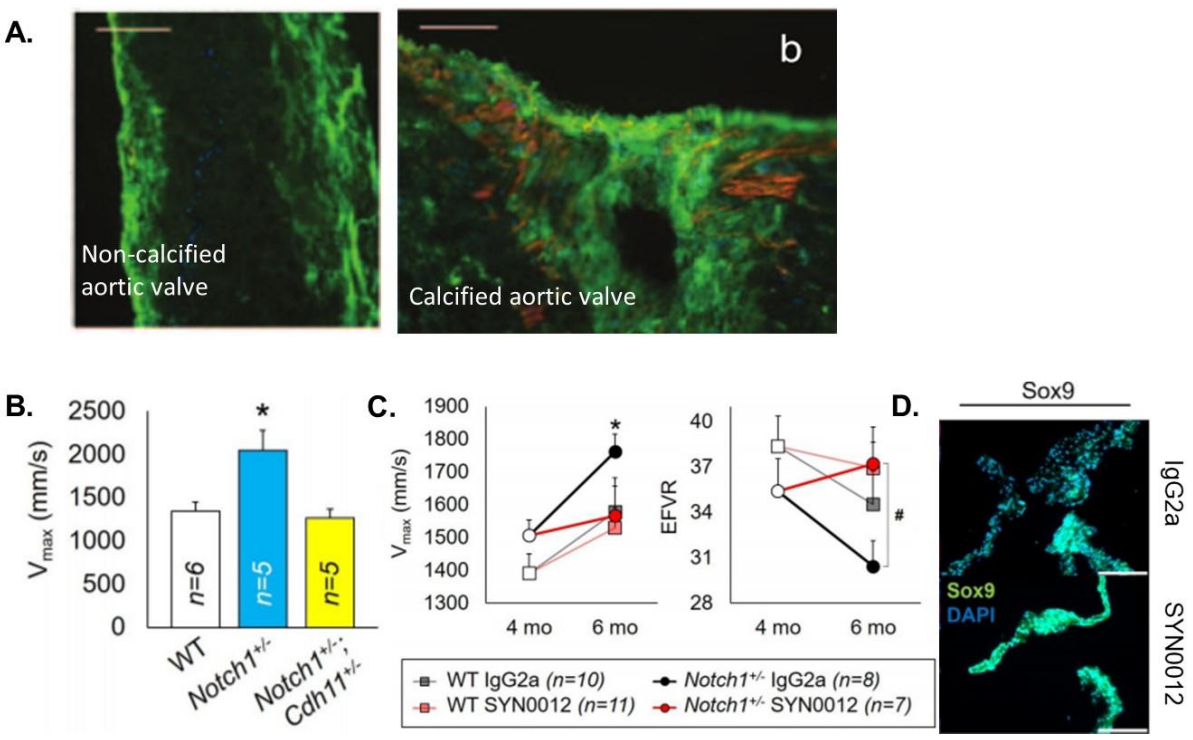

Figure 3.3 Cadherin-11 involvement in CAVD
CDH11 is significantly overexpressed in calcified human aortic valves (A, green), as is αSMA (red staining in A-b). In a *Notch1*$^{+/-}$ model of valve disease, genetic ablation of *Cdh11* reduces hemodynamic signs of stenosis (B), as does functionally blocking CDH11 function (C). CDH11 blocking increases SOX9 expression, suggesting anti-osteogenic effect (D). (Reprinted with permission from [154] and [150])

3.3.3 Mechanical stimuli

The mechanical environment of the aortic valve is extremely dynamic and, when compared with most other tissues in the body, quite harsh. While the structure of the aortic valve is uniquely adapted to this environment, mechanical factors have been shown to play a role in the progression of CAVD. Mechanical changes within the aortic valve occur most dramatically during development, when the loose ECM of the endocardial cushions is compacted into a mature valve structure, and during old age, which is coincidental with increased risk of CAVD and associated changes in hemodynamics [14], [155]. VECs exposed to high oscillatory fluid shear, similar to what occurs on the fibrosa side of the valve, increase expression of cellular adhesion molecules ICAM-1 and VCAM-1, possibly recruiting monocytes

and initiating an inflammatory process that results in disease [156]. The directionality of shear appears important too, as the pulsatile unidirectional flow similar on the ventricularis side does not result in similar levels of adhesion molecule upregulation. VECs align perpendicular to the direction of fluid shear, and the underlying ECM is likewise highly patterned [94], [95]. Altering shear profiles on the surface of the VECs may lead to adverse ECM remodeling which weakens the integrity of the valve. Increased mechanical strain is also known to lead to adverse remodeling of the valve, as shown in various *ex vivo* studies [58], [157]. These effects have been partially traced to VIC-driven calcification through *in vitro* work [150], [151]. Finally, changes in ECM mechanics as a result of minor remodeling or passive degradation may have significant impact on cellular behavior. Even passive cues such as substrate stiffness have been shown to regulate VIC phenotype and drive osteoblast differentiation [158], [159].

Mechanical properties of the cells themselves can also have implications on overall health of the valve. VICs from the four different cardiac valves are known to exhibit different mechanical properties which are thought to contribute to the valve deformability during valve closure and propensity for ECM secretion [160]. Changes in cell phenotype often correspond with alterations to the cells' cytoskeletal organization, which can be measured dynamically using a technique called micropipette aspiration.

3.3.4 LncRNAs and *H19*

Non-coding RNAs are increasingly being implicated in a variety of cardiovascular diseases, including CAVD. While the notion that ncRNAs have no function has long-since been discarded, identifying precisely what their function is remains a challenge. Various ncRNAs, including miRNAs and lncRNAs, have been clearly shown to play a role in CAVD.

Sequencing based cohort studies have identified several miRNAs with significantly altered expression in CAVD, including miR-106a, miR-148a, miR-204, miR-181b, and many more [161]. Some have been identified as potential drivers of disease, such as miR-214, which

regulates TGFβ expression in response to shear [162]. Others, such as miR-204 and miR-486, act downstream of TGFβ and BMP2 and have been shown to mediate CAVD in stimulated VIC cultures [163]. Still others have been proposed as biomarkers without clearly defined function [164]. In any case, a plethora of miRNA species are clearly part of CAVD disease progression, with more being identified on a regular basis [165].

H19 is one of the only identified lncRNAs known to be involved in CAVD [161]. Despite the extensive body of research into the imprinting mechanism and regulation of *H19* expression, its role in states of disease is only recently coming to light. Dysregulation of *H19* is thought to occur primarily through hypomethylation of the paternal allele, losing the imprint established after birth and allowing for expression from the paternal allele [166]. Such dysregulation has been observed in a variety of cancers as well as cardiovascular disease [167], [168]. In cancer, the role of *H19* is the subject of much debate, with various reports showing both an oncogenic and a tumor suppressing role for the lncRNA [169]. In actuality, the mechanism of action of *H19* seems to depend on the developmental context in which it is studied. Raveh et al. make a compelling case for context-specific action of *H19* as an oncogene, primarily through its inhibition of tumor suppressor P53 activity [170]. Additional work from the same lab has also shown that *H19* is responsive to TGFβ stimulation, and was required for induction of SLUG and repression of epithelial cadherin, strongly suggesting a role for *H19* in EMT [171]. Similarly, *H19* was shown to increase metastasis in bladder cancer, also through downregulation of epithelial cadherin [172]. In cardiovascular diseases the role of *H19* is similarly complex, with some reports identifying it as a driver of disease and others indicating that it is responsive to injury and works to restore function [115], [173]–[177].

Recently, *H19* was directly implicated in CAVD. Hadji et al. found that sclerotic and stenotic human aortic valves had significant upregulation of *H19* [178]. This was associated with hypomethylation in the promoter region upstream of *H19*, correlating with other work by Agba et

al. showing hypomethylation and increased expression of *H19* with increased age in various rat tissues [179]. Most importantly, Hadji used *in vitro* VIC cultures to demonstrate that elevated *H19* bound the promoter region of *NOTCH1*, blocking the transcription factor P53 from inducing *NOTCH1* transcription. Functionally, this mimics *NOTCH1* deficiency, reproducing the phenotype of familial *NOTCH1* mutation, including upregulation of *BMP2*, *RUNX2*, and *CDH11*, leading to increased calcification (Fig 3.4).

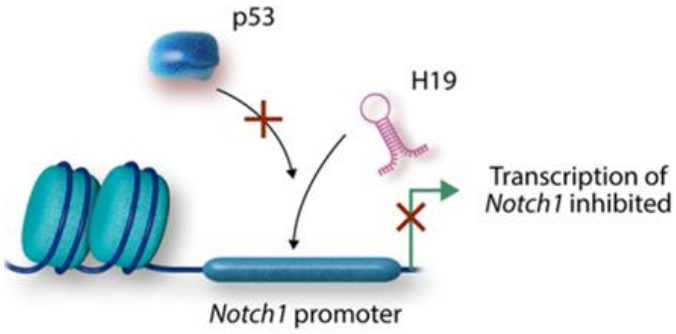

Figure 3.4 NOTCH1 repression by H19
H19 outcompetes P53 to bind the *NOTCH1* promoter, reducing expression of *NOTCH1* and mimicking a loss-of-function mutation. This induces classical CAVD pathogenesis.

3.4 Mouse models of valve disease

Mice are the most common model animal used to study CAVD. Mice present numerous advantages over other methods of research, including ease of breeding, short lifespans, low cost, and *in vivo* relevance. Unfortunately, mice do not naturally replicate many of the features of human disease. Importantly, mice do not have clearly tri-laminar valves, as is seen in humans [180]. Furthermore, wild-type mice on normal diets do not develop aortic stenosis or calcific remodeling at the same rate as humans do, necessitating external means of inducing CAVD symptoms [180], [181]. This is typically done with genetic mutant mouse lines targeting either developmental signaling or lipid regulating pathways, often in conjunction with a high-fat, high-cholesterol diet. Developmentally based models of CAVD include heterozygous loss-of-function mutations in *Notch1* or its effector protein RBPJk, which both cause calcification, fibrotic

remodeling, and immune cell recruitment without significant developmental abnormalities [149], [182]. Mice genetically deficient for apolipoprotein E or the low-density lipoprotein receptor are also used to induce hyperlipidemia and aortic valve disease. While these models have certainly advanced our understanding of human valve disease, they come with significant biases based on the specific mutation or treatment in the animal. Given that treatment of valve disease will likely depend on catching it in its earliest stages, development of a mouse model which replicates initiating factors of human CAVD would represent a major advantage over the models currently available.

3.5 Valve disease treatment strategies

Although treatment options for valve disease have advanced greatly in the past few decades, these options are still extremely limited. Treatments of pediatric valve disease are limited to post-birth intervention, and given the nature of these valve defects, surgical intervention is the only means to address congenital valve disease. While pharmaceutical targeting of valve disease is possible in older patients, no effective pharmaceuticals are known, and the disease is often asymptomatic and not discovered until it is fairly advanced. This leaves surgery as the only option for this patient population as well. The following section discusses current practices as well as attempted pharmaceutical strategies.

Currently, no pharmaceutical therapies to prevent or treat valve disease currently exist, though many potential options have been investigated [13], [154]. Drugs targeting lipid activity were among the first proposed to treat valve disease, based on the co-incidence of dyslipidemia and CAVD as well as a pathological similarity between CAVD and atherosclerosis [183], [184]. Various statins showed great promise in retrospective studies, and early clinical trials indicated potential benefits to hemodynamic metrics of valve health, but larger follow-up studies found no benefit to statin treatment outside of their known beneficial effects on vascular health [13], [185], [186]. Broader targeting of signaling pathways known to be involved in the initiation and

progression of CAVD have been proposed, including targeting TGFβ signaling and serotonin family members. Various studies in vitro and in vivo have yielded promising results, but overly broad targets such as these may cause more harm than benefit [16], [58], [187]. Recent results in mice suggest that functionally blocking CDH11 slows valve disease progression, though this has not seen clinical translation [154]. Nucleic acid targeting of dysregulated genes downstream of known mutations or dysregulation events is an emerging avenue of research, but these results also have years before sufficiently accurate targeting enables clinical use.

Given the inability to address valve disease pharmaceutically, surgical replacement is the only viable option to directly address many instances of disease. Current options for the replacement valve include mechanical valves, bioprosthetic replacements, or autograft replacement. Over 280,000 total valve replacements are performed annually worldwide, with nearly 100,000 replacements in the United States along. Replacement is indicated for cases of both congenital and age-related valve disease, though the implications for the recipient vary drastically between these two groups. Specific morbidities associated with each type of replacement as well as with the surgical means to implant the valves are discussed below.

Mechanical valve replacements were the first to be developed and successfully implanted in human patients. These valves are made of inert materials such as stainless-steel alloys and non-thrombotic polymers, but they still typically require anticoagulant treatment. Early models such as the ball-in-cage valves were found to damage blood cells, and were recently discontinued [188]. Other styles of mechanical valve, including the tilting disc and bi-leaflet valves, are less hemodynamically destructive, but still require low-level anticoagulants, which may impose restrictions on highly active pediatric recipients or increase the likelihood of a bleeding event in older valve recipients. Finally, mechanical replacement valves are structurally rigid, which limits their usefulness in pediatric patients who will be growing rapidly.

Tissue-based bioprosthetic valves are typically sourced from porcine valves or bovine pericardium [189]. These tissues are decellularized to reduce immunogenicity by a variety of methods and crosslinked, typically with glutaraldehyde [189], [190]. This process leaves behind a natural biomaterial composed of ECM, which can be implanted either mounted on a stent or sutured in place. Cadaveric valves (termed allograft or homograft valves) are less frequently used due to lack of source material. Bioprosthetic valve more closely recapitulate native valve hemodynamic profiles, and their low thrombogenicity avoids the need for anticoagulants. However, the decellularization and fixation processes required to obtain the valve structures are known to alter the mechanical properties of the remaining tissue, leading to unexpected cellular responses post-implantation. Cells may remodel and calcify the replacement valve in a process similar to CAVD, or the lack of native VICs and VECs to sustain the mechanical integrity of the valve may lead to degradation. Indeed, mechanical failure of bioprosthetic valves is a fact that is taken for granted. Lifespans on these valves range from 10-20 years, and many of those who receive these valves will require re-operation. In the case of elderly recipients, the need for re-operation comes at an especially unwelcome stage of life. Finally, as with mechanical valves, bioprostheses do not grow with pediatric recipients. Their relative flexibility may enable longer use before the need for replacement, but the higher hemodynamic activity and surrounding tissue growth is thought to accelerate degradation, such that bioprosthetic valves are typically not recommended for pediatric recipients.

Traditionally, replacement valves were implanted via open heart surgery. This traumatic procedure has extremely high morbidity, particularly in the very young and very old. Transcatheter implantation of aortic valves has recently been pioneered and is being utilized more frequently as a means to lower surgical morbidity. While secure placement of the valve using a catheter has been demonstrated, concerns over the durability and control of this implantation method linger. The Ross procedure is a relatively new surgical technique involving

the transplantation of the pulmonary valve into the aortic valve site, and the replacement of the pulmonary valve with a bioprosthetic valve. While this procedure has advantages for the aortic valve, replacement of the pulmonary valve with non-native tissue incurs many of the same problems previously discussed.

3.6 Tissue engineered heart valves

Tissue engineered heart valves have been increasingly looked to as a potential solution for the many issues which arise with other valve replacement strategies. In principle, a successful TEHV would combine the hemodynamic advantages of a tissue-based prosthesis with the long-term durability of a mechanical valve while also enabling the potential for active growth and remodeling which would be of great advantage to pediatric recipients. In practice, development of a successful TEHV would require the perfect combination of material (either synthetic or biological) and cellular resident, the candidates for which are discussed below.

