Valve cell dynamics in developing, mature, and aging heart valves

Dissertation

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Abstract

Heart valve dysfunction affects ~2.5% of the population, and this incidence increases significantly in the elderly, affecting up to 13.2% of people over the age of 75. While the pathology has been well defined, mechanisms underlying progressive valve deterioration are still unknown. The mature, healthy valve structure is composed of two main cell types, the overlying valve endothelial cells (VECs) that form an uninterrupted monolayer surrounding the valve, and valve interstitial cells (VICs) that make up the core structure and secrete specialized layers of extracellular matrix (ECM) proteins. These specialized layers are arranged in a tri-laminar fashion, providing all the necessary biomechanics to withstand hemodynamic forces. In contrast to healthy young valves, aging valves are associated with deterioration of the ECM organization, leading to alterations in biomechanical properties that can result in stiffened or stenotic valves which fail to fully open. Although the pathogenic changes in ECM organization have been well described, little is known about how age-related changes in valve cell populations contribute to the progression of degeneration in the elderly. Here we provide insight as to how changes in the phenotype and function of valve cell populations occur throughout maturation and aging, which likely contribute to age-related valvular dysfunction. We identify key cellular processes within VECs that decline with age, including metabolism, endothelial to mesenchymal potential, proliferation and barrier function. Furthermore, using a novel...
method to isolate murine VECs, we perform RNA-sequencing analysis and report the differential and common transcriptomes of VECs at embryonic, post-natal, young-adult and aging-adult stages, revealing the age-dependent heterogeneity of these cells. In addition to resident valve cell populations, we show that circulating, CD45+ cells incorporate into the valve structures beginning at embryonic stages and continuing into adulthood. Further, we provide evidence to show that the majority of these cells are nonclassical patrolling monocytes. Furthermore, using bone marrow transplants, we show that both the phenotype and ability of the transplanted CD45+ cells to incorporate into the valve structure is dependent on the age of the cell and the age of the surrounding tissue environment. Collectively, these studies demonstrate that both the resident and extra-cardiac valve cell populations are highly sensitive to age-related changes and variable in their phenotypes. As aging is known to be a multifactorial process, this work has identified several key parameters that may contribute to impairment of the valve to maintain critical structure-function relationships; leading to degeneration and disease.
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**Fields of Study**

Major Field: Molecular, Cellular, and Developmental Biology
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Chapter 1: Introduction

1.1 Overview

Heart valves are dynamic structures designed to open and close over 100,000 times a day to facilitate blood flow through the heart to the systemic circulation. In healthy individuals this is largely achieved by structure-function relations between the cellular and extracellular components of the mature valve and the hemodynamic environment. There are two sets of cardiac valves: the atrioventricular (AV) valves including the mitral and tricuspid that separate the atria from the ventricles on the left and right sides respectively; and the aortic and pulmonic semilunar valves that separate the ventricles from the great arteries. It is the coordinated movement of these valvular structures that maintain unidirectional blood flow during the cardiac cycle. Perturbations in heart valve structure-function can result in valvular dysfunction, in which the valve is unable to efficiently open.
or close, leading to serious complications including heart failure[1, 2]. Aging is a significant risk factor for acquiring valvular dysfunction in which ~13.2% of those of those over 75 years old are affected[3]. At present, surgical intervention remains the only effective treatment and this is largely attributable to the current lack of understanding of the molecular mechanisms involved in heart valve maintenance and age-associated degeneration, thereby limiting therapeutic advancements.

1.1.2 The Mature Heart Valve Structure

The mature heart valve leaflets are connective tissues composed of a stratified extracellular matrix (ECM) and two major cell populations. These populations include the fibroblast-like valve interstitial cells (VICs) and the valve endothelial cells (VECs). VICs are the most abundant population within the valve and are located throughout the ECM whereas VECs form a monolayer over the valve surface, encapsulating the leaflet[4]. The valve ECM is highly organized into three layers, the fibrosa layer, primarily composed of collagen fibers, the spongiosa layer, constituted of mainly proteoglycans, and the atrialis/ventricularis layer, highly enriched in elastin. The composition and organization of these layers provides the biomechanical properties necessary to withstand the surrounding hemodynamic forces and is highly conserved across species[2]. In contrast to healthy valves, disruptions to the endothelial cell layer, ‘activation’ of otherwise quiescent VICs, and changes in the organization and contribution of ECM components is often associated with valve dysfunction that can lead to progressive heart failure[5-7]. The cause and effect of changes in the cellular components of the valve are not clear, however significant
changes in the ECM lead to deformations in the valve leaflets and alterations in the biomechanical properties.

1.1.2 Valve ECM and Biomechanics

As mentioned, the valve is composed of three highly organized layers of ECM arranged according to blood flow (Figure 1). The fibrosa is located furthest away from blood flow and predominantly composed of collagen fibers that provide tensile strength to the valve leaflet during opening, while transmitting forces to promote coaptation of the leaflets in the closed position[8-10]. Adjacent to the fibrosa is the spongiosa, with a lower abundance of collagens and high prevalence of proteoglycans. This composition provides a more compressible matrix, allowing the valve to geometrically ‘flex’ and absorb high forces[11, 12]. Finally, the layer adjacent to blood flow, termed the atrialis (AV) or ventricularis (semilunar), largely consists of elastin fibers that facilitate tissue movement by extending as the valve leaflet opens and recoiling during closure[13, 14]. Stratification of the ECM layers is necessary for proper valve function and disruption of the tri-laminar structure is highly correlated with valve pathogenesis including disease states such as mitral valve prolapse (MVP) and calcific aortic valve disease (CAVD)[7, 15]. It is therefore essential to maintain ECM stratification for efficient valve function and this is largely mediated by two valve cell populations.
1.1.3 Valve Cell Populations

The ECM architecture within the mature valve leaflets is maintained in the adult by valve interstitial cells (VICs). Mature VICs are a quiescent, fibroblast-like population whose primary role is to produce, degrade, and regulate the organization of ECM proteins within the healthy valve leaflets[4]. VICs are a highly heterogeneous population in which several factors including location within the valve, sex, species, age, and disease states can influence their gene expression[16-18]. Studies in mice have shown that this heterogeneity is evident starting in embryonic remodeling stages when distinct subsets of VICs can be detected by their differential expression of mesenchyme and myofibroblast markers including, Twist1, Periostin, Vimentin, and α-SMA, as discussed later in this chapter[16]. Although these markers are downregulated in mid post-natal stages, by 6 weeks of age, almost all quiescent VICs re-express Vimentin, similar to early stages and rare α-SMA+ VICs are observed throughout adulthood. Moreover, it has been demonstrated that porcine VICs isolated from various anatomical locations within the mitral valve are highly heterogeneous in their metabolic, secretory, and adherence properties[19]. Thus, VICs are phenotypically dynamic in their function and gene expression; however, the mechanisms underlying these differences and how this heterogeneity affects ECM architecture are still not understood.

In addition to the VIC population, the encapsulating VECs serve as a physical barrier to protect ECM and VICs against the constant changes in hemodynamic forces and circulatory risk factors[20, 21]. Although the function and phenotypic markers of VECs are similar to vascular endothelial cells, inherent differences have been observed, rendering
VECs as a unique, specialized population. For example, unlike vascular endothelial cells that align in a parallel fashion in response to hemodynamic flow, VECs align perpendicularly and this property has been observed both in vivo and in vitro[22, 23]. This phenotypic difference is also reflected in the differential transcriptional profiles observed between porcine aortic valve endothelial cells (pAVECs) and vascular endothelial cells (pAECs) exposed to laminar shear stress, in which pAVECs were found to be express significantly less proinflammatory genes[24]. Additionally, distinctive hemodynamic flow conditions found on either side of the valve significantly influence VEC phenotypes in a side specific manner. The spatial localization of VECs on the underside and overlying surface of the valve leaflets allow for differential exposure to mechanical stimuli. In the aortic position, VECs on the underside (ventricularis) experience laminar shear and low stress, while those on the overlying surface (fibrosa) are subjected to oscillatory flow and high pressure in diastole[25]. In a study using VECs isolated from the aortic vs ventricular side of porcine aortic valves, distinctly different gene expression profiles were found between the two populations[26]. Interestingly, VECs on the aortic side of the valve correlated with reduced expression of anti-calcific molecules including eNOS[26, 27]. Moreover, a recent study has reported that ECM protein content is regulated in a side specific manner in response to hemodynamic flow patterns suggesting side-specific mechanosensory mechanisms of VECs[28].

In addition to side specific diversity, studies have also found heterogeneity in the ability of VECs to undergo an endothelial to mesenchymal transition (EMT) in response to TGFβ1 and TNF-α; and only subpopulations of VECs that undergo EMT have the ability
to subsequently differentiate into chondrogenic and osteogenic lineages[29, 30]. Collectively, these studies indicate that VECs are highly heterogeneous in their function and molecular profiles.

VECs are in direct contact with the hemodynamic environment and are therefore exposed to changes flow, circulating risk factors and inflammatory cells, yet variations in these environmental cues are also associated with alterations in VIC biology. As VECs are the intermediate link between the hemodynamic environment and VICs, they likely serve as mechanosenors translating external stimuli into signaling pathways to subsequently regulate VICs. In vitro studies from several labs have shown that VECs molecularly communicate with VICs through paracrine signaling[27, 31-35]. Regulatory pathways involved in this communication include, Notch1, TGFβ1, and nitric oxide (NO) and disruptions in these signaling axes leads to valvular pathologies and congenital defects as described in mouse models of valvular stenosis, bicuspic aortic valve (BAV), and CAVD[34, 35]. Recent evidence also points to reciprocal interactions in which VICs can regulate VECs by suppressing VEC calcification and EMT[32, 33]. However, the molecular mechanisms underlying this reciprocal regulation are unclear and much more work is needed to understand the complexity of the VEC and VIC interplay.