3.6.1 Materials used for TEHVs

Many synthetic materials have been proposed and tested for use in TEHVs. Typically, polymer-based biomaterials have been leading candidates, as they offer a high degree of similarity to the properties of the native valve. Polymers also have the advantage of highly tunable material properties, chemical properties, and degradation characteristics [191]. Synthetic materials also do not require animal or human sourcing and can be generated at will to any size desired. While structural integrity of the implanted valve is obviously crucial from the first moment of implantation, so too is the means by which this structure will be changed and remodeled by the cellular residents. Most synthetic materials used for tissue engineering are designed to be bioresorbable, so that the recipient's body can replace the implant with native tissue [189]. To achieve this, materials must be properly functionalized for cell adhesion and invasion. Ultimately, many synthetics have been shown to fail in this respect, either by not

replacing the resorbing implant fast enough, leading to failure, or by not resorbing quickly enough, resulting in fibrotic response and restenosis.

Many approaches to construction of a TEHV also rely on biological materials, either from a xenogenic source or constructed from purified ECM components. As with bioprosthetic implants, decellularization is a key requirement for use of xenogenic materials. Detergents used in this process may alter the structural properties of the valve tissue, and fixation is often cytotoxic to cells seeded onto the valve, though methods have been developed to address this problem [192], [193]. Allograft valves do not require fixation but are in limited supply. Other approaches utilize purified ECM components to build a valve construct from the ground up. Collagen, fibrin, fibronectin and various GAGs have all been used with varying degrees of success. One of the more promising aspects of this approach, however, is the ability to utilize many ECM components in a similar patterning to the native valve. This capability has been demonstrated using 3D bioprinting, though has not been tested *in vivo* or even in a bioreactor setting [194], [195]. Significantly, these ground up approaches also enable the encapsulation of cells, another crucial component of a successful TEHV.

3.6.2 Cell sources for TEHVs

In attempting to recreate the native valve in a TEHV setting, the final goal would be to have a resident cell population similar to VICs. Given the complexity of VIC phenotypes, this is an obviously complex task. Many cell sources for TEHVs have been investigated and used in research settings, though none fully recapilulate the active homeostatic maintenance that VICs are capable of. Mesenchymal stem cells (**MSCs**) are a popular choice, as they share a mesenchymal lineage with VICs and are capable of differentiation into cells of various phenotypes similar to VICs [196]. Unfortunately, this phenotypic instability is thought to also enable pathogenic differentiation of MSCs, particularly when implanted into an environment known to be prone to calcification [197]. Endothelial progenitor cells are also an attractive

candidate, given their differentiation capacity and availability from peripheral blood. In particular, endothelial progenitors can become endothelial cells, potentially replicating a VEC phenotype, and can undergo EMT to acquire a mesenchymal phenotype like VICs [198]–[200]. Additional native cells have also been considered, including VICs and VECs themselves [201]. However, procurement of these cells would be extremely limited, and collectable sources would likely be from diseased valves in the first place. Other mature lineages such as vascular smooth muscle cells, endothelial cells, and fibroblasts have been used, but these cell types are generally not suited for the high mechanical stresses of the aortic valve [197].

Table 3-1 Design considerations for TEHVs

		Advantages	Disadvantages
Biomaterial	*Synthetic*	• Tunable mechanical / chemical / degradation characteristics • High consistency • On-demand accessibility • Material approval from FDA already exists	• Non-native materials • Tough to pre-seed cells • Fewer animal trials/less clinical data
	Xenogenic tissue	• Existing tissue architecture and mechanical properties • Natural ECM enables cell-based remodeling • Similarities to existing replacement strategies accelerate regulatory approval	• Decellularization may change material properties • Fixation is cytotoxic • Less accessible
	Soluble ECM	• Encapsulating cells • High degree of spatial control • Customizable geometry	• Difficulty achieving high mechanical integrity • Untested *in vitro*
Cell Source	*Mesenchymal stem cells*	• Similar to VICs • Phenotypic flexibility allows for adaptation to valve environment • Readily remodels ECM	• Propensity to differentiate to osteoblastic lineage • Bone marrow derived cells are tougher to acquire
	Endothelial progenitor cells	• Able to generate VEC lining on valve exterior • Readily accessible from peripheral blood	• Encapsulating cells • High degree of spatial control • Customizable geometry
	Native cell populations	• Defined phenotype and function • VEC-mediated suppression of VIC calcification	• Low availability • Cells from cadaveric or donor sources may have pre-existing disease symptoms

3.7 iPSC derived endothelial cells as a potential cell source for TEHVs

The use of patient-derived iPSCs driven down a similar developmental pathway as VICs is one potential method for generating a suitable cell line for populating a TEHV. Human iPSCs

were first generated roughly a decade ago by reprogramming fully differentiated fibroblasts via introduction of four factors [202]. Since this Nobel Prize winning discovery, iPSCs have been heralded as a means to create any tissue type from an inexhaustible supply of donor cells. Their pluripotency enables them to be differentiated to any cell type (excluding extra-embryonic cells), and generating patient-specific iPSCs from donor cells such as dermal fibroblasts is a relatively straightforward process that ensures genetic similarity to the recipient, thereby lowering the risk of immune rejection following transplant [203]. Differentiation protocols have been established for fibroblasts, vascular smooth muscle cells and cardiomyocytes. Perhaps more relevant to VIC development, however, are the various methods for generating endothelial cells. The endocardial origin and EMT process which gives rise to VICs may be fundamental to the heterogeneous phenotype of VICs and are certainly crucial components of using *in vitro* cell culture to replicate embryonic development of this cell line.

Current strategies to create a novel differentiation scheme focus on cytokine and small molecule inhibitor treatments to drive differentiation, and many existing principles could certainly be applied to the development of a VEC-to-VIC differentiation strategy. Several protocols have outlined methods for the generation of vascular endothelial cells, a lineage similar to endocardial cells [204], [205]. These methods generally use a stage of mesoderm induction via Wnt activation followed by endothelial specification through VEGF treatment and purification of the resultant endothelial cells. Optimizing existing protocols for the development of endocardial specific cells could be done via the addition of growth factors known to be secreted from the myocardium, which develops in close proximity to the endocardium. In practice, creating a novel differentiation strategy by modulating the soluble signaling environment is the most straightforward approach, but this alone may not be sufficient to specify a VEC and subsequent VIC lineage [206].

Mechanical cues are known regulators of stem cell fate, and have been shown to play a role in differentiation to different lineages [159], [207]. Given the dynamic mechanical environment of the developing heart valves, it is entirely plausible that mechanical stimulation may be a requirement for accurate differentiation of endocardial cells. Indeed, later stages of valve development have been shown to be dependent on mechanical forces, including EMT and ECM remodeling as the endocardial cushions are formed into the mature valve structures [15], [208], [209]. Indeed, the sole report of iPSC-derived endocardial cells mention the lack of mechanical stimulus as a major shortcoming of the work [210].

Chapter 4

Cyclic strain promotes H19 expression and vascular tube formation in iPSC-derived endothelial cells

Text for Chapter 4 was adapted from **Vander Roest MJ**, and Merryman WD, Cyclic strain promotes *H19* expression and vascular tube formation in iPSC-derived endothelial cells. *Submitted*

4.1 Abstract

Introduction: IPSC-derived endothelial cells have the potential for therapeutic application in several cardiovascular diseases. Mechanical strain is known to regulate both endothelial cell behavior and stem cell differentiation and may play a role in directing endothelial differentiation of iPSCs. *H19*, a lncRNA, is known to affect endothelial cells in several mechanically relevant pathologies and may play a role in this process as well. Therefore, we investigated expression changes of *H19* resulting from mechanical stimulation during iPSC differentiation, as well as functional effects on endothelial tube formation.

Methods: iPSCs were subjected to 5% cyclic mechanical strain during endothelial differentiation. Reverse transcription PCR (**RT-PCR**) and flow cytometry were used to assess changes in mesoderm differentiation and gene expression in the final endothelial cells as a result of strain. Functional outcomes of mechanically differentiated cells were assessed with a tube formation assay and changes in *H19*. *H19* was also overexpressed in human umbilical vein endothelial cells (**HUVECs**) to assess its role in non-*H19*-expressing cells.

Results: Mechanical strain promoted mesoderm differentiation, marked by increased expression of brachyury 24 hours after initiation of differentiation. Strain also increased expression of *H19*, *CD31*, VE-cadherin, and *VEGFR2* in differentiated endothelial cells. Strain-differentiated endothelial cells formed tube networks with higher junction and endpoint density than statically-differentiated cells. Overexpression of *H19* in HUVECs resulted in similar patterns of tube formation.

Conclusions: H19 expression is increased by mechanical strain and promotes tube branching in iPSC-derived endothelial cells.

4.2 Introduction

Endothelial cells line the interior of the circulatory system and are critically involved in a variety of cardiovascular functions and diseases. During healthy function, the endothelium regulates nutrient and oxygen exchange, mediates immune cell infiltration from the circulatory system to target tissues, maintains vascular tone, and is crucial for the formation of new blood vessels [211]. The endothelium is also directly involved in numerous cardiovascular diseases, such as atherosclerosis, coronary artery disease, or valvular heart diseases, and induced pluripotent stem cell (iPSC)-derived endothelial cells are increasingly being considered for cell therapy approaches to replace the dysfunctional endothelium. Therefore, therapeutic success with iPSC-derived endothelial cells will require a detailed understanding of the factors that influence their differentiation and the functional impacts on the final cells.

Several methods to generate iPSC-derived endothelial cells have been reported [204], [205], [212], [213]. Protocols vary, but the progression from pluripotency through a mesodermal lineage with subsequent endothelial specification and purification is quite consistent. Several studies demonstrate methods to generate subtype-specific endothelial cells, including arterial and venous endothelial cells, blood brain barrier endothelial cells, and endocardial cells. These approaches have largely relied on alterations in the makeup, concentration, and timing of the

delivery of soluble factors which drive the differentiation process. While soluble signaling will likely remain a key component of iPSC differentiation, additional factors such as the mechanical environment have also been shown to affect stem cell differentiation and should be better understood for a complete picture of endothelial differentiation.

Passive mechanical cues such as cell substrate stiffness, stiffness gradient, and cellular shape confinement have been shown to direct stem cell fate towards different lineages [159], [207]. Active mechanical stimulation, including cyclic strain, fluid shear, or applied torque have also been investigated as regulators of stem cell differentiation, with mixed results [214]–[216]. In the context of endothelial development, fluid shear stress has been the major focus of *in vivo* developmental studies, as well as most *in vitro* work, though strain is another major mechanical signal imparted to the developing endothelium [217], [218]. In this study, we investigate the role of cyclic mechanical strain, such as would be imparted by cardiac contraction, on the endothelial differentiation of iPSCs.

Several genes known to regulate endothelial development such as NOTCH1 and members of the Wnt family are reexpressed in states of cardiovascular disease, particularly pathologies characterized by extensive remodeling of the ECM and alterations to the mechanical environment such as atherosclerosis and calcific aortic valve disease [219]–[224]. Recently, lncRNA *H19* has been implicated as a potential driver of these diseases [115], [176], [225]. *H19* is a developmentally expressed lncRNA that is imprinted after birth, with very low expression in most mature tissues [226], [227]. *H19*'s reemergence in disease states suggests a similarity to other developmentally active genes, prompting our interest in its activity during endothelial cell differentiation. Furthermore, it's involvement in mechanically altered disease states raises questions about its ability to react to mechanical stimulation.

The aim of this study was to investigate the role of mechanical forces on endothelial differentiation of iPSCs, specifically focusing on *H19* expression and downstream effects. The

effects of mechanical strain were examined in the context of mesoderm differentiation of iPSCs as well as the expression profile and functional changes to the resultant endothelial cells. Functional effects of alterations in *H19* expression were assessed using an *H19* overexpression model in HUVECs, in which *H19* was normally minimally expressed.

4.3 Methods

Cell culture

Human iPSCs (line DF19-9-11T, WiCell) were maintained on six-well plates coated with growth factor reduced Matrigel (Corning) in mTeSR media (StemCell Technologies) changed daily and passaged with ReLeSR passaging reagent (StemCell Technologies) according to manufacturer's instructions. All experiments were performed with cells between passage 30 and 50. HUVECs (ATCC) were maintained on gelatin coated dishes in EGM-2 (Lonza) and passaged with Trypsin at confluence. All HUVEC experiments were performed with cells between passage six and ten.

Endothelial cell differentiation

EC differentiations were carried out as described by Patsch et al., with minor modifications to allow for cell adhesion and differentiation under strain conditions [204]. A schematic representation of the differentiation protocol (Fig 4.1 A) and representative phase contrast images at significant timepoints are shown in Figure 4.1. Prior to differentiation, untreated BioFlex plates (Flexcell International Corp.) were coated with 10μg/cm^2 growth factor reduced Matrigel diluted in DMEM/F12 (Gibco) and incubated at 37°C for a minimum of four hours. iPSCs were allowed to reach 90% confluency and passaged with ReLeSR onto the coated BioFlex plates at a 1:10 split ratio in mTeSR with 10μM ROCK inhibitor Y-27632 (Tocris Biosciences). Cell clump size during passaging was kept at 10-30 cells per clump to ensure cell

adhesion and survival during differentiation (Fig 4.1 B). After 24 hours, media was changed to N2B27 mesoderm induction media, consisting of a 50:50 mixture of DMEM/F12 and neurobasal media (Gibco) supplemented with N2 (Gibco), B27 (Gibco), 7μM CHIR99021 (Tocris) and 25 ng/mL BMP4 (Peprotech). At this point, cells undergoing strain were subjected to 5% equibiaxial strain on the FT-4000 tension system (Flexcell International Corp.) for the duration of the differentiation while statically-differentiated cells were differentiated on unstrained BioFlex plates. After 72 hours without media change, media was replaced with endothelial induction media, consisting of StemPro-34 SFM medium (Gibco) with 200ng/mL VEGF (Genscript) and 2μM Forskolin (Tocris), changed daily. Cells immediately after beginning endothelial induction are shown in Figure 4.1C. After 48 hours of endothelial differentiation, cells were detached with Accutase (Innovative Cell Technologies) and enriched for endothelial cells using magnetic cell separation for VE-cadherin-positive cells, following manufacturer's instructions (Miltenyi Biotec). VE-cadherin-positive cells were either used immediately for RT-PCR or plated in StemPro-34 supplemented with 50ng/mL VEGF onto dishes coated with human fibronectin (Corning) for later use in Matrigel tube assays (Fig 4.1 D).

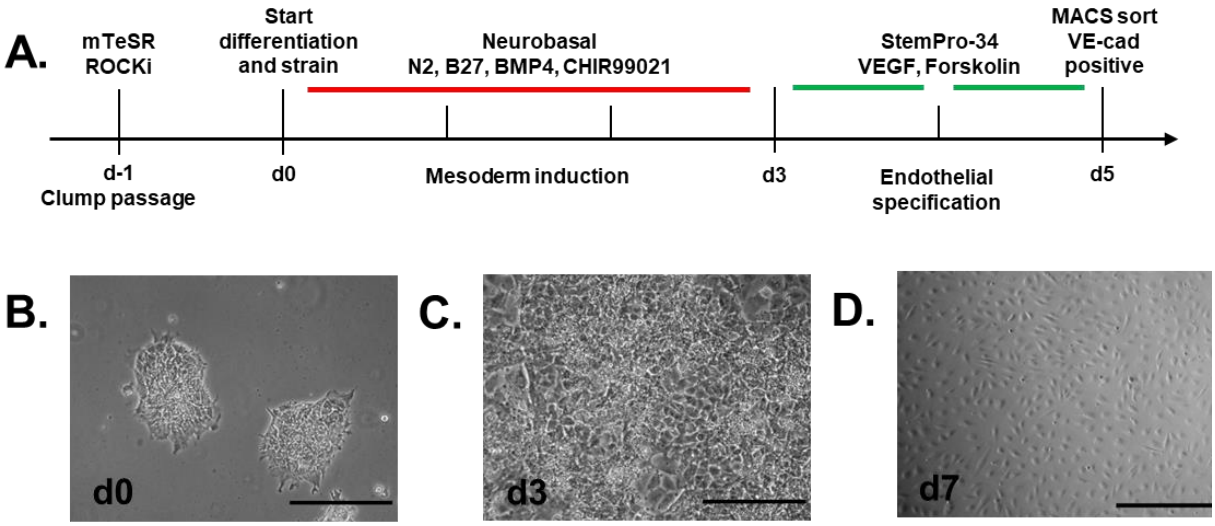

Figure 4.1 Differentiation timeline and cell morphology
iPSCs were differentiated to endothelial cells according to the timeline in A. Cells were passaged as clumps to promote adhesion to the flexible substrate (B). After three days of mesoderm induction, cells formed a confluent monolayer. Following two days of endothelial specification and magnetic sorting for VE-cadherin positive cells, purified endothelial cells formed traditional endothelial monolayers (D).

RT-PCR

Total RNA was purified from pelleted cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. For HUVEC transfection experiments, RNA was purified from the Trizol using a Direct-zol RNA MiniPrep kit (Zymo Research) to allow for DNAse treatment to ensure removal of remaining plasmid DNA. Equivalent amounts of RNA were reverse transcribed using the Superscript IV First-Strand Synthesis System (Life Technologies) and the supplied oligo(dT) primers. RT-PCR was performed with equal amounts of cDNA using iQ SYBR Green Supermix (Bio-Rad) and gene specific primers listed in Table 4.1. Gene expression was normalized to GAPDH.

Table 4-1 RT-PCR primer sequences

Gene Name	Forward Primer*	Reverse Primer*
Gapdh	CAGCCTCAAGATCATCAGCA	ATCCACAGTCTTCTGGGTGG
H19	ACACAAAACCCTCTAGCTTGG	GTCTTTGATGTTGGGCTGATG
CD31	TCTGATTGGCTAACTGAACCC	CAGACACCATTCCAAAACCAG
VE-cad	CAGATCTCCGCAATAGACAAGG	TATGCTCCCGGTCAAACTG
VEGFR2	TCTTTTGGTGTTTTGCTGTGG	TGGTCTGGTACATTTCTGGTG

*Primer sequences are 5' to 3'

Immunostaining

Undifferentiated iPSCs were plated onto Matrigel coated coverslips and differentiated following the previously described protocol. Low adhesion to the coverslips resulted in smaller colonies, though cells were found to be morphologically similar to differentiations performed on FlexCell plates. Likewise, fully differentiated iPSC-derived endothelial cells were plated onto fibronectin coated coverslips for immunostaining. At the indicated differentiation timepoint or when purified endothelial cells reached confluency, cells were fixed and permeabilized with 4% paraformaldehyde and 0.1% vol/vol Triton in a solution of 5% bovine serum albumin (BSA) in PBS. Cells were washed with BSA buffer and incubated in primary antibody (Cell Signaling Technology, #81694, clone D2Z3J) in 1% BSA solution for 4 hours, then washed twice and incubated in secondary antibody dilutions for 1 hour. Cells were then washed twice in BSA buffer and mounted onto slides with Prolong Gold with DAPI (Invitrogen P36931).

Flow Cytometry

Cells were dissociated with Accutase and collected in FACS buffer consisting of 3% fetal bovine serum in DPBS. Cells were fixed in 2% formaldehyde in PBS for 30 minutes at room temperature, then pelleted and permeabilized in 0.1% Triton-X for 15 minutes at room temperature. Primary antibody against brachyury (Cell Signaling Technology, #81694, clone

D2Z3J) and fluorescently labeled secondary antibody (ThermoFisher, A21245, Lot 1837984) were then used to label the cells, which were then analyzed on a 3-laser LSR-II analyzer.

Tube Formation Assay

Growth factor reduced Matrigel was thawed on ice, and 125μL per well was added to a 48-well plate and allowed to gel for 30 minutes at 37°C. Endothelial cells (either transfected HUVECs or iPSC-derived cells) were dissociated with Accutase and seeded on top of the gels at 40,000 cells per well in 200μL of the respective growth media. After four or eight hours (for HUVECs and iPS-derived endothelial cells respectively), media was replaced with 200μL of media containing 2μM Calcein-AM (ThermoFisher Scientific) for 20 minutes. Gels were then fluorescently imaged using a 488nm excitation wavelength. Full frame images were cropped for regions in focus and analyzed using AngioTool [228]. All metrics were normalized to image area. Data came from 3-4 images per gel of 2 gels seeded from 4 independent differentiations or transfections.

Micropipette aspiration

Micropipette aspiration was used to measure the elastic modulus of individual cells using previously reported methods[160], [229]–[231]. Capillary tubes (Fisher) were coated with Sigmacote (Sigma), rinsed and dried. Coated tubes were then pulled into micropipettes with an internal orifice diameter of approximately 6 μm (Fig 4.2A). Mechanical analysis was performed using a custom-built pressure regulator system with Fluigent controller and a secondary fluid reservoir built around an inverted microscope. Differentiated iPSC-derived endothelial cells were collected with Accutase at the indicated timepoint, rinsed, resuspended in complete media, and kept on ice until testing within 2 hours of lifting cells. Resuspended cell solutions were placed in a 35mm dish under the microscope and analyzed with the micropipette aspiration system.

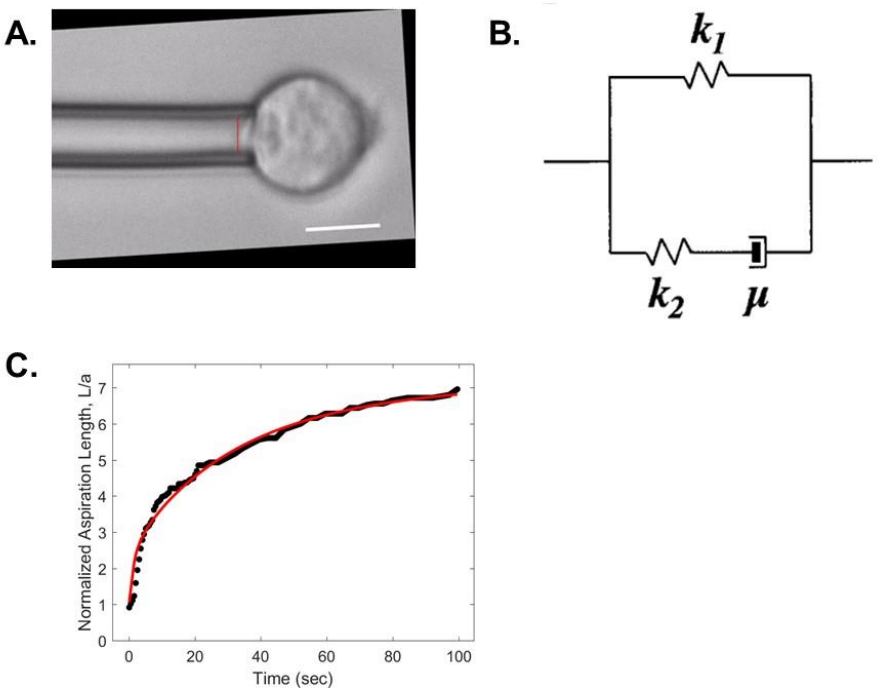

Figure 4.2 Micropipette aspiration

Cells were anchored to the tip of the micropipette using a low negative pressure (~50Pa). After solid attachment, a step pressure of 400Pa was applied to the cells while collecting continuous video. A custom MatLAB analysis script was used to register all images of the video and detect the leading edge of the cell inside of the micropipette, as well as to measure pipette and cellular diameters. These measurements were fit to a standard linear solid viscoelastic mechanical model as shown in Figure 4.2B. Elastic parameters (k1 and k2) and apparent viscosity (μ) were calculated from the "rigid punch" model for the displacement of the cell's leading edge as a function of the time

$$L(t) = \frac{\varphi a \Delta P}{\pi k_1}\left[1 - \frac{k_2}{k_1 + k_2} e^{-t/\tau}\right]$$

where L is the displacement of the cell leading edge, a is the inner diameter of the micropipette, ΔP is the applied pressure, t is time, and τ is a time constant (Fig 4.2C). Apparent viscosity (μ), instantaneous elastic modulus and steady-state elastic modulus of the model were determined as

$$\mu = \frac{\tau k_1 k_2}{k_1 + k_2}$$

$$E_0 = \frac{3}{2}(k_1 + k_2)$$

and

$$E_\infty = \frac{3}{2}k_1$$

respectively.

Statistics

All data is presented as mean ± standard error. Statistical significance of RT-PCR fold change was assessed with a one-sided t-test on normalized Ct values, and statistical significance of tube assay formation metrics was assessed with one-way ANOVA with Tukey's post-hoc test. In the event that data was not normally distributed, significance was assessed using ANOVA on ranks. $P<0.05$ was considered to be significant.

4.4 Results

4.4.1 EC differentiation of iPSCs is possible on FlexCell plates with cyclic strain

Using minor modifications to a previously published endothelial differentiation protocol, we were able to differentiate iPSCs to a vascular endothelial lineage. To allow for more robust adhesion to the FlexCell plates and continued attachment during application of cyclic strain, plates were incubated in Matrigel coating media overnight and iPSCs were seeded as clumps of 10-30 cells rather than single cells. At the start of mesoderm induction, cells were also subjected to 5% cyclic, equibiaxial strain at a 1Hz duty cycle. After 72 hours, a dense monolayer similar to previously reported protocols was obtained, and media was switched to VEGF-

containing endothelial induction media, changed daily. Endothelial cells were enriched by (magnetically assisted cell sorting) **MACS** sorting for vascular endothelial VE-cadherin, and the resulting cell populations formed typical endothelial monolayers and stained positively for CD31 and VE-cadherin (Fig 4.3A-D).

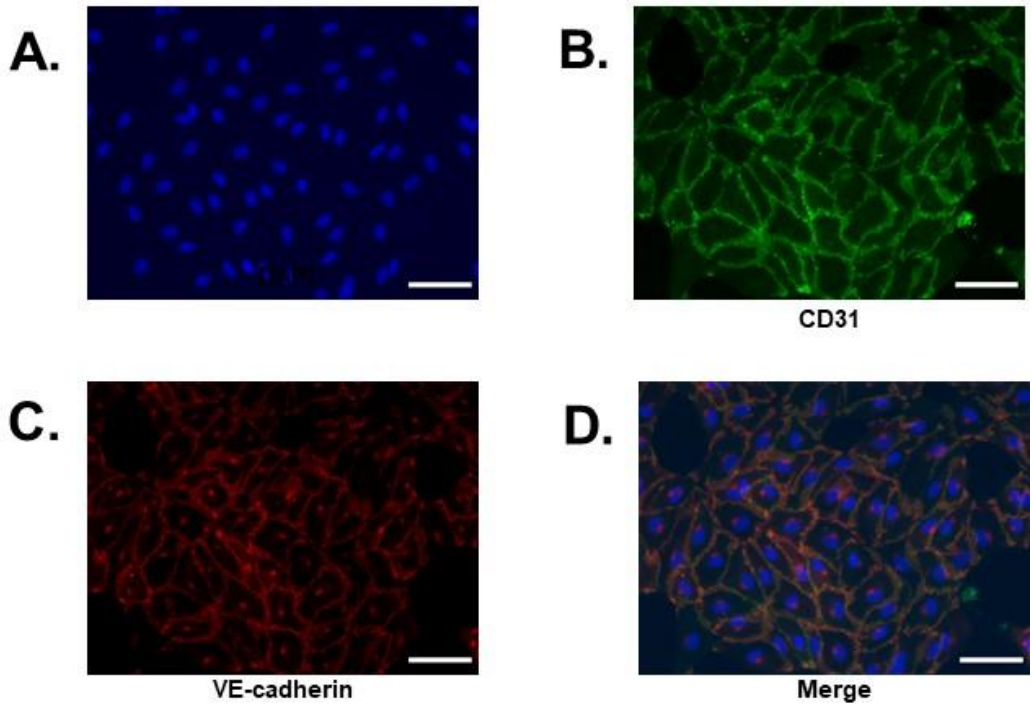

Figure 4.3 – Fully differentiated endothelial immunostaining
Following VE-cadherin magnetic purification and 2 days of growth on fibronectin coated coverslips to reach confluency, endothelial cells were stained for DAPI (A), CD31 (B), and VE-cadherin (C). Co-localization of CD31 and VE-cadherin on the periphery of the cell shows classic endothelial phenotype and confirms the identity of the purified cells.

4.4.2 Strain accelerates mesoderm induction and H19 expression

Knowing that mechanical forces are capable of affecting stem cell differentiation, we also probed the effects on mesoderm differentiation and found an accelerated shift from pluripotency to a mesoderm lineage, marked by elevated transcription of brachyury at 24h with strain compared to statically-differentiated cells (Fig 4.4A, 24h $p=0.047$). In both conditions, brachyury transcription peaked between 24 and 36 hours before a strong decrease prior to endothelial induction. This finding was investigated at the translational level using immunostaining. Image

quality of adherent cells on FlexCell plates was poor, but a rough timeline of brachyury translation was obtained using iPSCs differentiated on glass coverslips. Fluorescent intensity peaked between 24 and 48 hours, matching transcriptional data (Fig 4.4B). Based on these results, we investigated expression of brachyury using flow cytometry of cells 24h after starting differentiation. At this timepoint, we hoped to capture a difference between the two conditions either in fraction of cells expressing brachyury or in expression level per cell. We found minimal change in percentage of brachyury-positive cells, though a robust increase in median fluorescence intensity in the strain-differentiated cells compared with the statically-differentiated, suggesting higher levels of brachyury translation per cell (Fig 4.4C,D).