1.2 Valve Disease and Age-Related Degeneration

During valve disease, significant changes in the production and/or degradation of ECM proteins leads to deformations in the valve leaflets or cusps and alterations in the biomechanical properties. For example, mitral valve prolapse (MVP) is associated with
increased collagens and proteoglycans, resulting in thickened valve leaflets that ‘bulge’ or prolapse into the atrium, thus preventing valve closure resulting in regurgitation[36-38]. Alternatively, calcific nodule formation generally leads to stiffened, or stenotic valves that fail to fully open[39]. Whether the valve is regurgitant or stenotic, the secondary effects on cardiac function can be detrimental to the affected individual. The observed changes in ECM content can be due to abnormal production of the ECM components, or aberrant expression of ECM remodeling proteins but it is likely the combination of these two processes that result in pathological remodeling.

Similar to disease, aging heart valves undergo progressive changes in ECM composition and organization, altering their biomechanical properties[40-43]. Most notably, the number of collagen fibers and collagen crosslinks significantly increases in both aging human and porcine valves (Figure 2)[40, 41]. This has largely been attributed to age-related changes in the expression of collagen modifying enzymes and matrix metalloproteinases[44, 45]. Further, this noted expansion in collagen content has been shown to contribute to the age-associated increase in valve thickness and stiffness[42, 46]. In addition to collagens, reduced elastin and increased glycosaminoglycan content further promotes stiffening of the aging valve[44, 47, 48].

On a cellular level, diseased valves are associated with VIC activation and dysfunction and damage to the overlying valve endothelium[49, 50]. Deterioration of the physical barrier likely increases exposure of the underlying VICS and ECM to physical forces evoked by the hemodynamic environment, in addition to circulating risk factors, hence the association of VEC denudation with ECM degradation and altered cell
turnover[5]. In addition to physical damage, disruptions in intrinsic and extrinsic signaling pathways in VECs leads to pathological changes in VICs and ECM[34, 35].

During disease and age-related degeneration, a subpopulation of VICs becomes activated, similar to myofibroblasts, in which they aberrantly begin to express ECM and remodeling proteins, leading to disorganization of the tri-laminar structure[41, 44]. Studies in humans,[7, 51, 52] large animals,[53-55] and mice[56-59] have demonstrated that during activation, VICs upregulate the myofibroblast marker α-SMA, which is thought to recapitulate embryonic phenotypes. Notably, VIC activation is not ubiquitous to all resident VICs further supporting heterogeneity amongst the population and there is a diverse profile of ‘activated’ markers that are increased in subsets of VICs depending on disease, age, sex and species[16, 17]. The mechanisms behind VIC activation are still poorly understood but studies have indicated the involvement of a combination of molecular and biomechanical cues. For example, VICs cultured in the absence of VECs are ‘activated’, transdifferentiate towards a myofibroblast-like cell type[32], and undergo premature spontaneous calcification[34]. These pathologic phenotypes can be rescued by co-culture with endothelial cells, or treatment with an NO donor[27, 31, 32, 34], further highlighting the importance of the NO signaling pathway in maintaining function of the endothelium. In another example, a recent in vivo study from our lab identified Tgfb1 as a critical growth factor secreted by VECs to VICs to prevent calcification, by promoting nuclear localization of the transcription factor, Sox9[35]. Together, these data demonstrate that a disruption in VEC signaling often leads to deregulation of VIC homeostasis, resulting in the onset of disease states. VIC activation is also influenced by biomechanics. In vitro
studies have shown that substrate stiffness can induce VIC activation[16, 60-63]. Interestingly, this activation can be reversed by transferring cells to a soft substrate[60], or adding polyunsaturated fatty acids to the growth medium[64], highlighting the plasticity of cultured activated VICs and potential reversibility of activation. As valve stiffness progressively increases with age, the integrated role of biomechanics and VIC dysfunction likely plays a significant role in age-associated pathology.

Very little is known about the impact of aging on VECs. Work from the vascular field has shown that aging is associated with vascular endothelial cell dysfunction, ultimately contributing to the onset of cardiovascular diseases such as arterial hypertension and atherosclerosis[65]. Mechanisms underlying this dysfunction include alterations in endothelial barrier function, increased oxidative stress, and altered pro- and anti-thrombotic properties. Similar to vascular endothelial cells, a recent study has shown that hemostatic protein regulation in porcine VECs is also affected by aging resulting in the differential expression and mislocalization of thrombic and anti-thrombic proteins in aging porcine valves[66]. Additionally, increased oxidative stress and decreased NO signaling and have been implicated in heart valve disease and thought to be specific to valve endothelial cells similar to the process described in atherosclerosis[34, 67-69]. This is supported by the observed excess production of superoxide in VECs which is spatially localized with calcific lesions in human stenotic aortic valves[68] and hypercholesterolemic mice[70]. However, the role of VEC oxidative stress and NO signaling during aging remains elusive. From these studies it is apparent that there is some overlap between the mechanisms underlying age-associated dysfunction in the vascular
and valvular systems; however, it is known that there are inherent differences between vascular and valvular endothelial cells and therefore much more work is needed to confirm the conservation of these processes.

1.3 Valve Development, Maturation, and Maintenance

1.3.1 Embryonic development

Heart valve development begins when regions within the atroventricular canal (AVC) and outflow tract (OFT) of the embryonic heart tube form swellings known as endocardial cushions. These regions are first identified by localized deposition of proteoglycans and hyaluronan known as cardiac jelly. Following cushion formation, a subpopulation of overlying endothelial cells undergo an endothelial-to-mesenchymal transition (EMT) in which they lose cell-cell contact, delaminate and transform into mesenchyme cells that migrate, proliferate and invade the cushion[71, 72]. This essential process is initiated in the mouse at embryonic day (E) 9.5 and continues until ~E14.5[73]. The resulting mesenchyme cells serve as precursors to VICs in the mature valve structures[74, 75]. The process of EMT is complex and relies on an intricate network of growth factors, transcription factors, and intermediate signaling molecules that crosstalk between multiple cell types to tightly regulate each step.

Although the majority of mesenchymal VICs that form the endocardial cushions are derived from endothelial EMT[74, 75], contribution of non-endothelial cell lineages during development has been demonstrated. For example, lineage tracing in mice using the Wt1/IRES/GFP-Cre and the Wt1CreERT2/;Rosa26mTmG model (induced at E10.5),
demonstrates significant contribution of epicardial-derived cells to the AV valves following endocardial cushion formation[76, 77]. The Wnt1-cre transgene fate maps cells originating from the neural crest cell lineage and studies in mice show contribution of these cells to both the OFT and AV endocardial cushions as well as aorticopulmonary septal structures[75, 78]. Further, fate mapping of the secondary heart field lineage using the Mef2c-Cre model identified an additional cell population contributing to endothelial cells of the outflow tract cushions[79]. These collective studies demonstrate that the valve precursor cell pool is derived from multiple sources, however the role of these differential cell lineages to valve development and disease remain largely unknown.

1.3.2 Embryonic Remodeling

Following EMT, the developing valve undergoes a remodeling process characterized by condensation of the mesenchyme cushion cells that remain proliferative at the leading edge to promote elongation[20]. At this time, mesenchyme cells begin to express myofibroblast markers including, Twist1, Periostin, Vimentin, and α-SMA in which they become activated and mediate the ECM remodeling process by breaking down primitive ECM proteins and replacing them with collagen, proteoglycan, and elastin fibers that will later be organized into the stratified layers[2, 16].

1.3.3 Post natal Growth and Maturation

In the first few days after birth, the murine immature heart valve remains highly proliferative in which the replicating cells contribute to overall valve growth and
elongation[80]. Concurrently, ECM remodeling continues, in which the major matrix proteins begin to organize and condense into a tri-laminar arrangement, which becomes established in the AV valves by post natal day 7 (PND7) in the mouse. This paired condensation and proliferation results in the thinning and elongation necessary to form the mature fibrous leaflet. By mid post natal stages, mesenchymal and myofibroblastic markers are downregulated as the immature mesenchyme cells differentiate into mature VICs that take on the quiescent phenotype, a non-proliferative minimally active state in which they will remain throughout adulthood in the absence of disease.

As mentioned earlier, multiple cell lineages contribute to the heart valve structures during embryonic development. Although there have not been reports of cardiac lineage contribution after embryogenesis, contribution of an extra-cardiac lineage, derived from the bone marrow has been reported in adult valves[81, 82]; however, the timing of this contribution is currently unknown and further studies are needed to determine if this population could play a role during post natal remodeling.

1.3.4 Adult Valve Maintenance

During mature adult stages, the valve cell populations remain minimally active. Although the dynamic processes observed during embryonic and post natal stages, such as proliferation, metabolism, and ECM remodeling have considerably subsided by adulthood, the valve cells remain highly sensitive to the surrounding hemodynamic and tissue environment[44, 66, 83] Consequently, pathological stimuli or changes in hemodynamics can trigger the valve cell populations to undergo significant phenotypic changes, mediating
pathological ECM remodeling resulting in biomechanical failure. Therefore, both preventative upkeep and reparative mechanisms of valve cell populations is critical for maintaining a functional valve throughout adulthood. However, the mechanisms underlying how these populations maintain themselves both in response to normal “wear and tear” and to pathological events is still largely unknown.

One plausible mechanism of adult valve cell maintenance is EMT. In addition to undergoing EMT to establish VIC progenitors during development, VECs demonstrate degrees of ‘plasticity’ in their ability to undergo EMT during adulthood as well (Figure 3). It has been proposed that this dynamic characteristic may serve to replenish the VIC population throughout life. In vitro studies have shown that subsets of mature VECs have the potential to undergo EMT in response to TGFβ1 or inflammatory cytokines (interleukin-6, tumor necrosis factor-α)[84]. Additionally, in vivo, mesenchyme-like cells have been observed in sub-endothelial locations in large animal models of mitral valve regurgitation[85] and a mouse model of calcification (ApoE−/−)[33], suggesting EMT. The ability of VECs to undergo EMT in adult valves indicates that there is sub-population potentially capable of self-renewal that may serve to continually replenish the mature VIC population and whose activity is enhanced during the disease process[86].