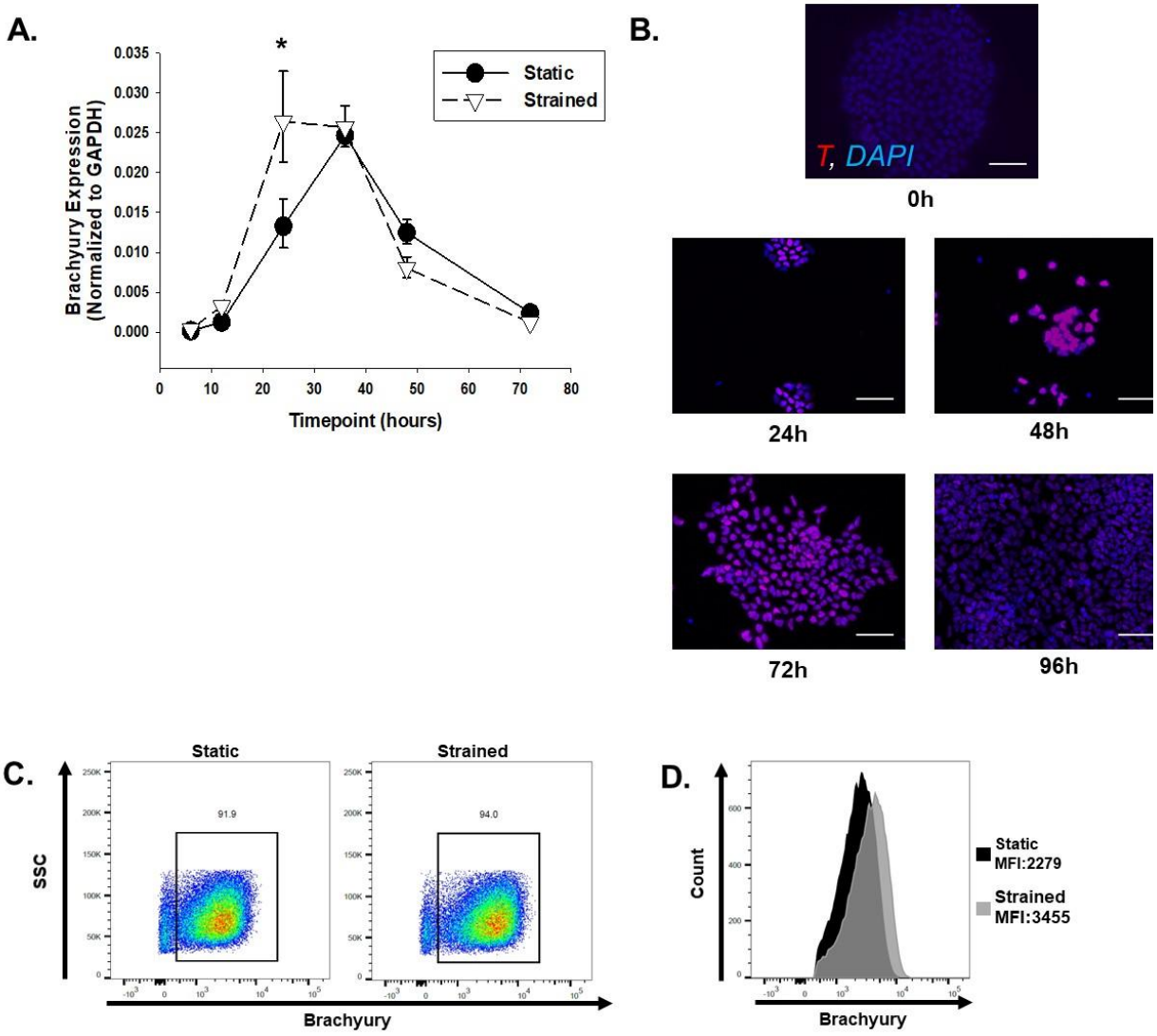

Figure 4.4 Accelerated mesoderm differentiation with strain

Strain accelerated mesoderm differentiation, marked by increased expression of brachyury at 24h after initiation of differentiation (A, p=0.047). Translation of brachyury in differentiations conducted on glass coverslips to allow for staining showed similar trends as transcriptional data (B). Flow cytometry showed a minimal increase in fraction of brachyury positive cells (C) and modest increase in fluorescence intensity in strained cells (D). SSC – side scatter; * = p<0.05 by ANOVA

Mesoderm differentiation and loss of pluripotency was further confirmed by a loss of *OCT4* expression over the duration of mesoderm specification (Fig 4.5A). Interestingly, *H19* expression was also found to be quite low during initial stages of mesoderm differentiation, but increased dramatically by 48 and 72 hours (Fig 4.5B).

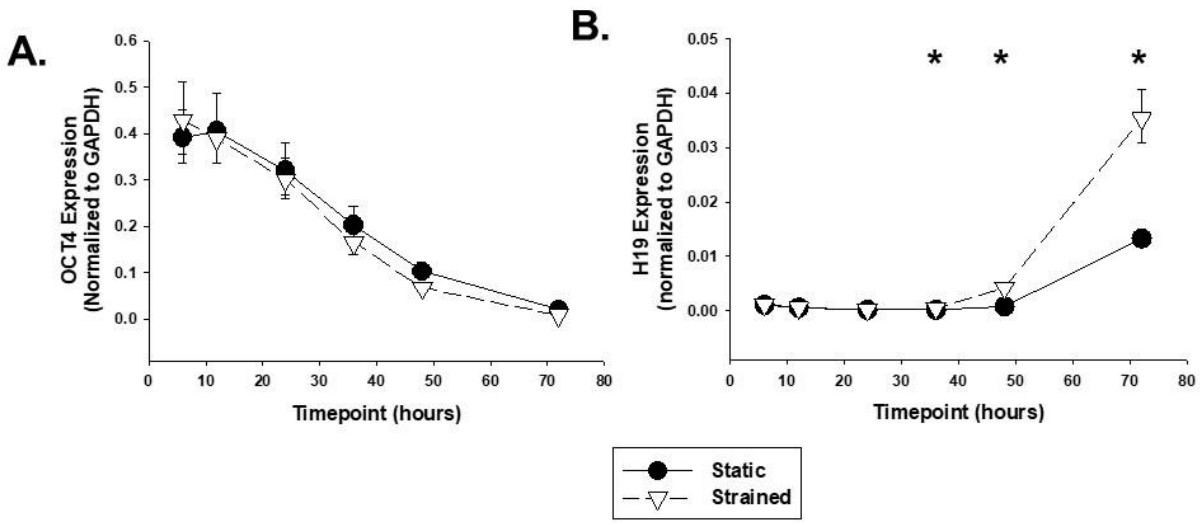

Figure 4.5 Strain induces increased *H19* expression during mesoderm differentiation Both strained and unstrained differentiations showed robust loss of OCT4 expression, indicating loss of pluripotency and commitment to a mesoderm lineage (A). Additionally, expression of *H19* was significantly increased in the strained differentiations by 36h (p=0.014), and persisted to the end of the mesoderm induction stage of the differentiation (B, p=0.014 at 36h, p=0.008 at 48h, p=0.002 at 72h). * = p<0.05 by ANOVA

4.4.3 Strain induces transcriptional increase in H19 in iPS-derived endothelial cells

After VE-cadherin enrichment, the resultant cells were assessed for transcriptional differences between strained and unstrained differentiations. As with mesoderm differentiation, cyclic strain induced 2.2-fold higher expression of *H19* in fully differentiated endothelial cells (Fig 4.6A, p=0.012). This was also accompanied by higher transcription of endothelial markers *CD31*, VE-cadherin, and VEGF receptor 2 (*VEGFR2*) (Fig 4.6A, p=0.0006, p=0.0009, p=0.036 respectively).

4.4.4 Strain-differentiated endothelial cells exhibit higher tube formation capacity

Functional outcomes of the increased levels of *H19* and other endothelial markers were tested using a Matrigel tube assay. Strain-differentiated cells were found to compact into tube-like structures more quickly, such that at 4 hours post-seeding, the majority of the cells had formed tubes. Conversely, statically-differentiated cells were slower to form tubes and at 4 hours were seen to remain in monolayer on the surface of the Matrigel in many areas of the well. By 8 hours, both conditions had compacted into more mature tube-like structures (Fig 4.6B). Analysis of tube networks showed that strain-differentiated endothelial cells had higher junction density, resulting in shorter average tube length and higher endpoint density (Fig 4.6C-E).

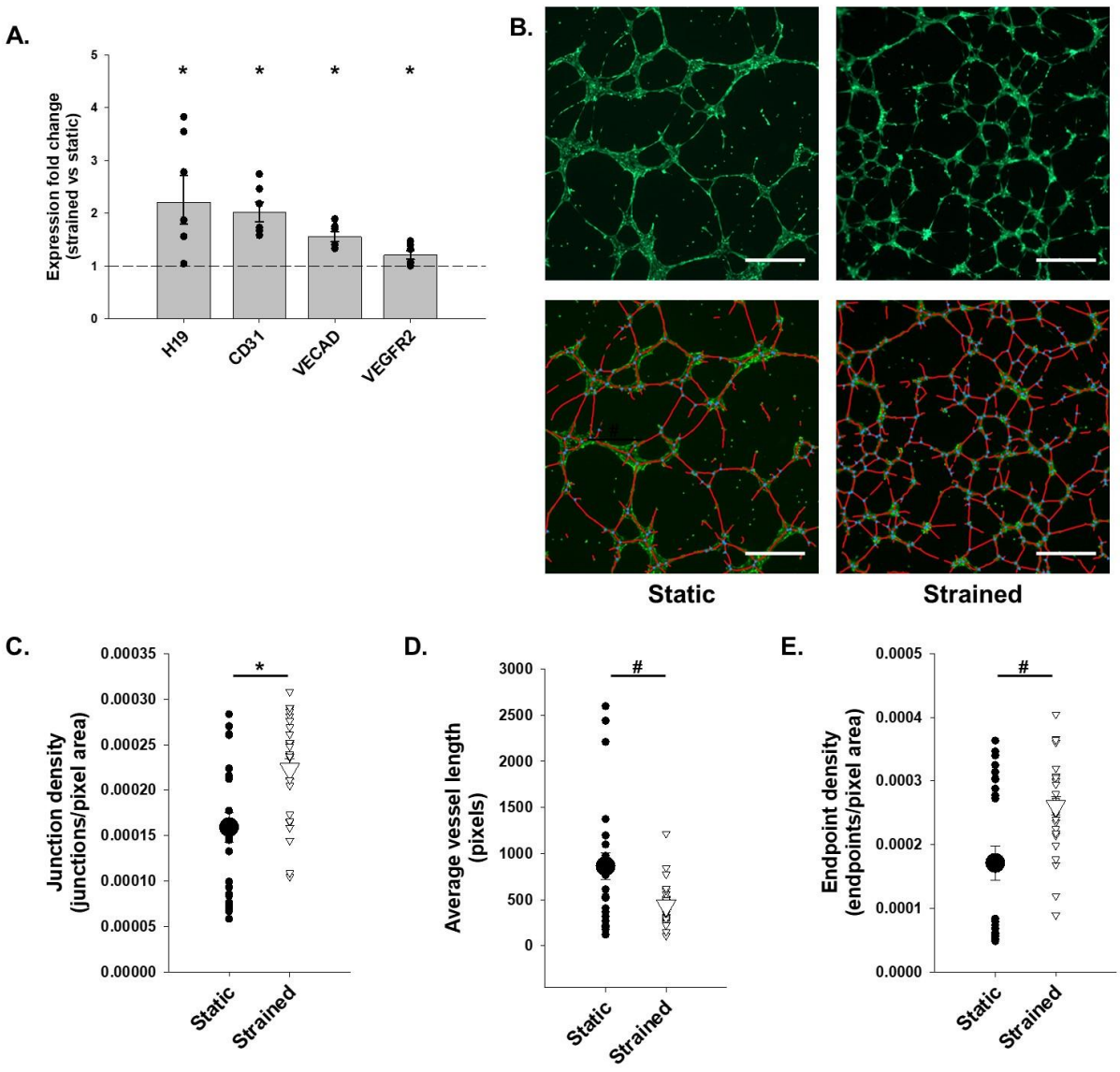

Figure 4.6 Strain-induced transcriptional and functional changes

Strain-differentiated endothelial cells had elevated expression of *H19*, CD31, VE-cadherin, and VEGFR2 compared to their statically differentiated counterparts (A, p=0.0123, p=0.0006, p=0.0009, p=0.0363 respectively). Strain-differentiated and statically-differentiated endothelial cells were used in a Matrigel tube assay and analyzed with AngioTool (B) Top images in B show calcein-AM stained tube networks and lower images show AngioTool analysis, with tubes labeled in red and junctions labeled in blue. Strain-differentiated iPSC-derived endothelial cells formed tube networks with higher junction density, lower average vessel length, and higher endpoint density (C-E, p=0.002, p=0.020, p=0.035 respectively). Data points from 3-4 images per gel of 2 gels seeded from each of 4 independent differentiations. Scale bar = 500 μm; *=p<0.05 by ANOVA; #=p<0.05 by ANOVA on ranks

4.4.5 H19 is a driver of increased tube formation capacity in HUVECs

To assess the role of *H19* in the observed increase in tube formation in strain-differentiated cells, HUVECs were utilized as a model system which does not normally express *H19*. HUVECs were transfected with an *H19* overexpression construct as a proxy for strain-induced *H19* upregulation. Overexpression was confirmed with RT-PCR and at levels comparable to iPSC-derived endothelial cells; *H19* was unexpressed in HUVECs transfected with empty vector control plasmid (Fig 4.7A). Expression of other endothelial markers was not altered by overexpression of *H19* (Fig 4.7A). In a tube formation assay with HUVECs, *H19* overexpression was found to mirror the effects seen in strain-differentiated iPSC-derived cells, namely, increased junction density and endpoint density with shorter average tube length (Fig 4.7B-E).

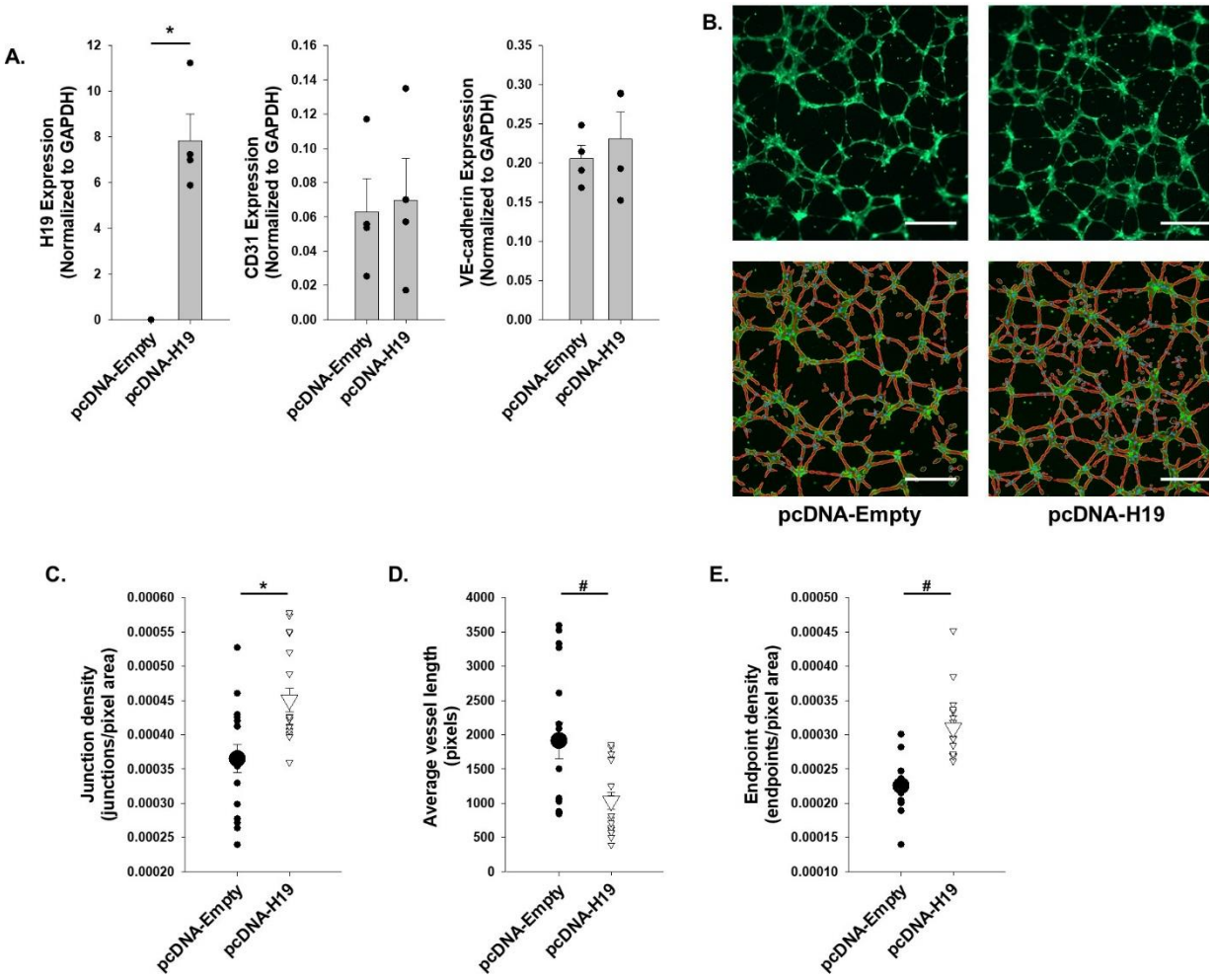

Figure 4.7 *H19* expression in HUVECs replicates strain-differentiation in a tube assay
HUVECs were transfected with either a pcDNA3.1 *H19* overexpression construct or empty pcDNA3.1 as a control. *H19* was found to be highly expressed in the overexpression condition and was not detected in control transfections, while CD31 and VE-cadherin expression were unchanged (A, p<0.001). As with iPSC-derived endothelial cells, *H19* expression led to more compact tube formation (B) with higher junction density, lower average tube length, and higher endpoint density (C-E, p=0.003, p=0.005, p<0.001 respectively). Data points from 3-4 images per gel of 2 gels seeded from each of 4 independent transfections. Scale bar = 500 μm; *=p<0.05 by ANOVA; #=p<0.05 by ANOVA on ranks

4.4.6 Strain differentiated endothelial cells exhibit unique mechanical properties

Measurement of individual cell mechanical properties with micropipette aspiration revealed interesting differences between strain-differentiated and statically-differentiated

endothelial cells. While the populations were indistinguishable at the time of sorting, strain-differentiated endothelial cells trended towards lower stiffnesses at 24 and 48 hours post sort (Fig 4.8). Low sample number and high variability between samples prevented meaningful statistical comparison, but these results indicate potential changes in cell mechanical properties as a result of strain during differentiation.

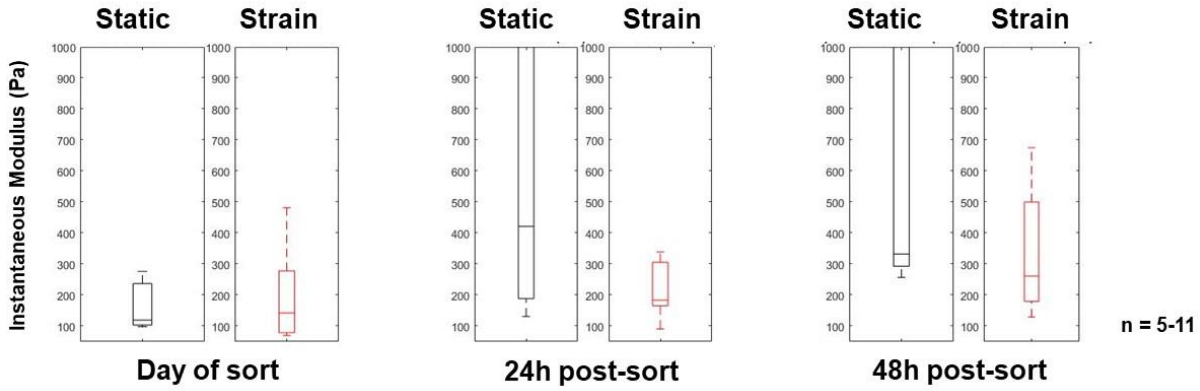

Figure 4.8 Mechanical properties of iPSC-derived endothelial cells
Mechanical properties of differentiated endothelial cells were tested using micropipette aspiration. No differences were found at the time of endothelial purification, but strain-differentiated endothelial cells trended towards lower instantaneous elastic modulus up to 48 hours after sorting.

4.5 Discussion

This study demonstrates a role for active mechanical force in endothelial differentiation of iPSCs and *H19* expression and a functional outcome of increased *H19* in endothelial cells. While other studies have shown crosstalk between mechanical forces and *H19* expression in various disease contexts, this is the first study to investigate this relationship in endothelial differentiation of iPSCs. By applying cyclic, equiaxial strain to differentiating iPSCs, mesoderm differentiation was accelerated, as shown by increased expression of mesoderm marker brachyury. Over the same timeframe, strain led to increased expression of *H19*, which persisted through endothelial differentiation, leading to increased tube formation in a Matrigel tube assay.

We found that mechanical strain induces mesoderm differentiation of iPSCs, shown by accelerated expression of mesoderm marker brachyury. Interestingly, other studies have shown that strain over 10% acts to maintain pluripotency and prevent spontaneous differentiation of

human embryonic stem cells in conditioned (pluripotency maintenance) media [232]. However, lower strain rates and application of unconditioned, differentiation-permitting media did not act to prevent differentiation, suggesting a synergistic effect of both chemical stimuli and mechanics in the induction of differentiation. The present work reinforces this concept and shows that in the context of chemically driven endothelial differentiation, strain actually serves to promote a more differentiated phenotype.

One of the major findings of this work was the impact of strain during differentiation on the ability of iPSC-derived endothelial cells to form tube networks. Mechanical forces have long been known to affect vascular network formation and have been studied developmentally and with a variety of *in vitro* methods. Generally, physiologically relevant mechanical stimuli are thought to promote vasculogenesis, though most studies show this relationship using fully differentiated endothelial cells or developmental models [217], [218]. Strain applied to differentiating embryoid bodies has been shown to promote vasculogenesis, suggesting a role for mechanical stimuli earlier in the developmental process [233]. In particular, strain was shown to promote branching points, a finding which we observed in the present work, with evidence that *H19* may mediate this effect.

H19 is known to be developmentally active, and has recently been implicated in a variety of cardiovascular diseases involving the endothelium [110], [115], [176], [227], [234]. This correlates with a number of other pathways that are active in both development and disease, including the Wnt family, the BMP family and Notch pathway[14], [219]–[224]. Crosstalk between *H19* and these pathways has also been observed, with *H19* repressing *NOTCH1* transcription and promoting BMP2 activity in CAVD disease and activating Wnt/β-Catenin signaling and mediating BMP9 activity in osteoblast differentiation and glioma [225], [235]–[237]. These interactions make *H19* a promising candidate for cardiovascular research and prompted our interest in it for this study.

Intersections between various mechanical stimuli and *H19* expression have been previously shown, including in a pressure overload model of cardiac hypertrophy, a hindlimb unloading model of osteoporosis, and tension induced osteogenesis [238]–[240]. In these studies, strain correlated with expression of *H19*. Similarly, *H19* is known to be upregulated in CAVD, a pathology characterized by increased valvular strain which augments valve calcification [225]. Our work shows a similar upregulation of *H19* expression with increased mechanical strain. While some studies suggest that loss of imprint and upregulation of *H19* expression may be driving certain cardiovascular diseases, our work indicates that alterations in the mechanical environment may precede *H19* dysregulation. In any case, *H19* has been shown to have downstream functional impact in both *in vivo* and *in vitro* models, and a thorough understanding of how the mechanical environment impacts its expression will be necessary for development cell-based or other therapies.

Our results show that along with increasing *H19* expression, mechanical strain during endothelial differentiation also impacts endothelial tube formation, though further work is needed to demonstrate whether this pattern is causal or coincidental. Direct modulation of *H19* expression in iPSCs and iPSC differentiations was attempted, but was not consistently feasible, leading us to utilize HUVECs as a proxy cell model to investigate functional impacts of *H19* expression in endothelial cells. Strain also promoted expression of endothelial markers *CD31* and VE-cadherin, which are known to regulate tube formation, though we did not see similar changes as a result of *H19* expression in HUVECs [241]. One study of glioma-associated endothelial cells showed that *H19* promotes angiogenesis, potentially through suppression of microRNA-29a, though work points to microRNA-181a as a more likely target in microvascular endothelial cells [234], [237]. Despite the possibility of parallel mechanisms through which strain may alter tube formation, it was clear that increased *H19* expression lead to similar tube

formation capacity in HUVECs, suggesting that *H19* is a potential mediator of these changes in iPSC-derived cells.

Mechanical properties of endothelial cells are known to differ from source to source. Cells from different cardiac valves are also known to exhibit different mechanical properties, and this is thought to lead to different responses to stress within the different valves [14], [160]. Here we identified potential differences between endothelial cell mechanical properties as a result of differences in strain while they differentiated. These findings have implications for the differences between endothelial subtypes which develop in different mechanical environments within the circulatory system (microvascular compared with great vessels compared with endocardial cells, etc.).

This study shows that cyclic strain promotes earlier mesoderm differentiation of iPSCs and that endothelial cells differentiated under strain conditions have increased branching in a tube formation assay. *H19* was investigated as a mediator of this effect and was found to have similar functional outcomes in an overexpression model in HUVECs. While further work is needed to clearly identify the downstream effects of *H19* expression in iPSC-derived endothelial cells, it is clear that the mechanical environment impacts endothelial function and expression of *H19*.

Chapter 5

H19 is not hypomethylated or upregulated with age or sex in the aortic valves of mice

Text for Chapter 5 was adapted from **Vander Roest MJ**, Krapp C, Thorvaldsen JL, Bartolomei MS, and Merryman WD. *H19* is not hypomethylated or upregulated with age or sex in the aortic valves of mice. *Physiological Reports*, 7(19) (2019):e14244 [242]

5.1 Abstract

Epigenetic dysregulation of long non-coding RNA *H19* was recently found to be associated with CAVD in humans by repressing *NOTCH1* transcription. This finding offers a possible epigenetic explanation for the abundance of cases of CAVD that are not explained by any clear genetic mutation. In this study, we examined the effect of age and sex on epigenetic dysregulation of *H19* and subsequent aortic stenosis. Cohorts of littermate, wildtype C57BL/6 mice were studied at developmental ages analogous to human middle age through advanced age. Cardiac and aortic valve function were assessed with M-mode echocardiography and pulsed wave Doppler ultrasound, respectively. Bisulfite sequencing was used to determine methylation-based epigenetic regulation of *H19*, and RT-PCR was used to determine changes in gene expression profiles. Male mice were found to have higher peak systolic velocities than females, with several of the oldest mice showing signs of early aortic stenosis. On the other hand, the imprinting control region of *H19* was not hypomethylated with age, and *H19* expression was lower in the aortic valves of older mice than in the youngest group. These

results suggest that age-related upregulation of *H19* is not observed in murine aortic valves and that other factors may initiate *H19*-related CAVD in humans.

5.2 Introduction

CAVD is an increasingly prevalent source of cardiovascular morbidity in the elderly, but identifiable genetic causes only explain a small fraction of disease cases [9], [131], [243]. *NOTCH1* loss of function mutation is one of the most widely studied genetic causes of CAVD, although such a mutation is not found in the majority of disease cases [143], [244]. Despite this discrepancy, alterations in the *NOTCH1* signaling pathway offer a proven mechanism for CAVD, and downstream mechanisms for valve calcification are similar for *Notch1*-driven CAVD in mice and idiopathic CAVD in humans [13], [151], [154]. As a result, upstream signaling or alternative mechanisms to mimic *NOTCH1* loss of function have been sought as an explanation for idiopathic CAVD.

Recently, long non-coding RNA *H19* has been found to be highly upregulated in stenotic and sclerotic human aortic valves [178], [225]. Furthermore, *H19* was shown to competitively bind to the promoter region of *NOTCH1* in aortic VICs, preventing P53 recruitment and subsequent *NOTCH1* transcription (Fig 5.1A). This effectively suppresses *NOTCH1*, leading to calcification even in the absence of a *NOTCH1* mutation. However, this makes the means by which *H19* expression becomes so dramatically upregulated a critical question to understand disease initiation.

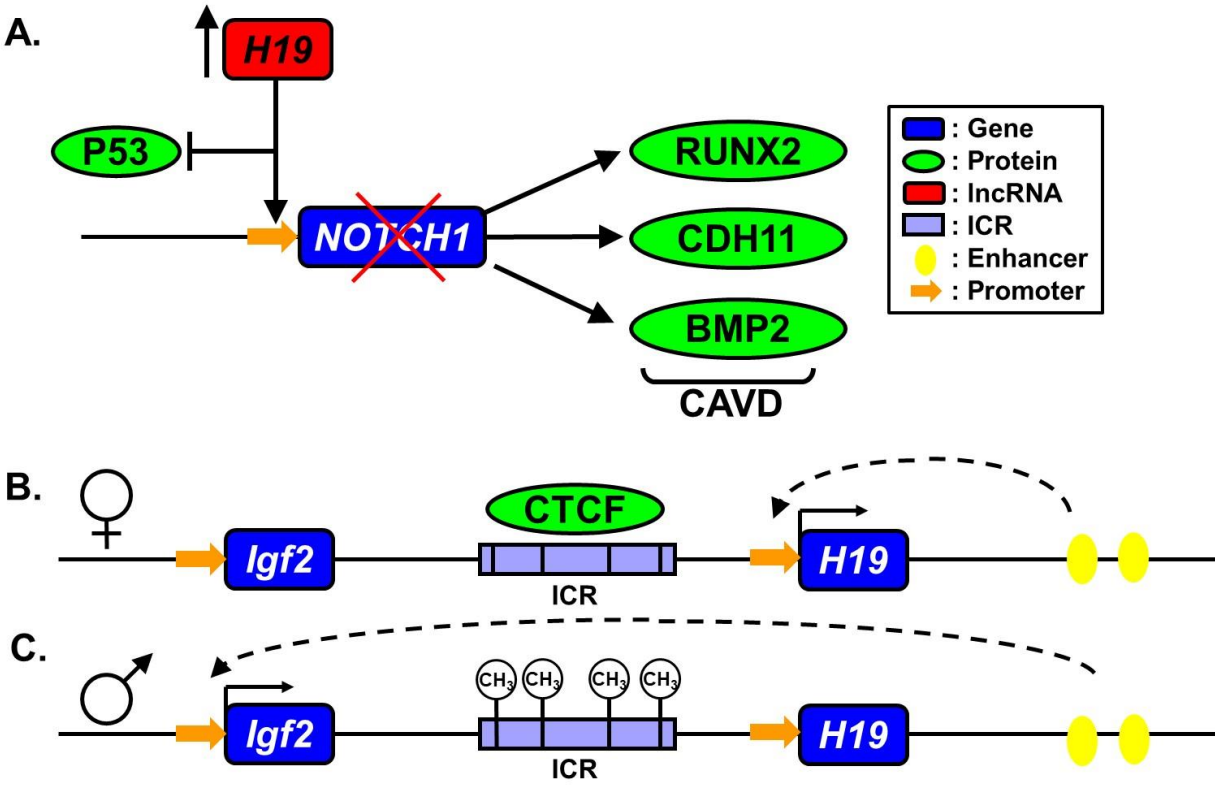

Figure 5.1 *H19* imprinting and effect on CAVD

High levels of *H19* compete with P53 to bind the *NOTCH1* promoter, decreasing expression of *NOTCH1* and mimicking a loss-of-function mutation known to lead to CAVD (A). The *H19*/*IGF2* locus contains a differentially methylated domain in the intergenic space. On the maternally inherited allele (B) CTCF binds to a series of four 21bp repeats, resulting in interaction of downstream enhancers with the *H19* promoter and expression of *H19*. CTCF also acts as an insulator, keeping the downstream enhancers from promoting *IGF2* expression. On the paternal allele (C) methylation of the differentially methylated domain prevents CTCF from binding, enabling enhancer interaction with the *IGF2* promoter. *IGF2* expression is increased, while *H19* expression is almost entirely stopped. Hypomethylation in the ICR can lead to increased *H19* expression.