Another possible mechanism of cell maintenance during adulthood is the reported contribution of bone marrow derived cells to the adult VIC population (Figure 1.3)[81, 82, 87-89]. Bone marrow transplant studies have shown that cells of bone marrow origin integrate into the VIC compartment in both human[87, 89] and murine adult valves[81, 82]. Bone marrow transplants in mice show that the majority of transplanted cells express
the hematopoietic marker, CD45, and once incorporated into the VIC compartment, become fibroblast-like in their ability to express pro-collagen 1[81, 82]. Further, the integration of hematopoietic cells into the valves appears to be a normal homeostatic process, as CD45+ cells are also detected in the valves of wild-type non-transplanted adult mice. However, both the function and contribution of these cells during valve development, aging, and disease pathogenesis is still unknown.

1.3.5 Closing

As aging is a multifactorial process, the age-associated changes discussed here are likely the result of many factors including changes in hemodynamics, increasing circulatory risk factors, and the development of other age-related physiological conditions. These factors then affect the highly sensitive valve cell populations, causing degenerative changes in the ECM components. Therefore, it is the combination of these factors with varying genetic susceptibility that likely sets the stage for age-associated degeneration and disease. Thus, improving our knowledge of how the valve cell populations function to maintain the healthy valve state during adulthood and how these mechanisms degenerate during aging will provide the platform necessary for the development of new therapeutics to target age-associated valve disease.
Figure 1. Overview of the heart valve structure. The atrioventricular (mitral (A), tricuspid) and semilunar (aortic (B), pulmonic) valves are highly organized structures composed of cellular and extracellular components. Each valve leaflet or cusp is surrounded by a continuous, single layer of valve endothelial cells and interspersed by valve interstitial cells. The extracellular matrix is highly organized and largely composed of elastin fibers in the atrialis/ventricularis (dark grey), proteoglycans in the spongiosa (blue) and collagens (yellow) in the fibrosa and these are arranged according to blood flow (red arrows).
Figure 2. Structural and molecular changes in aging heart valves. (A) The young, healthy adult heart valve is a highly organized structure. (B) Within the aging-adult heart valve, VICs become activated and aberrantly express matrix remodeling enzymes associated with increased collagen bundles and glycosaminoglycans, and decreased elastins leading to increased thickness of the valve leaflet (bracket). The aging valve is also associated with increased stiffness. Stiffness has been shown to induce VIC activation and activated VICs express more matrix leading to an even stiffer substrate, resulting in a continuous cycle (arrows). In addition, VEC disruption has been correlated with degenerative valves.
There is data to suggest that mature VECs can undergo rare endothelial to mesenchymal transition (EMT) events during adulthood which could serve as a mechanism to replace adult VICs over time (blue arrows). Additionally, bone marrow-derived cells (green) from the bloodstream have been detected in the adult valve leaflets and may also serve to replenish the valve cell populations (black arrows).

**Figure 3. Proposed mechanisms of adult valve cell maintenance.**
Chapter 2: Isolation of Murine Heart Valve Endothelial Cells

2.1 Introduction

Normal valve structures consist of stratified layers of specialized extracellular matrix (ECM) interspersed with valve interstitial cells (VICs) and surrounded by a monolayer of valve endothelial cells (VECs). VECs play essential roles in establishing the valve structures during embryonic development, and are important for maintaining lifelong valve integrity and function. In contrast to a continuous endothelium over the surface of healthy valve leaflets, VEC disruption is commonly observed in malfunctioning valves and is associated with pathological processes that promote valve disease and dysfunction. Despite the clinical relevance, focused studies determining the contribution of VECs to development and disease processes are limited. The isolation of VECs from animal models would allow for cell-specific experimentation.

Previous work by several labs has successfully isolated VECs from porcine and ovine models[22, 30, 90]. Due to the large size of these valves, isolations through swabbing and/or enzymatic digestion, followed by a number of different isolation methods including

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Figure 1. Schematic representation of the isolation process for murine valve endothelial cells.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{Figure 2. Flowchart illustrating the isolation steps for murine valve endothelial cells.}
\end{figure}

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magnetic bead cell separation and single cell clonal expansion has been effective to generate pure populations[22, 90, 91]. However, these models can be restrictive due to the incomplete annotation of the pig and sheep genomes limiting the availability of molecular tools in addition to the high costs. Therefore, experimentation of porcine and ovine VECs after isolation can be restrictive. Mouse models are preferable due to the many possibilities for genetic manipulation and molecular tools in the embryo and adult, but to date, no VEC isolations in small animal models have been reported. This is likely due to the difficulty of working with small tissue samples that include a minority cell population that currently lack unique identity of VEC-specific markers, thereby preventing antibody-based isolation methods.

In this chapter, we report a new method for direct isolation of murine VECs at embryonic and adult stages. This protocol takes advantage of Tie2-GFP mice, which express green fluorescent protein (GFP) in all endothelial cell types and have been extensively used to study endothelial cell populations[92]. However, the novelty of this current study is that these mice, for the first time have been utilized to isolate endothelial cells from the valves. By careful dissection of the valve tissue and a series of nine enzymatic digestions followed by fluorescence activated cell sorting (FACS), VECs can be isolated and used for various experimental techniques including RNA extraction and culture, directly following sorting.
2.2 Protocol

2.2.1 Preparation of Equipment and Solutions

- Sterilize the dissection tools - fine tissue scissors to extract adult hearts and 2 fine forceps for dissection of the valvular region - by autoclaving in a covered instrument tray. Spray tools with 70 % ethanol (ETOH) prior to dissection.

- Prepare all solutions immediately prior to the experiment and sterilize solutions by passing them through a sterile 0.2 μm filter. Keep solutions on ice until use.

**Sterile Dissociation Buffer** (15 mL total/sample). Combine 1.2 mL collagenase IV, 300 μl 2.5% trypsin and 150 μl chick serum. Bring volume up to 15 mL with Hank’s Balanced Salt Solution (HBSS).

**Sterile Sorting Buffer** (12 mL total). Combine 25 μl 0.5M Ethylenediaminetetraacetic acid (EDTA) and 12 μl DNase I (RNase-free). Bring volume up to 12 mL with HBSS.

**VEC Culture Media:** Mix endothelial growth media according to the manufacturer’s instructions (see material spreadsheet) by adding the aliquoted components from the kit to 500 ml of EBM2 media.

**GFP Negative Culture Media** (non-endothelial cell media): Mix 445 mL of Medium 199 1X with 5 mL of Penicillin/Streptomycin (Pen/Strep) (1 % final concentration) and 50 mL FBS (10 % final concentration).
2.2.2 Dissection of the Valvular Region from Adult mice and E14.5 embryos

*Tie2-GFP* mice were purchased from Jackson Laboratories (stock number 003658)[92] and were maintained as homozygous genotypes on an FVB/N background, and therefore ensuring that all off-spring express GFP in Tie2-positive endothelial cells.

For FACS sorting, prepare one age matched negative sample that does not contain GFP such as *C57/Bl6* to set GFP gate parameters for FACS sorting. NOTE: This protocol and representative results are based on using three, four-month-old adult mice or one litter at embryonic day (E) 14.5.

- Adult Mice: Immediately following sacrifice by CO₂ anoxia, use dissection scissors to gently open the chest cavity and expose the heart and lungs. Once exposed, grip the heart with forceps at the great arteries and pull away from the body. Remove lung tissue if intact, and place hearts in cold HBSS to rinse. Remove the ventricle and pull away the atria leaving the atrioventricular canal ‘ring’ and aortic regions intact. Make one incision down the side of atrioventricular canal to open up the ‘ring’ and expose the valvular structures. Remove the myocardium and proximal aorta regions by gently teasing away with forceps. Identify the valve leaflets as white, dense tissue over the pink myocardium. Gently detach the chordae tendinae from the atrioventricular valves and remove as much of the remaining myocardium as possible. Be cautious to not pull or scrape the valve leaflets during this process since VECs may be dislodged. NOTE: Careful dissection is critical to eliminate non-valvular endothelial cells that could
contaminate the sample. Place trimmed valvular regions in a 1.5 mL eppendorf tube containing 1 mL HBSS and keep on ice.

- Embryos: Sacrifice female mice 14.0 days after copulation plug is observed (morning of copulation plug = 0.5 days).

- Immediately following sacrifice, dissect the uterus (containing embryos) and wash in cold HBSS. Dissect out individual embryos from the uterus and remove the embryonic yolk sac surrounding each embryo. Remove the hearts from each of the embryos and follow step 2.1. (NOTE: gently squeezing the embryo with forceps just below the upper appendages should help to expose the heart and make removal easier.)

2.2.3 Cell Dissociation and Preparation for FACS

- Using sterile pipette tips, remove HBSS from the eppendorf tube containing the valvular regions.

- Replace with 1 mL dissociation buffer and 4 μl DNase I.

- Rotate the tubes at 37 °C for 7 mins.

- Pipette up and down 3 times and then let the sample settle for 15 sec. Collect the supernatant (containing the dissociated cells) into a 15 mL conical tube.
• To stop the collagenase reaction, add 125 μl of horse serum to the collection tube. Keep collection tube on ice.

• Repeat all dissociation steps starting with the addition of dissociation buffer nine times to collect nine fractions of supernatant.

• Pass the fractioned collection through a 70 μm nylon filter into a new collection tube to ensure a single cell suspension by removing any debris and clumps.