H19 is highly conserved among mammals and is found in an imprinted locus near *IGF2* [245]. This locus is epigenetically regulated by an ICR, located between the two genes (Fig 5.1B,C) [245]. On the maternally inherited allele, the ICR binds CTCF, which serves as an insulator between *Igf2* and enhancer elements downstream of *H19*. As a result, shared enhancers promote expression of *H19* while *Igf2* is silenced (Fig 5.1B). On the paternally inherited allele, methylation of the ICR prevents CTCF binding, allowing the downstream enhancers to activate *Igf2* expression while *H19* is silenced (Fig 5.1C) [246]. This imprint is

established during embryonic development and persists through the life of the organism, though disruptions in this epigenetic signature could lead to rapid changes in *H19* expression [247].

Hadji et al. showed that hypomethylation in the promoter region of *H19* was associated with increased expression in calcified human aortic valves, even though the imprint was maintained [225]. More recently, Agba et al. showed evidence for age-associated hypomethylation in the *H19*/*Igf2* ICR of rats, suggesting a loss of imprint which correlated with increased *H19* expression [179]. Together, these findings suggest an epigenetic mechanism by which *H19* may become upregulated with advanced age and lead to CAVD via the NOTCH1 pathway, even in the absence of a genetic mutation.

The goal of this study was to determine if these findings were replicated in mouse aortic valves, and if age-related *H19* expression is a mechanism for CAVD in a mouse model. Because *H19* is known to be involved in other cardiovascular diseases, expression levels were also assessed in ventricular tissue and the aortic arch, as well as the liver, which is known to express higher levels of *H19* and was previously shown to exhibit age-related loss of imprint. While many existing mouse models of CAVD consider only male mice, CAVD is also highly prevalent in women, thus motivating our investigation into the effect of sex on *H19*-driven calcification [248], [249]. Because our primary interest was in the effects of age and sex (rather than genetic mutation or chronic injury through diet) on *H19* expression in the aortic valve, these experiments were conducted in healthy C57BL/6 mice maintained on a normal diet.

5.3 Methods

Mice

Groups of five male and female C57BL/6 mice were purchased from Jackson Laboratory at 26, 52, and 78 weeks of age. Mice were assessed via echocardiography and euthanized for sample collection within two weeks of receipt. All procedures were performed in accordance

with protocols approved by Institutional Animal Care and Use Committee at Vanderbilt University.

Echocardiography

Mice were anesthetized with isofluorane, and a Vevo 2100 imaging system was used to acquire parasternal, short axis M-mode images of the heart and pulsed-wave (PW) Doppler images of the aorta immediately distal to the aortic valve. An exemplary PW Doppler scan and corresponding flow profile traces are shown in Figure 5.2 A and B. VevoLAB software was used to analyze M-mode cardiac cycles (~9 per image) and extract PW Doppler images. A custom MATLAB script was used to isolate and average PW Doppler cardiac cycles (~10-30 per image) in order to compute the systolic transvalvular pressure gradient and peak systolic velocity (**PSV**) [250]. Ejection fraction to velocity ratio (**EFVR**), a metric used as an indicator of valve disease, was calculated as EFVR = (ejection fraction)/(4*(PSV)^2) [251].

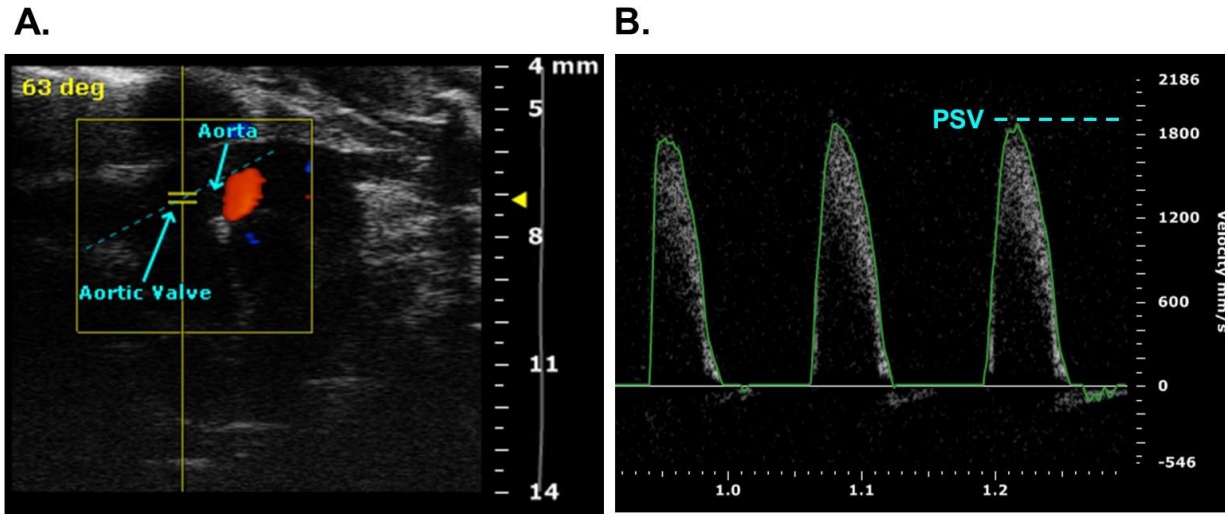

Figure 5.2 Sample Doppler ultrasound scan
Doppler ultrasound scans of aortic flow immediately distal to the aortic valve were collected and analyzed as shown in A. Representative flow profiles of aortic flow is shown in B. Green curves from B were extracted and analyzed with a custom MatLAB script.

Microdissection and sample collection

Mice were euthanized by carbon dioxide inhalation and promptly dissected. The systemic and pulmonary circulatory systems were flushed with sterile PBS, and the aortic valve leaflets – connected to a minimal annulus of aorta – were dissected away from the ventricles. Samples of liver, left ventricle, and ascending aorta were also harvested and cleaned of external connective tissue and fat. Samples were flash frozen in liquid nitrogen and stored at -80°C until RT-PCR analysis.

Nucleic acid purification

Tissue samples were thawed at room temperature and bead homogenized in 400 µL of RLT-plus buffer with Reagent DX to reduce foaming (Qiagen, Hilden, Germany) in Lysing Matrix D tubes (MP Biomedicals, Santa Ana, CA) until no visible tissue remained. RNA and gDNA were purified using the Qiagen AllPrep Micro kit following manufacturer's instructions and were stored at -80°C until further analysis.

Real-time PCR

Reverse transcription was performed using the SuperScript IV Reverse Transcriptase kit with oligo(dT) primer (ThermoFisher Scientific, Waltham, MA). RT-PCR was performed with equal amounts of cDNA using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and gene specific primer sequences (Table 5.1). Gene expression was normalized to the geometric mean expression of *Gapdh*, *Tuba1b*, and *Actb*, which was found to be more stable than any housekeeping gene in isolation. For visual clarity, gene expression was normalized to the highest expressing sample of each gene.

Table 5-1 RT-PCR primer sequences

Gene	Forward Primer*	Reverse Primer*
Gapdh	ATGACAATGAATACGGCT	TCTCTTGCTCAGTGTCCTTG
Tuba1	CCGGTGTCTGCTTCTATC	CCATGTTCCAGGCAGTAGA
Actb	CAAGCAGGAGTACGATG	AACGCAGCTCAGTAACAGT
H19	GGAATGTTGAAGGACTGA	GTAACCGGGATGAATGTCT
Igf2	CGCTTCAGTTTGTCTGTT	GCAGCACTCTTCCACGATG
Notch1	ATGTCAATGTTCGAGGAC	TCACTGTTGCCTGTCTCAAG
Bmp2	TTATCAGGACATGGTTGT	GGGAAATATTAAAGTGTCAG

*Primer sequences are 5' to 3'

Methylation Analysis

Pyrosequencing was performed as previously described with the following modifications. 40 ng of bisulfite treated DNA was used as input, and 5 µL of the biotinylated PCR product was used for each sequencing assay [252].

Statistics

All values are presented as mean ± standard error. Two-way ANOVA with post-hoc Holm-Sidak test for multiple comparisons was used to detect differences between age and sex. In the event that conditions of normality or equal variance were not met, one-way ANOVA on ranks was used to detect differences due to age within each sex, and the Mann-Whitney rank sum test was used to detect difference due to sex at a specific age. Potential correlations between measured variables were assessed by the Pearson product-moment correlation r. For all statistical tests, a value of $P<0.05$ was considered significant.

5.4 Results

5.4.1 Aortic valve health diverges, but doesn't worsen with age

Analysis of PW Doppler images revealed no changes with age in PSV or peak transvalvular pressure gradient, though as whole cohorts by sex, male mice had higher PSV than females (P=0.032) (Fig 5.3A,B). While typical values for aortic PSV in healthy BL6 mice fall below 150 cm/s, we identified one 78-week-old male mouse had a PSV over 200 cm/s, indicative of aortic stenosis, and several older male and female mice had velocities between 150 and 200 cm/s, indicative of early stenosis [180]. Male mice also had higher systolic gradients (P=0.032). None of the mice showed signs of left ventricular hypertrophy, indicating that observed hemodynamic changes were early stage, prior to extensive cardiac remodeling. Curiously, the 52- and 78-week-old male mice had two- and three-times larger variance in PSV than younger males, indicating a wide divergence of overall valve health with increased age.

5.4.2 Cardiac function is preserved with age

Ejection fraction was extremely consistent with age in male mice (Fig 5.3C). Female mice exhibited increasing ejection fraction with age, such that 26-week-old females had lower ejection fraction than 26-week males (P=0.042) and 78-week-old females had higher ejection fractions than 78-week males (P=0.005). 78-week-old females also had higher ejection fractions than 26- and 52-week-old females (P=0.017, P=0.025). No differences were detected in EFVR, though males again showed trends toward divergent valve health with age (Fig 5.3D).

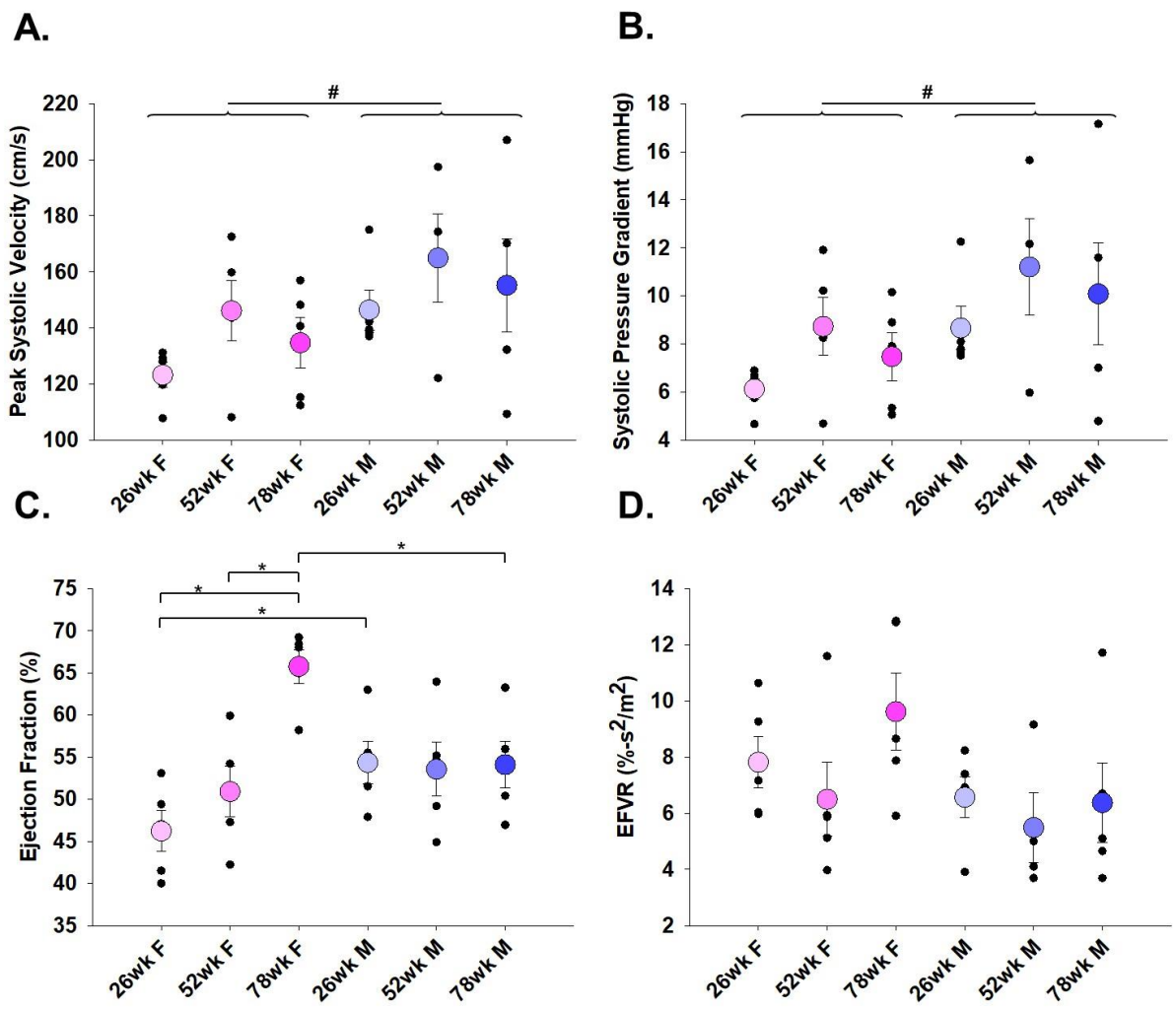

Figure 5.3 Valve health in male mice diverges with age
Echocardiography showed elevated PSV and transvalvular pressure gradient in male mice (p=0.032, P=0.032) and trends of higher and more divergent velocities and pressure gradient with increasing age in male mice (C, D). Ejection fraction was consistent for males but increased with age in females (E). EFVR showed similar trends as PSV and pressure gradient (F). *P<0.05 between individual groups by two-way ANOVA; #P<0.05 between sex by two-way ANOVA; PSV: peak systolic velocity; EFVR: ejection fraction to velocity ratio

5.4.3 H19 imprint and expression are not altered by age or sex in the aortic valve

Pyrosequencing of the *H19* ICR in mouse aortic valve gDNA revealed no change in average methylation fraction due to age or sex (Fig 5.4A). Likewise, there was no observed trend towards increased *H19* expression in the valve with increasing age nor correlation

between methylation fraction and *H19* expression (Fig 5.4 B,C). Rather, 26-week-old mice had higher average *H19* expression than either the 52- or 78-week-old mice (P=0.025; P=0.017), fitting the trend of elevated expression in development with reduced expression due to imprint in later life.

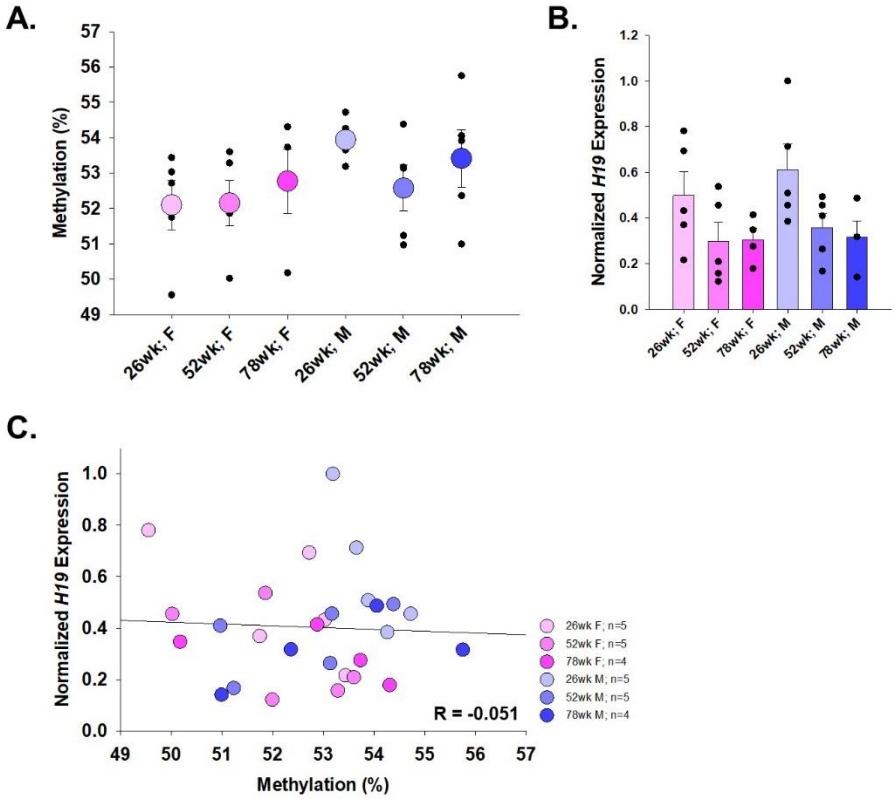

Figure 5.4 Methylation and expression of *H19* is unchanged with age and sex
H19 ICR methylation was unchanged in all samples studied (A) and did not correlate with *H19* expression (B,C). Expression of *H19* was significantly higher in 26wk old mice than in 52 wk old mice (p=0.011) and 78wk old mice (p=0.012), matching a pattern that might be expected from development of an imprint in youth without hypomethylation-related dysregulation.

5.4.4 Downstream genes are unaltered with age or by H19 expression

Expression of *Igf2*, which is regulated by the *H19/Igf2* ICR, and *Notch1*, which was previously shown to be repressed by *H19*, also showed no clear age or gender effect, though individual comparisons reached statistical significance (Fig 5.5A). *Bmp2*, a driver of osteogenic calcification that has been found to be increased in calcified valves, was higher in male mice

than in females overall (P=0.002) and specifically at 26 and 78 weeks of age (P=0.015; P=0.017). To examine if *H19* may still be suppressing *Notch1* on an individual level that is not revealed in group-averaged data, we also correlated expression of *H19* and *Notch1* in individual mice, but no significant effect of *H19* expression on *Notch1* expression was detected (Fig 5.5B).

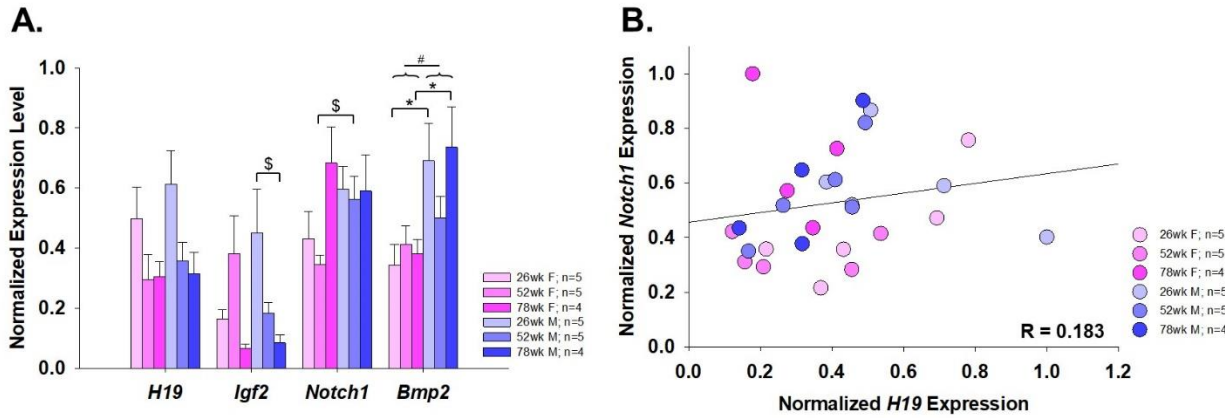

Figure 5.5 Downstream signaling unchanged with age or *H19* level
Expression of genes coregulated with *H19*, downstream of *H19*, or known to be active in the progression of CAVD were unchanged with age or sex as a whole, though individual comparisons did reach statistical significance (A). *Bmp2* expression was higher in males as a whole than in females (p=0.002). No correlation was found between *H19* expression and *Notch1* expression, suggesting a potential threshold effect for significant suppression of *Notch1* by *H19* (B). *P<0.05 between individual groups by two-way ANOVA; $P<0.05 between individual groups by ANOVA on ranks or Mann-Whitney; #P<0.05 between sex by two-way ANOVA

5.4.5 Liver and aortic tissue show increased H19 expression in oldest mice

Based on other studies showing *H19* upregulation in aortic aneurysm and cardiac ischemia, as well as a study that found age-related loss of imprint in rat liver, we also probed *H19* expression changes in the liver, left ventricle, and ascending aorta [174], [179], [253] (Fig 5.6). The 78-week-old male mice had higher *H19* expression than other age males in both the liver and ascending aorta. *H19* expression trended upwards with age in female liver, while the ascending aorta of 78-week-old female showed higher expression than the other female age groups and the 78-week male mice. In left ventricular tissue, no clear pattern of age-related *H19*

upregulation was observed, though the 78-week females had significantly lower *H19* expression than other female age groups, and 52-week males had higher expression than other male age groups.

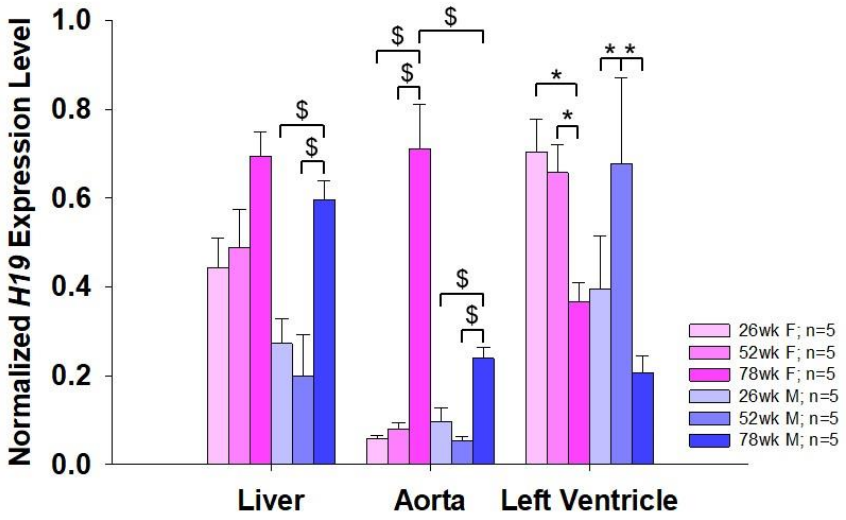

Figure 5.6 *H19* expression in other tissues showed potential age-related dysregulation
H19 expression was higher at 78 weeks in the liver and aorta of male mice and in the aortas of female mice. No clear trend in *H19* expression was found in the left ventricle, though 78-week females had lower expression (P=0.005 78 week to 26 week; P=0.014 78 week to 52 week) and 52-week males had higher expression (P<0.001 52 week to 26 week; P<0.001 52 week to 78 week) than other aged mice of the same sex. *P<0.05 between individual groups by two-way ANOVA; $P<0.05 between individual groups by ANOVA on ranks or Mann-Whitney; #P<0.05 between sex by two-way ANOVA

5.4.6 $H19^{DMD\text{-}9CG}$ mutant mice do not exhibit functional hallmarks CAVD

To study the impact of direct alterations in *H19* expression on the development of CAVD, we performed the same echocardiographic and molecular analyses on a small cohort of $H19^{DMD\text{-}9CG}$ mice. These mice have 9 CpG-dinucleotides mutated in the CTCF binding regions of the

ICR, enabling CTCF to bind the paternal allele thereby inducing *H19* expression (Fig 5.7A). All mice tested were 52-week-old littermates and consisted of two females and one male with a paternally inherited $H19^{DMD\text{-}9CG}$ allele and one female and one male with a WT paternal allele as controls.

Echocardiographic analysis of the aortic valve showed very little variation in PSV and pressure gradients between mutant and wildtype mice (Fig 5.7 B,C). PSVs for all mice tested were all less than 100 cm/s, well below the 52-week-old C57BL/6 mice and below any level that would indicate valve stenosis. Ejection fraction and EFVR values were also tightly grouped with no apparent differences between mutant and WT mice (Fig 5.7 D,E).

5.4.7 $H19^{DMD\text{-}9CG}$ mutant mice do not have molecular signatures of H19 driven CAVD

Though low sample numbers preclude meaningful statistical analysis, RT-PCR did not show significant upregulation of *H19* with a paternally inherited $H19^{DMD\text{-}9CG}$ allele (Fig 5.7 F). Indeed, mutant female mice had slightly lower *H19* expression than the WT control. While male mice followed the expected *H19* expression trend, both the mutant and WT males had lower *H19* expression than the female mice. The downstream impact of *H19* expression on *Notch1* and *Cdh11* expression was similarly inconsistent.

As with the C57BL/6 mice, expression patterns of classical calcification markers in $H19^{DMD\text{-}9CG}$ mice were inconsistent (Fig 5.7 G). *Runx2* was higher in all $H19^{DMD\text{-}9CG}$ mutant mice than in their WT littermates, though given the absence of such a pattern in *H19* expression, it's unlikely that this trend is directly linked to *H19* levels.

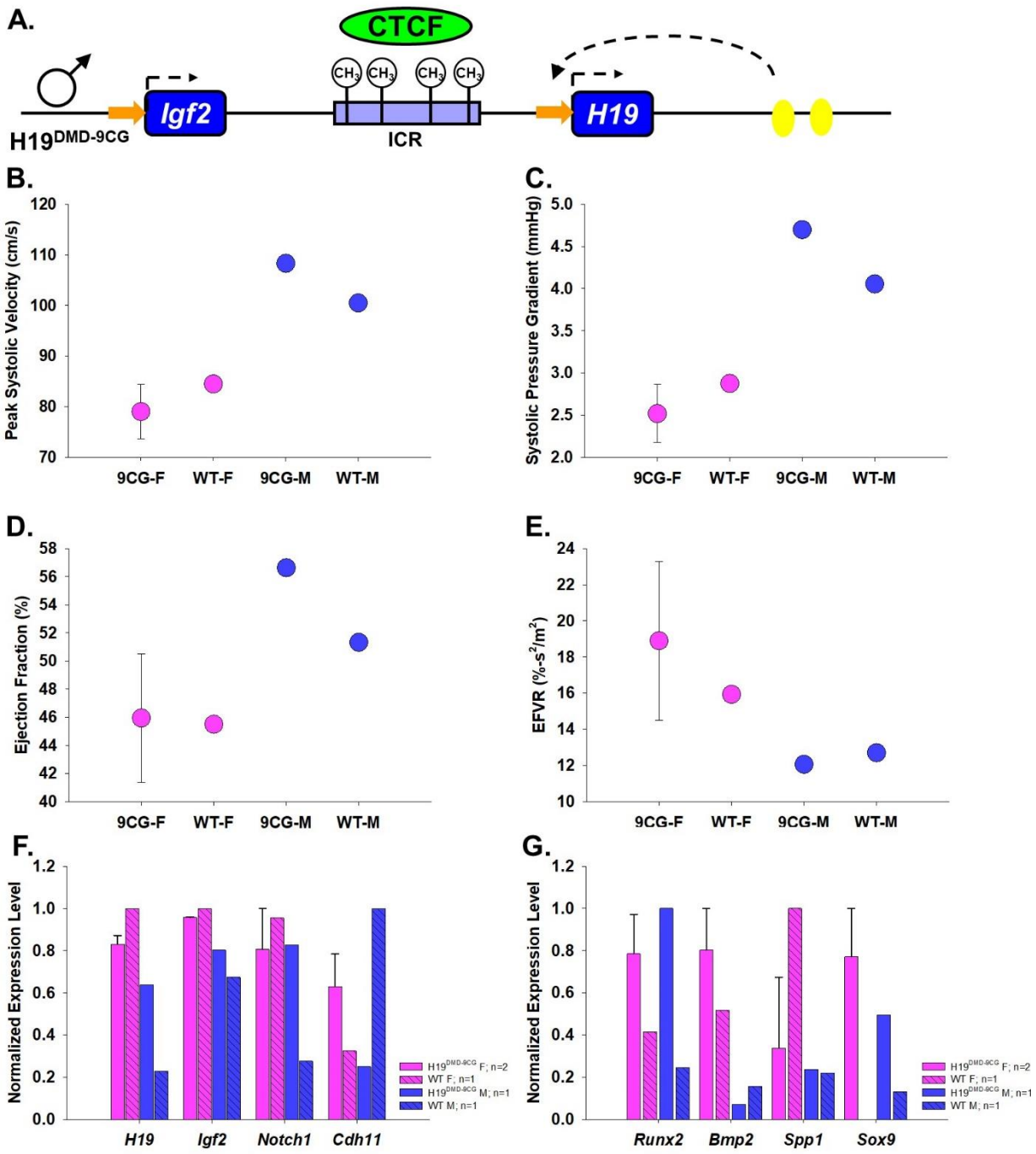

Figure 5.7 *H19*$^{DMD-9CG}$ mutant mice do not show signs of CAVD

The *H19*$^{DMD-9CG}$ allele (A) mutates nine CpG sites within the *H19/Igf2* ICR, enabling CTCF to bind the allele. When paternally inherited, this allele has been reported to reduce *Igf2* expression and increase *H19* expression. However, there was no pattern of functional change (B-E) or changes in gene expression (F, G) which followed the proposed mechanism of *H19*-driven CAVD in the aortic valves due to the *H19*$^{DMD-9CG}$ mutation. EFVR: ejection fraction to velocity ratio; n.d.: not detected.

5.5 Discussion

This study investigated the effects of age and sex on hemodynamic function and *H19* expression in the aortic valve. To our knowledge, this is the first cross-sectional study of a potential CAVD-initiating mechanism in both male and female mice that did not have a CAVD-associated mutation or receive a chronic hypercholesteremic treatment. Furthermore, this study isolated nucleic acids from individual aortic valves, enabling expression profiling and methylation analysis without requiring sample pooling. This approach represents a novel approach to probe subtle signaling which may precede or initiate valve disease in mice without introducing the biases of specific models, whereas most studies (including our own [154]) typically utilize specific mutations, diet, or cardiovascular injury to induce symptoms of CAVD, which may bias or obscure subtle signaling changes early in disease progression.

We found that the *H19*/*Igf2* ICR does not undergo age-related hypomethylation and *H19* is not upregulated in mouse aortic valves with age alone. This contrasts with expression data from other tissues such as liver and ascending aorta, as well as with prior studies in rats that showed loss of ICR methylation and *H19* upregulation in a tissues such as brain and skin [179]. The rats used in that study and the mice used here included animals comparable to 60 to 70 year-old humans, well within a timeframe at which early signs of CAVD could be expected. These results suggest a mechanism for preserving *H19* imprinting and low expression levels that differs by tissue type or species.

In addition to the lack of a robust increase in *H19* expression, we did not observe substantial correlation between expression of *Notch1* and *H19* in the aortic valve. Previous studies have shown that elevated *H19* can repress *NOTCH1* in human aortic valve interstitial cells and mouse brain tissue [225]. A potential explanation for this discrepancy is a threshold effect, in which *H19* expression must reach a certain critical level before measurably repressing

Notch1. Further work with a titratable *H19* overexpression system may be a useful tool to answer this question.