• Spin the suspension at 400 g for 5 min to pellet the cells.

• Resuspend the cell pellet in 1 mL Sorting Buffer and keep on ice until FACS.

2.2.4 FACS Sorting GFP positive VECs

• Set gates for forward and side scatter to exclude debris and capture single cells; exclude doublets by using doublet discrimination gating. Record the negative control sample and define gate settings in order to compare and accurately define the GFP-positive cell population in the Tie2-GFP sample.

• Analyze GFP positive cells via FACS and refine gate settings.
• Sort the *Tie2-GFP* sample and collect both GFP-positive and GFP-negative cells. If cells will be used for RNA extraction, sort directly into the Sorting Buffer. To culture cells after FACS, collect GFP positive cells in a sterile tube containing 1 mL VEC media with 1 mL FBS, and collect GFP negative cells in 1 mL of non-endothelial media with 1 mL FBS. Use this 50 % media/50 % serum combination to improve cell viability. Keep on ice until post FACS analysis.

2.2.5 *Post FACS Analysis*

• **RNA Extraction**

  o Immediately following FACS, centrifuge GFP positive and negative cells at 1500 g for 8 mins.

  o Carefully pipette the supernatant (sorting buffer) and discard.

  o Resuspend the cell pellet (pellet is not visible for the sample sizes recommended here) in 200 μl Trizol.

  o Freeze at -80 °C or begin standard phenol-chloroform extraction to isolate total mRNA as previously described by our lab[93].
○ Use 50 – 200 ng of RNA for cDNA synthesis using the Mastermix according to the manufacturer’s instructions.

○ Subject cDNA to quantitative PCR amplification using IDT Primetime qPCR probes against mouse endothelial cell markers (CD31, *von Willebrand Factor* (vWF)), valve interstitial cell markers (*α-smooth muscle actin* (α-SMA), *Periostin* (Postn)), myocyte markers (*Mhc6, Mhc7*) and GAPDH.

- **Culturing Murine VECs**

  ○ After FACS sorting and cell collection (step 4.3), centrifuge cells at 300 g for 5 min.

  ○ Carefully pipette off the supernatant (sorting buffer + media) and discard.

  ○ Resuspend the GFP-positive cell pellet in 1 mL of VEC media and the GFP-negative pellet in 1 mL non-endothelial media.

  ○ Plate each sample in one well of a plastic chamber slide and grow until confluent. Culture for about 1 week for GFP negative cells to become confluent and more than 2 weeks for GFP positive cells to become confluent (from a sample of 3, adult mice). Change media every two days.
**Immunofluorescent Staining**

- Remove media from cells and fix in 4% paraformaldehyde (PFA) for 30 mins at room temperature. Wash fixed cells three times for 5 mins each in Phosphate Buffered Saline Solution (PBS).

- Remove chamber wells from slide and block in 5% bovine serum albumin/1xPBS for one hour at room temperature.

- Incubate slides overnight at 4°C with primary antibodies (CD31, 1:1000 or α-smooth muscle actin (α-SMA), 1:100). Wash for 5 mins in PBS, 3 times.

- Dilute the secondary antibody (Alexa-Fluor 568 Goat anti-Rat) 1:400 in PBS, add to slides, and incubate one hour at room temperature. Rinse slides for 5 mins in PBS, 3 times.

- Mount in Vectashield-containing DAPI and incubate 1 hour at 4°C prior to viewing.
2.3 Representative Results

2.3.1 Tie2-GFP cells co-localize with endothelial cell markers in the embryonic and adult heart valves.

In order to confirm the specificity of Tie2-GFP expression in VECs from embryonic and adult mice, immunofluorescence was performed to determine co-localization with the endothelial cell marker, CD31 in tissue sections prepared from E14.5 and 3 month old adult Tie2-GFP mice using methods previously published by our lab [94]. As shown in Figure 4, VECs co-express GFP (Fig. 4A, B, E, F) and CD31 (Fig. 4C, D, E, F) at both adult (Fig. 4B, D, F) and embryonic (Fig. 4A, C, E) stages, therefore validating our model for subsequent VEC isolation.

2.3.2 FACS analysis identified distinct GFP-positive and GFP-negative cell populations in embryonic and adult heart valves isolated from Tie2-GFP mice.

Wild type C57/Bl6 mice were used to set GFP parameters and approximately 2% of single-gated cells from Tie2-GFP mice show a significant enrichment in GFP-positive cells by FACS analysis in both embryonic and adult samples (Fig. 5). Based on co-localization studies shown in Figure 4, these cells are considered VECs and yield an average of 61,800 total cells (samples range from 39,000 – 77,000 cells/sample) in adult samples (n=3), and 8,928 cells (8,015 - 11,000 cells/litter) from one litter of E14.5 embryos.
2.3.3 PCR analysis confirms enrichment of endothelial cell markers in GFP-positive cells and valve interstitial cell markers in GFP-negative cells isolated from Tie2-GFP mice.

Gene expression analysis of GFP-positive and –negative cell populations by qPCR following FACS show distinct molecular profiles. Compared to GFP-negative cell populations isolated from Tie2-GFP embryos and adults, GFP-positive cells are enriched for expression of endothelial cell markers CD31 and vWF, (Figure 6). Further, expression of myocyte markers (Myh6, Myh7) in these cell populations is very low, demonstrating minimal contamination of non-endothelial cells. In contrast, GFP-negative cells isolated from adult Tie2-GFP mice and E14.5 embryos are enriched for valve interstitial cell (VIC) markers, α-sma and Periostin (POSTN) relative to GFP-positive cells. Enrichment of these markers is higher in GFP-negative cells isolated from E14.5 samples compared to adults due to the quiescent phenotype of adult VICs and therefore low expression of activated markers.

2.3.4 GFP-positive cells isolated from Tie2-GFP adult mice can be cultured in vitro.

The ability to culture GFP-positive and GFP-negative cells isolated from adult samples was examined based on cell morphology and immunohistochemical staining. Using protocols previously described by us[95] we show in Figure 7 that GFP-positive cells appear round in morphology (Fig. 7A) and co-express GFP with the endothelial cell marker CD31 after two weeks in culture (Fig. 7C). In contrast, non-endothelial cells are negative for GFP, display a mesenchymal-like morphology (Fig. 7B) and express the VIC marker α–SMA after nine days (Fig. 7D).
2.4 Discussion

Here we describe for the first time, a novel method for the isolation of embryonic and adult murine VECs from Tie2-GFP mice. While this mouse line has been extensively used for the isolation of endothelial cell populations, this is the first report showing selective isolation of VECs. Due to the fragility of the VEC population, we have developed a stringent protocol that allows for single cell isolation of GFP-positive (and GFP-negative) cells from heart valves of embryonic and adult mice. Compared to the original publication using whole embryos or organs from Tie2-GFP mice[92], we have optimized collagenase and dissection steps based on the low abundance of this fragile endothelial cell population to isolate a select population of cells.

VEC isolations have previously only been reported in large animal models with limited genetic and biomolecular tools. These tools are well established in mice and therefore, the ability to isolate murine VECs allows for an expanded set of experimental designs to be used for heart valve research. Therefore, a significant advantage of this approach is that VECs can be isolated temporally from wild type mice, and models of valve disease and injury. A second advantage is that VECs can be isolated and analyzed almost immediately after dissection, preserving expression patterns that reflect the in vivo situation more accurately. Further, this isolation protocol is sufficient to eliminate cell culture for number expansion and therefore potential phenotypic changes induced by the in vitro environment are prevented.
Despite the novelties and experimental benefits of this protocol, we recognize that limitations still exist. First, the size of the VEC population within murine valves is very small and therefore, multiple timed embryonic litters are needed in order to generate sufficient RNA for gene expression analysis. While this can be overcome using multiple breeders, it could have an impact on the application of some post-isolation analysis tools. This limitation has been a challenge particularly for establishing confluent cultures of GFP-positive VECs in order to perform more thorough analyses of VEC phenotypes including molecular profiles and functional assays. Therefore, we acknowledge that not all difficulties have been overcome by our approach but this is an area of interest that we are striving to overcome.

This approach of isolating VECs from valvular regions introduces the possibility of contamination from Tie-GFP-positive, non-valvular endothelial cells of the endocardium, or vascular structures within the ventricular myocardium. To date, molecular distinction of VECs from other cardiac endothelial cell populations have not been identified. However an enhancer region of the \textit{Nfatc1} gene has been identified and shown to specifically label VECs that do not undergo EMT, and no other endothelial cell population within the heart[96]. As a \textit{Cre} model (\textit{Nfatc1}^\textit{enCre}) is available, future studies could utilize the specificity of this line to minimize contamination risks. In addition to non-valvular \textit{Tie2-GFP}-positive cells, there is always the possibility of contamination from myocytes at the point where the valve leaflets attach to the annular region of the septal and mural myocardial walls. In data not shown here, we initially began studies to isolate VECs from dissected valvular regions using antibody-conjugated beads coated with anti-GFP and
anti-CD31. While this approach was successful for isolating the endothelial cells, we experienced significant cell clumping from adjacent, non-endothelial cell types and therefore VICs and myocardial cells contaminated our experimental sample. This has been avoided using FACS analysis as parameters have been set to isolate only single cell suspensions and PCR analysis to detect expression of myocyte-specific genes has controlled for this limitation (Figure 6). While our contamination can be deemed as minimal, it remains a potential experimental complexity which could be avoided in the future with the double selection of GFP and endothelial cell-specific surface markers.

Using this protocol, we have successfully isolated VECs from embryonic and adult mice and provided examples of how this approach can be used for RNA isolation and cell culture of GFP-positive and GFP-negative cell populations. However, this approach is not limited to these applications and can be used for a plethora of molecular, cellular and functional approaches. In addition, the Tie2-GFP background can be bred with genetic mouse models that will allow for comparative studies of VEC populations in health and disease. The development of this novel methodology will, for the first time, allow for focused studies examining the contribution of VECs in valve development and maintenance and could reveal previously unappreciated mechanisms of endothelial-dependent valve disease.
Figure 4. Tie2-GFP-positive cells co-localize with CD31 in embryonic and adult heart valves. Immunofluorescence to show association of (Tie2-)GFP expression (A, B) with the endothelial cell marker, CD31 (C, D) in the septal leaflets of the mitral valve at E14.5 (A, C, E) and adult (B, D, F) stages. The valve region is highlighted in A, C, E. (E, F) Merged images.
Figure 5. Tie2-GFP-positive VECs can be identified by FACS. Compared to age-matched C57/Bl6 controls (A, C), valves isolated from Tie2-GFP embryos (B) and adults (D) contain a distinct GFP-positive cell population, as indicated in green. GFP-negative events, shown in red were collected as a control. Numbers in B and D indicate the average % of GFP-positive cells from the total number of events sorted by FACS (n=3).
Figure 6. GFP-positive cells are enriched for endothelial cell markers whereas GFP-negative cells express genes associated with valve interstitial cells. Representative qPCR of endothelial cell markers (CD31, von Willebrand Factor (vWF)) and adult (Myh6) and fetal (Myh7) myocyte markers in GFP-positive cells isolated from valvular regions of E14.5 (A) and adult (B) Tie2-GFP mice. Note enrichment of endothelial cell markers and very low levels of myocyte-associated genes. (C, D) Fold changes in expression of valve interstitial cell markers α-sma and Postn in isolated GFP-negative cells from E14.5 (C) and adult (D) Tie2-GFP mice.
Figure 7. Tie2-GFP-positive cells maintain expression of endothelial cell markers in vitro. Following FACS, GFP-positive (A, C) and –negative (B, D) cell populations from adult mice were cultured until confluent and subject to phase contrast imaging (A, B) and fluorescent immunostaining (C, D). GFP-positive cells express CD31 (red) (C) after 10 days of culture. In contrast, GFP expression was not detected in the negative cell population, which stained positive for α-SMA, a marker of activated VICs (D).
Chapter 3: Growth and maturation of heart valves leads to changes in endothelial cell distribution, impaired function, decreased metabolism and reduced cell proliferation

3.1 Introduction

The mature heart valve leaflets are highly organized structures that open and close over 100,000 times a day to regulate unidirectional blood flow through the heart. Movement is largely facilitated by three highly organized layers of extracellular matrix (ECM) that provide all the necessary biomechanics to respond to changes in the hemodynamic environment during the cardiac cycle. In contrast, disruption to ECM organization is often associated with insufficiency that can lead to progressive heart failure[2]. Homeostasis of the valve ECM is mediated by a heterogeneous population of fibroblast-like valve interstitial cells (VICs), and previous studies have shown that VIC function is regulated by a single layer of valve endothelial cells (VECs) that line the surface of the leaflets[20, 32-35, 67]. In addition to regulating VIC behavior, the valve endothelium

3 Lindsey J. Anstine, Chris Bobba, Samir Ghadiali, and Joy Lincoln. Growth and maturation of heart valves leads to decreased endothelial cell distribution, impaired function, metabolism and reduced cell proliferation. JMCC. 2016.
serves as a physical barrier between the blood and the inner valve tissue; thereby preventing excess infiltration of circulating factors associated with risk and inflammatory cells[20, 21]. A murine wire injury model suggests that physical denudation of the aortic valve endothelium is sufficient to induce disease[97], consistent with histological findings reporting loss of endothelial cells in diseased human valves[5, 6, 50]. In addition, VEC-specific disruption of essential signaling pathways can alter ECM organization and lead to dysfunction in mice[34, 35, 98-101]. Therefore, integrity and function of the valve endothelium appear to be essential for maintaining structure-function relationships throughout life.

Aging is a significant risk factor of heart valve dysfunction, affecting up to 13.2% of people over the age of 75[3]. Age-related disorders of the vascular system are attributed to progressive endothelial cell dysfunction and pathological landmarks and mechanisms of this process have been used as early predictors of cardiovascular disease and targeted for therapeutic treatments respectively[102-105]. These studies have largely been focused on the association of vascular endothelial cell dysfunction with impaired endothelial nitric oxide (NO) synthesis. Similar to vascular endothelial cells, VECs also require NO to maintain function as reduced bioavailability of endothelium-derived NO leads to morphological defects at birth and dysfunction in adults[34, 101, 106-109]. While the requirement for NO is conserved, previous studies have noted several differences between vascular and valvular endothelial cells largely in their molecular and phenotypic response to biomechanical stress[24]. Therefore, it is not clear if determinants of age-related
endothelial cell dysfunction in vascular disease can account for failure of the valve to maintain structure-function relationships later in life.

To address this deficit, we investigated VEC histology, function and molecular profiles at key stages of valve development, growth, maturation, and aging. Our findings demonstrate that maintenance of the valve is a multifactorial process, and aging is associated with changes in VEC density, decreased function, reduced proliferation, and diverse molecular profiles. These findings highlight the potential mechanisms that may be abrogated in later stages of life, and potentially diseased valves that lead to impaired homeostasis.

3.2 Methods

3.2.1 Mice

*Tie2GFP (Tg(TIE2GFP)287Sato/J)* and wild type *C57BL/6J* were obtained from Jackson Labs. Mice aged to E14.5 (embryonic) post-natal day 1-3 (PN) 4 months old (young adult), and >12 months old (aging adult) were used for all studies unless otherwise stated in the text. All animal procedures were approved and performed in accordance with IACUC and institutional guidelines provided by The Research Institute at Nationwide Children’s Hospital.

3.2.2 Isolation of VECs from Tie2GFP mice

Murine VECs were isolated from E14.5, PND2-3, 4 month-old and 12-15 month-old *Tie2GFP* mice as previously described by our lab[110]. Briefly, valvular tissue from
both semilunar and atrioventricular valves was dissected and dissociated using collagenase IV for 7 mins at 37°C. The supernatant, containing the dissociated cells was collected and kept on ice. This process was repeated nine times in order to collect an enriched population of endothelial cells. Isolated cells were pelleted and resuspended in HBSS containing EDTA and DNaseI (RNase-free) and subjected to flow cytometry to collect the GFP+ endothelial cells as described below. All samples collected consisted of multiple biological replicates pooled together as shown in Table 1.

**Table 1. Number of GFP+ VECs collected and RNA yield**

<table>
<thead>
<tr>
<th>Sample</th>
<th># animals/litters pooled</th>
<th># GFP+ cells collected</th>
<th>RNA yield (ng)</th>
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</thead>
<tbody>
<tr>
<td>E14.5.1</td>
<td>1 Litter</td>
<td>8,015</td>
<td>8.0</td>
</tr>
<tr>
<td>E14.5.2</td>
<td>2 Litters</td>
<td>22,000</td>
<td>1.9</td>
</tr>
<tr>
<td>E14.5.3</td>
<td>2 Litters</td>
<td>18,000</td>
<td>15.7</td>
</tr>
<tr>
<td>PN.1</td>
<td>1 Litter</td>
<td>53,000</td>
<td>5.6</td>
</tr>
<tr>
<td>PN.2</td>
<td>1 Litter</td>
<td>30,000</td>
<td>38.4</td>
</tr>
<tr>
<td>PN.3</td>
<td>1 Litter</td>
<td>49,000</td>
<td>4.48</td>
</tr>
<tr>
<td>4 mo. Adult.1</td>
<td>3 mice</td>
<td>62,000</td>
<td>8.5</td>
</tr>
<tr>
<td>4 mo. Adult.2</td>
<td>3 mice</td>
<td>77,000</td>
<td>4.78</td>
</tr>
<tr>
<td>4 mo. Adult.3</td>
<td>3 mice</td>
<td>59,000</td>
<td>3.36</td>
</tr>
<tr>
<td>12-15 mo. Adult.1</td>
<td>3 mice</td>
<td>53,000</td>
<td>2.32</td>
</tr>
<tr>
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<td>3 mice</td>
<td>50,000</td>
<td>1.36</td>
</tr>
<tr>
<td>12-15 mo. Adult.3</td>
<td>3 mice</td>
<td>57,034</td>
<td>2.04</td>
</tr>
</tbody>
</table>
3.2.3 Histology

Bright-field and immunofluorescence

Whole embryos and whole hearts from embryonic, PN, young adult, and aging adult Tie2GFP or C57BL/6J mice were dissected and fixed overnight in 4% PFA/1xPBS at 4°C and subsequently processed for paraffin or cryo embedding. Paraffin tissue sections were cut at 7μm and subjected to Pentachrome staining according to the manufacturer (American MasterTech). Cryo-embedded tissue sections were cut at 7μm and stored -20° before immunofluorescent staining. Briefly, cryo sections were blocked for 1 hour (1%BSA, 1% cold water fish skin gelatin, 0.1% Tween-20/PBS) followed by incubation with CD31 (BD Biosciences #553370 rat anti-mouse 1:1000) or CD45 (R&D Systems AF114 rabbit 1:200) diluted in 1:1 Block/1xPBS overnight. On the next day, slides were incubated with goat-anti-rat-488 or goat-anti-rabbit-568 Alexa-Fluor secondary antibody for one hour at room temperature, mounted with Vectashield containing DAPI, and imaged on an Olympus BX51 microscope. Quantification of CD45+ cells was reported as a percentage of total DAPI+ cells in aortic valves from E14.5, PN, young and aging adult C57BL/6J mice. Statistical significance was determined using the Student’s t-test between each time point for n=3.