Despite the lack of strong *H19* upregulation, a few of the oldest mice showed signs of early aortic stenosis as indicated by elevated PSV and transvalvular pressure gradient. These functional indicators of stenosis even in the absence of *H19* upregulation underscore the heterogeneous nature of CAVD and suggest that *H19* is not the sole initiator of disease. Nearly 30 different mouse models of CAVD exist, and while many of these models are convergent, it is clear that multiple distinct mechanism can initiate and drive CAVD [13], [254], [255].

Certain sex-related differences emerged in this study which corroborate known statistics of human disease. For example, male mice had higher overall PSV values that trended upwards with age and higher *Bmp2* expression, indicative of onset of stenosis with activation of calcific pathways. This matches with clinical data showing that men are more likely to develop CAVD than women and that features of the disease (fibrosis and calcification) differ between sexes [248], [249]. Still, CAVD is relatively prevalent in women, and the differences in disease progression between sexes may direct more personalized treatment strategies. Despite this, many studies either do not distinguish between male and female mice or use only males. The work presented here included both male and female mice in order to better capture any differences in *H19* regulation and valve stenosis.

Although age-related upregulation of *H19* was not found in the aortic valve, it was confirmed in the liver and reported for the first time in the ascending aorta. It was also not found to increase in the left ventricle, showing a high degree of tissue specificity in overall expression regulation. Many studies have identified *H19* as a biomarker or driver of other cardiovascular diseases such as aortic aneurysm, smooth muscle cell apoptosis, endothelial cell aging, and ischemic heart failure [115], [174], [253]. Our findings of tissue-specific differences in age-

related upregulation of *H19* may inform future work looking at disease initiating events in these other pathologies.

Results in the $H19^{DMD\text{-}9CG}$ mice did not shed much light on the role that *H19* epigenetic control plays in CAVD. While this mouse model has been used successfully to study imprinting mechanisms in the *H19*/*Igf2* locus, it appears that trends observed in those studies were not replicated. *H19* expression was not consistently increased, nor was *Igf2* expression diminished in the $H19^{DMD\text{-}9CG}$ mice, as has been described previously in neonatal tissues [246], [256]. This may reflect the generally lower expression of these genes in 52-week-old mice or alternative epigenetic mechanisms for maintaining a strong imprint in valve tissue. Whatever the cause, we did not observe consistent changes in either expression of other genes or echocardiographic metrics that would indicate the presence CAVD.

This study is not without its limitations. One of the biggest challenges was the sample size – both of individual aortic valves and the overall size of the cohort. To obtain testable quantities of RNA and DNA, valves were used entirely for nucleic acid extraction. Alternate methodologies such as histology, immunohistochemistry, or *in situ* hybridization may have revealed more information about the extent of valve remodeling and stenosis. Furthermore, the high cost of raising or purchasing aged mice prevented higher numbers in each cohort, which may have increased the statistical power and significance of these results. Finally, the methylation sites tested in this study were within the ICR, which is typically considered to be the site of most epigenetic regulation of *H19* expression and was shown to be hypomethylated with age in work by Agba et al. [179]. However, Hadji et al. report a stronger association of *H19* expression with a particular CpG site in the promoter region of *H19* [225]. Despite this difference, we did not observe the increase in *H19* expression that was clearly reported in their work.

This work shows a lack of age-related epigenetic dysregulation of *H19* in mouse aortic valves, even while other tissue types demonstrated consistent upregulation of *H19* expression. Nevertheless, echocardiographic metrics and changes in gene expression in individual mice showed signs of valve remodeling and early stenosis. Together, these results show that *H19* loss of imprint and subsequent upregulation are not common in the aortic valve of old mice, and that increased *H19* levels do not appear to be a prerequisite for early stage valve disease.

Chapter 6

Summary, broader impacts, and future directions

6.1 Summary and broader impact

This work investigated the role of lncRNA *H19* in the context of cardiac valve development and age-related disease. These studies were motivated by the pressing clinical need to understand initiating events in valve disease and to develop better, developmentally guided therapeutic strategies. To address these challenges, we focused on two stages in which the majority of cases of aortic valve disease manifest – embryonic development and old age – with a particular focus on the role of mechanical factors and lncRNA *H19*, which has been recently identified as a regulator of aortic valve disease. While a large body of existing work documents the role of various protein signaling pathways and genetic mutations in aortic valve health, roles of mechanical factors and non-coding RNAs have been under-investigated, despite growing evidence that they are key factors in many disease cases. The research presented here shows new interplay between these factors in a developmental context and clarifies the role of *H19* in the initiation of disease.

To study aspects of valve development, we utilized an *in vitro* model of endothelial development which enabled rapid testing of mechanical stimulation with less optimization of highly specific differentiation protocols. While the methodology used was perhaps more relevant to vascular endothelial differentiation, the close developmental relationship between vascular endothelial cells and endocardial cells means results obtained in the present work can likely be extrapolated to endocardial development. Application of cyclic mechanical strain was utilized to simulate cardiac contraction, which is known to regulate aspects of early valve development.

This work is the first to our knowledge to apply such strain to differentiating endothelial cells from the initiation of the differentiation protocol. Cells differentiated with strain showed accelerated induction of mesoderm differentiation, and the purified endothelial population of strained differentiations had higher expression of *H19* and classical endothelial markers. Functionally, strained differentiations resulted in cells with higher tube formation capacity. *H19* was investigated as a potential cause of this effect in a HUVEC line and found to cause similar effects.

These results advance our understanding of the role that mechanics play in cellular differentiation and function. Given the highly dynamic environment in which the valve develops and functions, these findings have implications for our understanding of valve development as well as our ability to recreate valve developmental processes *in vitro*. Furthermore, these results may have implications for tissue engineering purposes beyond aortic valves. The broader endothelial differentiation strategy that we employed was initially developed for vascular endothelial cells, which are an important cell type for development of tissue engineered vascular grafts or other vascularized tissues.

Recent results indicating that *H19* acts as an upstream regulator of *NOTCH1* in CAVD motivated our investigation into the pattern of expression and correlation with valve health in mice. Given the lack of a unifying explanation for idiopathic cases of CAVD and the shortcomings in existing mouse models of valve disease, we investigated whether *H19* was a necessary prerequisite for the development of CAVD in otherwise healthy mice. We found that despite signs of hemodynamic changes indicative of early sclerosis, *H19* did not undergo age- or sex-related hypomethylation and upregulation in the aortic valve. These results suggest that while *H19* may play a key role in suppressing NOTCH1 signaling in CAVD, there may be additional factors leading to its dysregulation. However, dramatically increased expression of *H19* in the oldest murine aortas corroborates previous studies in rats and indicates that

dysregulation of *H19* is still found in cardiovascular tissues. Finally, we performed preliminary investigation into a mouse line with genetic alterations in the *H19* ICR and identified no significant dysregulation of *H19*, suggesting that it is not a useful model of murine CAVD.

While this study did not identify *H19* as a clear “smoking gun” in the case of CAVD, it did clarify its role in early stages of murine valve pathologies. Collectively, these results suggest that *H19* upregulation may not be a necessary precursor to valve remodeling and CAVD but may instead be part of an aggressive response to early disease which exacerbates symptoms through *NOTCH1* suppression. Dysregulation of *H19* is still observed in some cardiovascular tissues, however, and may be a causal factor of idiopathic CAVD in humans. There is a higher degree of tissue complexity in the valve structures of humans (Chapter 2) when compared to mice, which may also contribute to the difference in effect of H19 on valve disease between species.

In total, this work identified a novel role for mechanoregulation of *H19* and downstream effects in stem cell-derived endothelial cells and clarified the expression patterns of *H19* in murine aortic valve remodeling. These findings could be useful in development of novel tissue engineering strategies involving endothelial or endocardial cells or in development of a novel mouse model of valve disease specifically targeting *H19*.

6.2 Future directions

The present work advanced our understanding of *H19* in the context of valve development and disease but also raised important questions that can be used to direct future research into this topic. In particular, identifying direct regulators of *H19* expression and direct downstream targets which effect functional change would provide much greater mechanistic insight into its role in the valve. Just as we investigated *H19* as a potential regulator of *Notch1*

signaling which may explain disease in the absence of *NOTCH1* mutation, upstream regulators of *H19* may shed more light on initiating factors of disease. These regulators may take the form of epigenetic modulators which change the methylation status of the *H19* ICR. We showed clear mechanical regulation of *H19* expression, but it would be a great step forward to identify mechanotransductive surface receptors or stress response mediators which are responsible for strain-mediated increase of *H19* expression. Likewise, clarifying downstream signaling events which mediate *H19*'s functional effects would improve our ability to predict outcomes of its expression. Several connections have already been established, but these are not universally observed, and it appears that *H19* may exert a variety of downstream effects in a highly context-dependent manner.

In the developmental work, we utilized a previously published vascular endothelial differentiation strategy to enable quick experimentation with a simple overall system. While this has the potential benefit of extended relevance to other tissue engineering goals, it loses some of the specificity to the valve and the unique elements of the endocardial cells. Since the initiation of this project, a more refined method for generating stem cell-derived endocardial cells have been published [210]. Investigating the role of *H19* in this differentiation scheme may be more directly relevant to valve biology. Similarly, inclusion of mechanical stimulation, which was not investigated in this recent publication, may have more significant impact on the valvular endocardial differentiation. Alternatively, one could utilize mouse *in utero* samples to capture bona fide endocardial cells. Such samples are technically challenging to acquire, though advancements in single cell analysis techniques would enable a high data yield to analyze *H19* in the developing valve [257].

Finally, establishing the role that *H19* plays in a more robust model of valve calcification may be a better way to discover impacts that it plays in disease processes. For our study, specific mutations and chronic diet-induced injury were avoided as initiating factors so as to

avoid the bias that these introduce. However, the low rate of disease in wildtype mice hindered our ability to clearly identify or disprove connections between *H19* dysregulation and CAVD. To more clearly show such connection, one could use alternative models of CAVD, strongly driven by known mutation or diet, and probe for changes in *H19*. Alternatively, though surely more complex, one could develop transgenic mouse models in which expression of *H19* can be targeted directly with tissue specific transgene activation. Besides technical difficulty, this approach may face difficulty due to the embryonic lethality associated with certain alterations in the *H19* locus [111], [112].

Collectively, the work presented in this thesis contributes to the long-term goals of developing a better understanding of the roles of mechanics and lncRNAs regulating development and disease in valves.

APPENDIX A

Development of Cdh11 and H19 overexpressing transgenic mice

Rationale

To further probe the role of *H19* and downstream effector proteins in the development and progression of CAVD, preliminary work to develop transgenic mouse models to overexpress *H19* and Cdh11 was undertaken. Because the intent of these transgenic models was to examine the effects of modulation of the gene on adult-stage disease, and because of the developmental effects resulting from global modification in either of these genes, we employed a design utilizing floxed stop sequences upstream of the transgene. In combination with inducible, tissue specific Cre mouse lines, the mice would be able to develop and grow as normal, but on induction with Tamoxifen containing chow would undergo transgene recombination and subsequent overexpression of the target molecule. Expression of the transgene would also be tied to expression of a fluorescent reporter, both to confirm successful recombination and to identify transgene expressing cells for potential sorting and expression analysis.

Methods

A full-length murine Cdh11 open reading frame was ordered from Addgene with complementary restriction sites to cline into pROSA26-PA, a vector containing homology arms to the Rosa26 ubiquitous expression locus. Fluorescent reporter CherryPicker (Clontech) was cloned into the plasmid downstream of Cdh11 with an IRES sequence between the two to promote equal expression of both transcripts (Fig A1-A). A similar construct was generated for *H19* with the addition of a reverse tetracycline response element to control expression of *H19*, enabling crosses with constitutively active Cre lines without significant overexpression until administration of tetracycline (Fig A1-B).

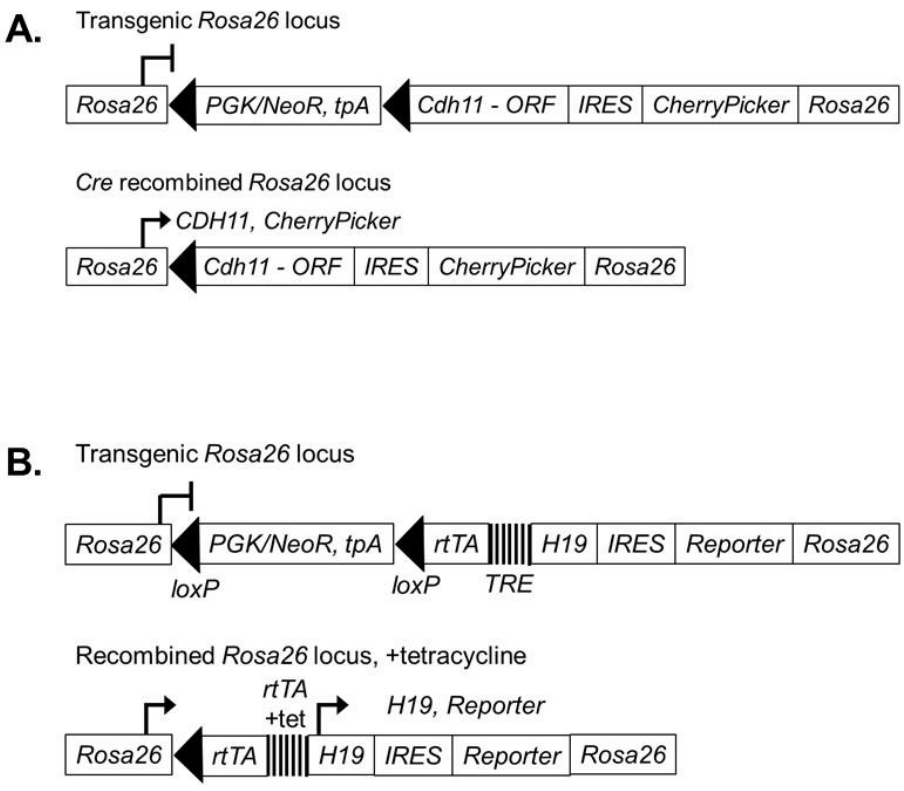

Figure A1. Transgene design

Construct design for the overexpression of Cdh11 (A) or *H19* (B) in mice. Homology arms on either end of the transgene promote insertion into the Rosa26 locus. A floxed selection cassette and transcriptional stop sequence can be removed when crossed with a cre-recombinase containing mouse line, leading to expression of the transgene and fluorescent reporter (bottom half of each panel).

Concurrently, a CRISPR/Cas9 construct targeting the Rosa26 locus at which transgenes would be inserted was also developed from plasmid PX458 (Addgene, [258]). Co-delivery of this plasmid allowed for higher insertion rate of the overexpression construct into the Rosa26 locus.

The Cdh11 overexpression construct was developed and tested in mouse embryonic fibroblasts in conjunction with the Rosa26 CRISPR targeting plasmid. The two constructs were co-delivered to mouse embryonic stem cells using a BioRad GenePulser, and cells were screened for neomycin resistance, indicating successful insertion of the transgene. From a

selection of 20 positive clones, one was positively identified for successful transgene insertion by PCR. Use of this clone was unsuccessful in generating a founder mouse.

Potential Impacts

While progress on development of these transgenic mice was halted due to lack of resources, these mice could significantly extend our understanding of *H19* signaling in adult valve disease. Indeed, a similar mouse model of Cdh11 overexpression was recently shown to be a valid model of CAVD. Given the hypothesized role of *H19* as an upstream regulator of *Notch1* and *Cdh11* expression, this mouse model would be expected to show similar results while also enabling investigation of *H19*-specific effects.

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