Quantification of VEC cell density

VEC density was quantified from at least 5 aortic valve sections taken from 3 biological replicates stained with Toluidine blue. Aortic valve cusps were divided into proximal (area between annulus and hinge region), mid (area between hinge and distal tip)
and distal (tip region denoted by increase in cross sectional area of the leaflet) regions based on morphology. For quantification, the number of endothelial cells on the cusp surface spanning the proximal, mid, and distal regions of young and aging adult aortic valves were counted and divided by the endothelial surface distance (µm) measured by ImageJ software. The number of VECs per 50µm of the valve surface was reported for each region. Significance was determined using the Student’s t-test between distal, mid, and proximal regions or between young and aging adult regions.

Transmission Electron Microscopy

Whole hearts from embryonic, PN, young adult, and aging adult C57BL/6J mice were placed in 2.5% glutaraldehyde in Millonig’s PO₄ with glucose for 24-48 hours at 4°C and then transferred into Millonig’s Buffer prior to aortic valve dissection. Dissected aortic valves were post fixed in 1% osmium tetroxide for 2 hours to overnight, rinsed in Millonig’s PO₄ buffer, and dehydrated in graded ethanol series and cleared in propylene oxide. Tissue was then infiltrated in 50-50 Epon/Araldite–propylene oxide for 4 hours followed by infiltration in full Epon/Araldite overnight. Samples were embedded in fresh Epon/Araldite and placed in a 60°C oven for 24-48 hours. Blocks were trimmed and 500 nm thick sections were cut and collected onto slides, which were stained with Toluidine blue and assessed for areas of interest. Selected blocks were cut at 60nm on a Leica Ultracut UCT ultramicrotome. Sections were collected onto CuPd grids and stained with uranyl acetate and lead citrate before viewing on a Hitachi H 7650 TEM. Observations
were concluded from imaging three independent aortic valve samples from each reported time point.

3.2.4 Culturing porcine aortic valve endothelial cells

Young and aging porcine aortic valve endothelial cells (pAVECs) were isolated from AoV cusps from 6-7 month-old juvenile pigs (young) or >2 year-old adult pigs (aging) (Animal Technologies) respectively as previously described[90]. Cells were cultured in DMEM containing 10% L-glutamine, 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin (pen/strep), and 50U/ml heparin sodium salt (Sigma). All experiments were carried out between passages 2-5 and aging and young pAVECs were always compared at the same passage number (p2-3).

3.2.5 DAR-4M AM Staining

Cultured pAVECs

Confluent young and old pAVECs were washed with PBS and treated with 10μM DAR-4M AM (CalBioChem 251765) for 30 minutes at 37°C. Cells were then rinsed, mounted in Vectashield containing DAPI (Vector), and imaged in the Texas Red channel. Alternatively, cultures were treated with an NO donor, 250 μM DETA NONOate (Cayman Chemicals 82120), under the same culture conditions, as a positive control. For quantification, color and brightness thresholds were set using ImageJ and integrated pixel density per field of view was measured. Statistical significance between DAR-4M AM+ young and aging pAVECs was determined using the Student’s t-test for n=4.
Murine aortic valves

*Tie2GFP* PN and aging adult mice were euthanized and gravity perfused through the left ventricle with 1xPBS until blood had been flushed out of the heart, followed by a 10 min perfusion with 0.01 mmol/L DAR4M- AM/PBS[111]. Hearts were then flushed with 1xPBS perfusion for 10 minutes followed by 4% PFA for 5 minutes. Hearts were removed and fixed in 4% PFA for 1 hour at room temperature before cryo-embedding. 7μm cryo sections were rinsed in PBS, mounted in Vectashield containing DAPI (Vector), and viewed in the Texas Red-568 channel. The number of DAR-4M AM+ cells per field of view was calculated in images of aortic valves from PN and aging mice and reported as a percentage of total DAPI+ cells.

3.2.6 *CellROX Staining for Reactive Oxygen Species (ROS)*

Hearts from PN and aging adult wild type mice were harvested and immediately cryo-embedded without fixation. On the same day, 7μm sections were cut and mounted. Slides were washed briefly with PBS and incubated with 5μM CellRox reagent (Fisher C10422) for 30 minutes at 37°C. Immediately following, slides were washed and mounted with Vectashield containing DAPI and imaged in the FITC 488 channel on an Olympus BX51 microscope. The number of CellROX+ cells, per field of view, was calculated in images of aortic valves from PN and aging mice and reported as a percentage of total DAPI+ cells for n=3.
3.2.7 EMT assay using pAVECs

Young and aging pAVECs were grown to confluency on plastic 24 well plates (for RNA extraction) or on Matrigen Softwell Custom collagen I coated, 8Kpa, hydrogel plates (for immunocytochemistry). Cells were treated with fresh medium, supplemented with TGF-β1 (2ng/ml) or with equal volumes of 0.1% BSA every 2-3 days for 7 days. Following culture, cells were fixed in 4% PFA for 30 minutes at room temperature and stored in PBS until immunocytochemistry or lysed in Trizol (Invitrogen) RNA extraction respectively (n=8). For immunostaining, cells were 4% PFA- (SMA) or methanol-fixed (VE-cadherin) for 20 minutes, blocked and incubated with primary antibodies against VE-cadherin (Santa Cruz SC-6458, 1:100) or SMA (Sigma A2546, 1:100) for two hours at room temperature. Secondary antibodies were applied as described above (3.2.3).

3.2.8 In Vivo Permeability With Evans Blue Dye (EBD)

5μg/μl/10g body weight Evans Blue Dye (Sigma) was injected via tail vein into PN and aging adult C57BL/6J mice. Twenty-four hours later, mice were sacrificed and subjected to whole body perfusion with 1xPBS, after which, hearts were dissected and immediately frozen in Tissue-Tek OCT Compound. Tissue was cut into 7μm sections and post-fixed in 4% PFA for 10 minutes at room temperature. Slides were then washed briefly in PBS and mounted in aqueous mounting medium. The presence of Evans blue dye fluorescence within aortic valves was imaged in the Texas Red channel on an Olympus BX51 microscope and overlaid with bright-field differential interference contrast (DIC) images (n=3).
3.2.9 In vitro permeability assay

In vitro permeability assays were carried out as described previously [112]. Briefly, young and aging pAVECs were plated on collagen-coated 6.5 mm diameter, 3.0μm pore size transwell inserts (Corning). Two monolayers were plated in each insert at a density of 1x10^5 over a period of 48 hours. 24 hours later Dextran-TMR (Life Tech D-1868) was added to the lower chamber of each well. 10μl of media was removed from the upper compartment at 15, 30, 60, 90, and 120 minutes after treatment, and diluted 1:10 in water. Diluted samples were analyzed with spectroscopy at 555-excitation/580-emission (n=4).

3.2.10 Cell stretching membrane injury assay.

Aging and young PAVECs were grown to 95-100% confluency on collagen coated 6-well BioFlex culture plates (FlexCell International). Directly before the experiment, fresh cell media (see above) was added containing 0.4mg/mL 4,000MW FITC-dextran. Cells were then subjected to 20 minutes of either 0% (no stretch) or 18% equibiaxial strain on Flexcell FX-5000 tension system. The plate was then washed twice with 1xPBS and incubated for 10 minutes in 4mM Ethidium bromide. Following two further 1xPBS washes, the membranes were cut out with a scalpel and placed on a dish with 1xPBS for imaging. Fluorescent images were captured with an Olympus IX81 microscope, using CY3 and FITC filters. 4 images were taken randomly within a 12.5mm radius of the center of each well using identical lamp intensity and exposure times. The number of FITC and Ethidium Bromide positive cells were then manually counted using ImageJ software over a total of three biological replicates. Under these conditions, FITC positive cells experienced plasma
membrane rupture followed by active membrane repair to retain the FITC dextran. Ethidium bromide positive cells have permanent plasma membrane rupture (dead cells). Injured and repaired cell fractions were calculated as the total number of FITC-dextran labeled cells divided by the total number of cells while dead cell fraction was determined by counting the number of EB labeled cells and dividing by the total number. Cells labeled with both EB and FITC-dextran were counted as dead. Areas of cell detachment were determined by outlining cell borders and calculating the cell area as a percentage of the total image area (n=3).

3.2.11 Cell Cycle Analysis

EdU injections and quantification

PND7, young adult, and aging adult mice were injected subcutaneously with 5μg/g body weight EdU (Invitrogen). 7 hours later, mice were sacrificed and hearts were harvested. Alternatively, mice were injected once a day for 7 days and tissue was harvested 2 weeks later. Tissue was fixed in 4% PFA overnight and processed for cryo-embedding. 7μm sections were subjected to Click-it EdU (Invitrogen) detection and immunofluorescent staining for CD31 (BD Biosciences rat #553370 anti-mouse 1:1000) according the manufacturer’s instructions. Proliferating VECs were detected by co-expression of CD31 and EdU. Every 6th tissue section spanning the aortic valve region was collected and used for quantification (n=3). Significance was determined using the student’s t-test between PND7 and young adult mice injected with a 7h single dose or between PND21 and young adult, PND21 and aging adult, or young adult and aging adult 7d pulse samples.
DNA content analysis of isolated murine VECs

Isolated VECs from PN and young adult mice, as described previously in 2.1 and by our lab[110]. Cells were then pelleted, resuspended in 4% paraformaldehyde for 10 minutes at 37°C, and chilled on ice for one minute. Fixed cells were stored in 1% sodium azide/PBS at 4°C until analysis. On the day of analysis, cells were permeabilized in 90% Methanol on ice for 30 minutes, rinsed, labeled with 1ug/ml DAPI (Pierce) with 0.1% Triton X-100, and incubated at 37° for 30 minutes. Cells were analyzed for DNA content using an Aria III cell sorter in which ~2,500 events were captured and Modfit software was used to determine DNA content after removal of aggregates (n=4). Student’s t-test was used to determine significance between GFP+ PN and young-adult cells in G1 phase or S/G2 phase, or between GFP+ and GFP- cells.

3.2.12 Valve Endothelial Cell Isolation and RNA-seq

VECs from embryonic, PN, young adult, and aging adult (12-15 months) mice were isolated as previously described by our lab[110] and described in Chapter 2. Isolated cells were stored in Trizol and submitted for RNA extraction and RNA sequencing at Ocean Ridge Biosciences (Palm Beach Gardens, Florida, USA). Sequencing was performed on a HISEq 2500 and all samples had a minimum of 83 million passed filter reads. Reads were aligned with 79% efficiency to the UCSC mm10 reference genome. Normalized counts of sequence reads (RPKM) were annotated to Ensembl genes and values were filtered to retain a list of genes with a minimum of approximately 50 mapped reads in one or more samples. Differential gene expression was assessed by Tukey test. Fold changes below the reliable
detection threshold (50 reads/gene) were reported at “NA”. Statistical significance and false discovery rates were calculated using linear regression and the Benjamini and Hochberg methods respectively. Pathway analysis was performed using WebGestalt software in which KEGG, Wiki Pathways, and GO Pathways are queried to report the statistically significant distribution of differentially regulated genes among functional biological pathways.

3.2.13 Quantitative Polymerase Chain Reaction

RNA was extracted from murine VECs and pAVECs with Trizol according the manufactures protocol (Invitrogen) and cDNA and PCR reactions were performed as previously reported by our lab[113]. Quantitative real-time PCR using a Step One Plus Real Time PCR system (Applied Biosystems) was used to detect changes in gene expression for Col4a2, Itga1, Rbm47, Chrdl1, Trim55, and Hrh2 using Primetime assays from Integrated DNA Technologies; normalized to GAPDH and for α-SMA, Snail, von Willebrand Factor (vWF), and Tie2 using Taqman probes; normalized to 18S. Fold change was determined relative to control and significance was calculated using student’s t-test (n=3-6).

3.3 Results

3.3.1 VEC density and cell-cell connections decrease with growth and maturation.

To examine age-associated changes in aortic valve structure, histological analysis was performed at embryonic (E14.5, post-EMT early remodeling), PN (PND1-3, growth
and elongation), young adult (4 months, maintained), and aging adult (>12 months, aging) stages (Figures 8A-D). As indicated by Movat’s Pentachrome stain, developing valves at embryonic stages are enriched in proteoglycans (blue, Figures 8A, B) and by the young adult stage, collagen (teal/yellow) and elastin (black) (Figures 8C and D) are more detectable consistent with previous reports detailing specific collagen types and elastin[2, 93]. In addition to the ECM, transmission electron microscopy analysis reveals temporal changes in valve cell morphology (Figures 8E-H). At the embryonic stage, VECs are cobble-stone like in shape, dense (arrows, Figure 8E), and in physical contact with underlying VICs (*, and arrowhead, Figure 8E). By PN stages, VEC morphology is more flattened (arrows, Figure 8F), and contact with VICs is observed (*, Figure 8F). In young adults, VECs exhibit long cytoplasmic extensions along the cusp surface (arrow, Figure 8G), and physical contacts with VICs are grossly rare. In the aging mouse, VEC nuclei are larger (arrow, Figure 8H) with extensive bundles of ECM fibers (bracket, Figure 8H) separating them from underlying VICs (arrowhead, Figure 8H). Toluidine blue staining of young and aging adult aortic valves highlights age-dependent changes in the spatial distribution of VECs in the ‘mid’ region of the cusp (Figure 8I-K) with a significant decrease associated with aging. Despite changes in VEC density, the relative percentage of VICs (~66.7%) and VECs (~33.2%) per field of view at embryonic through aging stages remains constant (data not shown).
3.3.2 VEC function declines with growth and maturation.

Reduced endothelial nitric oxide (NO) bioavailability and increased reactive oxygen species (ROS) production have been associated with valve disease onset, consistent with other cardiovascular systems[34, 65, 67-69]. At the post-natal stage, ROS was detected by CellROX (ROX) reagent in VECs and VICs at low levels (Figure 9A, C) and this was consistent with high NO production as indicated by DAR-4M AM staining (Figure 9D, F). In contrast, ROX was high (Figure 9B, C) and DAR-4M AM (Figure 9E, F) low at aging stages. To further support these findings, pAVECs isolated from >2 year-old aging pigs also demonstrated decreased NO production (Figure 9H, I) compared to pAVECS from younger (6 months) adult animals at the valve maintenance stage (Figure 9G, I). In association with oxidative stress, mitochondria within VECs from aging adult mice show mitochondrial disorganization (Figure 9K) compared to younger time points (Figure 9J). Together, this suggests that NO bioavailability and cell metabolism is impaired in aging VECs.

Previous studies have suggested that VECs undergo endothelial-to-mesenchymal transformation (EMT) to replenish the VIC population over a lifetime[32, 33, 84, 114]. To examine differences in EMT potential, young and aging pAVECs with cobblestone-like morphology (Figure 10A, B) were treated with 2ng/mL of the potent inducer, TGFβ-1. After 7 days, expression of the endothelial cell markers Tie2 and von Willebrand Factor (vWF) were decreased, while the mesenchyme marker smooth muscle α-actin (SMA) was increased more significantly in treated young pAVECs compared to BSA controls (Figures 10A, C, E) and aging pAVECs (Figures 10B, D, E).
In valves, the endothelium serves as a physical barrier to protect the underlying tissue from hemodynamic stress and circulating factors[20, 21]. To determine potential age-related changes in endothelial permeability, young adult and aging adult wild type mice were subject to tail vein injections of Evans Blue dye (EBD). In aortic valves from young mice, permeation across the endothelium was low and localized within the proximal region on the ventricularis side (arrowheads Figures 11A, C); consistent with low VEC density (Figures 11A, 1K). However, in the aging adult EBD was observed along the ventricularis (arrows, Figure 11D) and fibrosa (arrowheads, Figure 11D) surfaces at proximal and distal regions, consistent with reduced VEC density during aging (Figure 11B). This age-dependent increase in endothelial permeability correlates with increased infiltration of circulating CD45+ cells at the aging stage (Figures 11E-G). In support of in vivo findings, permeability assays in vitro demonstrate that compared to young, aging pAVECs are significantly more permeable (Figure 11H).

3.3.3 Young pAVECs are more resistant to stretch-induced injury in vitro.

To determine potential differences in cell membrane repair of young and old valve endothelial cells following physical injury, pAVECs were subjected to no stretch or 18% equibiaxial stretch at 1Hz for 20 minutes in the presence of FITC-labeled Dextran. As shown in Figure 12, the number of FITC-positive cells is higher in 18% stretched young pAVECs, compared to stretched aging pAVECs and no stretch controls (Figures 12A-C). pAVECs were treated with Ethidium Bromide to label cells with permanent plasma membrane breaks (necrosis), however negligible levels were detected in young and aging
cells (Figures 12A, B). In association, the number of detached cells after 18% stretch is greater in aging, compared to young, or unstretched pAVECs (Figure 12D). Taken together this data suggests that aging pAVECs have a greater cell detachment after stretch-induced injury, while young pAVECs are able to self repair their cell membrane to promote survivability.

3.3.4 Proliferation of VECs significantly declines after post-natal stages.

To examine potential changes in valve cell proliferation with aging, PN or young adult wild type mice were either injected with a single dose of EdU and harvested 7 hours later (7h single dose), or PN, young adult, or aging adult wild type mice were injected daily for 7 consecutive days (7d pulse) and harvested 2 weeks later (Figure 13A). Immunohistochemistry using anti-EdU on 7h single dose PN mice shows that ~6.2% of VECs (of the total VEC population) and ~3.3% of VICs (of the total VIC population) are proliferative (Figures 13B-D). These numbers are significantly higher than that observed in 7h single dose-treated young adult mice (~0.7% VECs, ~0.3% VICs), respectively. As expected, the 7d pulse approach captured a higher percentage of cells proliferating in all stages; however, proliferation rates of VECs and VICs were similar at the PN stage (Figure 13D). By the young adult stage, rates of both VECs (~2%) and VICs (~1.1%) were significantly lower than PN stages (~16.3% VECs, ~15.2% VICs), however, VECs (~2%) maintain a higher proliferative capacity than VICs (~1.1%). No significant differences in cell proliferation were noted between young and aging adult time points. To support EdU in vivo data, murine VECs from endothelial cell reporter (Tie2-GFP) mice were isolated
by flow cytometry and co-stained with DAPI to examine DNA content (Figures 13E-G). Consistently, a higher percentage of \emph{Tie2-GFP}+ cells were observed in S and G2 phases during PN stages when compared to young adults. In addition, the number of proliferating VECs at PN and adult stages was higher than \emph{Tie2-GFP} negative cells that are likely rich in the VIC population.

\subsection*{3.3.5 VECs have age-dependent mRNA profiles.}

In order to define the molecular profiles of age-related changes in VECs, RNA-seq was performed on VECs isolated from embryonic, PN, young adult, and aging adult \emph{Tie2-GFP} mice using published methods\cite{110}. Analysis revealed several mRNAs differentially expressed between each time point, with the largest change observed between embryonic and aging adults, and the smallest between young and aging adults (Figures 14A, B). This approach also revealed mRNAs that were unique and common to VEC populations at one, or more time points (Figure 14B), as well as distinct gene clustering patterns (Figure 14C) at each stage (Figure 14) (See Figure 15 for validation). As expected several ‘classic’ endothelial markers were expressed in VECs, many of which demonstrate age-dependent expression patterns (Figure 14D). Additional Wiki bioinformatics analysis highlight biological functions predicted to be affected by differentially expressed genes at each time point. Of the mRNAs downregulated between PN and aging adults (and other time points as indicated in Figure 13E), \emph{Cell Cycle} (orange, Figure 14E), \emph{Focal Adhesions} (green, Figure 14E) and \emph{Oxidative Phosphorylation} (blue, Figure 14E) pathways were enriched
supporting findings for reduced proliferation (Figure 13), increased permeability (Figure 11) and disorganized mitochondria (Figures 9G, H).

3.4 Discussion

This current study demonstrates that VECs undergo significant age-related changes related to morphology and distribution, function and gene expression. We observe that aging VECs have lower cell proliferation rates, attenuated nitric oxide production, increased ROS, disorganized mitochondria, decreased SMA expression in response to TGFβ1 treatment and reduced cell membrane self repair in response to stretch-induced injury. These functional deficits are accompanied by a decline in endothelial cell density that correlates with increased permeability in older valves. Moreover, VECs express age-dependent transcriptional profiles, many of which support the observed decline in function. Thus, this study has made a significant contribution towards defining the characteristics of endothelial cell dysfunction, and produced a large-scale data set to define the molecular traits of VECs that likely contribute to age-related valve dysfunction.

Aging is a significant risk factor of valvular insufficiency, however the underlying mechanisms of valve maintenance that decline with age are not known. Using single dose, or pulse chase EdU we show that VIC and VEC proliferation rates are highest at PN stages compared to young and aging time points (Figure 13), and this is supported by pathway analysis of RNA-seq data showing that proliferation is as equally high at embryonic stages (Figure 14). Consistent with a decline in valve growth and remodeling, proliferation
significantly decreases in both cell populations by young adulthood (VECs -14.3%; VICs -14.0%), with a further decline during aging (VECs -0.24%; VICs -0.18%). Our study demonstrates that VECs have higher relative proliferation rates than VICs (Figures 13D and G). It is not clear why, but we speculate that more VECs proliferate after birth to maintain ‘endothelial coverage’ over the valve surface as the cusp elongates and thins during post natal growth and remodeling. While our study did not examine changes in the absolute numbers of VECs and VICs with aging, we can conclude that generation and expansion of the VEC and VIC pools occur during embryonic and post natal development, and beyond this, turnover of resident cells is low although the ratio between VEC and VIC number remains constant.

Histological analysis shows that VEC density within the endothelium is age dependent. At the embryonic stage, the cobblestone-like VECs are in close contact with each other, however the ‘clustering’ or density of VECs decreases with age (Figures 8I-K). In addition, physical contact between VECs and underlying VICs also declines. As a result, molecular communication between these cell types may become dysregulated with age, which in turn, could have significant implications on maintaining homeostatic phenotypes[20, 32-35, 67]. Changes in cell-cell contact are consistent with overrepresentation of downregulated mRNAs associated with focal adhesions (Figure 3.7E) and increased permeability (Figures 11C, D) at older time points. At the young adult stage, VEC distribution within the endothelium is not uniform, but cells are notably more clustered in mid regions on the fibrosa surface away from laminar blood flow. As expected, regions associated with low VEC density (ventricularis surface, distal) are associated with
hyper-permeability particularly in aged valves (Figures 11A-D), which may relate to increased infiltration of pathological stimuli including risk factors and excess inflammatory cells from the circulation in the older population[115, 116]. Furthermore, the ability of individual VECs to self repair their plasma membrane following injury induced by equibiaxial stretch is reduced with aging and this is associated with increased cell detachment which may further impact permeability.

In this study we utilized non-biased, high-throughput analysis to provide a much-needed database of VEC transcriptomes for the continued pursuit of identifying endothelial-mediated mechanisms of valve pathology. Our data shows that 83% of differentially expressed mRNAs are common to VECs at all time points (Figure 14B) and as expected, these include ‘classic’ endothelial markers (Figure 14D). The greatest number of differentially expressed genes was observed between embryonic and aging adults (1088 up, 4140 down), reflecting significant molecular diversity between development and degeneration. The least number of differentially expressed genes was observed between young and aging stages (28 up, 167 down). This suggests that molecular traits of VECs during stages of valve maintenance (young adulthood) are not significantly different from that of aged valves. Despite fewer mRNA changes cell function is notably impaired between maintenance and aging stages. This could be attributed to defects in transmission of signaling pathways between neighboring cells as a result of decreased cell-cell interactions (see Figure 8). In addition, it is also considered that RNA-seq does not capture post-translational or epigenetic modifications that could occur with aging.
Figure 8. Valve endothelial cell morphology, distribution, and cell contacts change with growth and maturation. (A-D) Movat’s Pentachrome staining to detect the deposition and organization of collagen (yellow), proteoglycan (blue), elastin (black), and muscle (red) in aortic valves from wild type mice at embryonic (E14.5) (A), post natal (PN) (B), young adult (4 months) (C) and aging adult (14 months) (D) stages. (E-H) Transmission electron microscopy of aortic valves at indicated time points. Arrows indicate valve endothelial cells (VECs), arrowheads show valve interstitial cells (VICs), and *denote contact between VECs and VICs. Bracket in H indicates expanded extracellular matrix. (I, J) Toluidine blue staining of aortic valves from young (4 months) and aging (15 months) wild type mice to show VEC density on fibrosa (arrows) and ventricularis (arrowhead) cusp surfaces. (K) Number of VECs on the surface of aortic valve cusps in distal, mid and proximal regions as indicted, n=3. Statistical significance based on *P<0.05. AoV, aortic valve.
Figure 9. Nitric oxide availability and mitochondrial organization are impaired with growth and maturation. (A, B) Aortic valves from post natal (PND7) (A) and aging adult (25 months) (B) wild type mice stained with CellROX reagent to detect reactive oxygen species in VECs (arrows) and VICs (arrowheads). (C) Percentage of ROX-positive cells over total number of cells (n=3). (D, E) DAR 4M-AM staining denoting nitric oxide availability in PN (C) and adult aging (19 months) (D) wild type aortic valves. (F) Percentage of DAR-4M AM-positive cells over total number of cells (n=3). DAR-4M-AM staining in cultured porcine aortic valve endothelial cells from young (G) and aging (H) animals. (I) Percentage of DAR-4M AM-positive cells over total number of cultured cells per field of view (n=3). (J, K) Transmission electron microscopy to show mitochondrial organization (arrows) in VECS from young adult (4 months) (J) and aging adult (14 months) (K) aortic valves of wild type mice. AoV, aortic valve. Statistical significance based on *P<0.05 in aging adult valves compared to post natal (PN) stages (n=3)
Figure 10. TGFβ1-mediated EMT is decreased with growth and maturation. (A-D) Immunofluorescent staining against the endothelial marker VE-cadherin (A, B, E, F) and mesenchymal marker, α-SMA (C, D, G, H), in young (A, C, E, G) and aging (B, D, F, H) pAVECs treated with 2ng/ml TGFβ1 every 2-3 for 7 days (E-H) compared to BSA treated controls (A-D). (I) Quantitative PCR to show fold changes of Tie2, vWF (endothelial), Snail and α-SMA (mesenchyme) in young and aging TGFβ1-treated pAVECs compared to BSA controls. Fold change relative to BSA controls, n=8. *p=<0.05 compared to BSA control, #p=<0.05 compared to TGFβ1-treated young pAVECs.
Figure 11. The valve endothelium is more permeable with maturation. (A, B) Toluidine blue staining of young adult (4 months) (A) and aging adult (14 month) (B) wild type aortic valves to show VEC density over the surface of the fibrosa (arrows), compared to the ventricularis (arrowheads). (C, D) Evans blue dye to determine permeability of the valve endothelium in young adult (C) and aging adult (D) wild type mice. (E, F) CD45 detection to show expression of infiltrating hematopoietic cells in young adult (E) and aging adult (F) aortic valves quantitation shown in (G). (H) Quantitation of relative fluorescence of permeated Dextran-TMR in young and aging pAVECs, n=4. *p=<0.05 compared to young pAVECs.
Figure 12. Cell membrane repair following stretch-induced injury decreases with maturation. (A, B) DIC images to show morphology and distribution of young (A) and aging (B) pAVECs following 18% equibiaxial stretch at 1Hz for 20 minutes. Green staining indicates cell-retained FITC-Dextran, while Propidium Iodide is in red with negligible detection. Quantitation of FITC positive cells are shown in C, and D indicates the % of detached cells under each condition, n=3, p<0.05 in aging compared to young pAVECs.
Figure 13. VEC proliferation declines with maturation. (A) Experimental design of EdU labeling in PN, young adult (4 months), and aging adult (15 months) aortic valves. (B, C) Representative aortic valves labeled with EdU (red) and CD31 (green) from 7h single dose treatment in PN (B) and young adult (4 months) (C) wild type mice. Arrowheads indicate EdU+ VECs, EdU+ VICs are marked by arrows. (D) Quantification of EdU+ VECs and VICs in aortic valves at indicated time points. n=3, *p<0.05 compared to PN, #p<0.05 compared to EdU+ VECs, +p<0.05 compared to 7h. (E, F) Flow cytometry to show DNA content of isolated VECs from PN (E) and young adult (4 months) (F) Tie2GFP mice, n=3. (G) Quantification of Modfit DNA content curves of isolated VECs (GFP+) and non-VECs (GFP-).
Figure 14. Age-dependent mRNA profiles of VECs. (A) Summary of the number of differentially expressed mRNAs between two comparative time points. (B) Venn diagram to show the number of protein-coding mRNAs (4852 total) expressed between time points based on ANOVA P-score <0.01 and max fold change >2. (C) Heat map to show hierarchal clustering between 3 biological replicates at each time point based on RPKM values for Continued
Figure 14 Continued

10,024 significant genes, with a false discovery rate (FDR) of <0.1. (D) Graph to show average RPKM values of detected endothelial cell markers. p<0.05 compared to E14.5 (*), PN (#) and 4 months (+). (E) WikiPathway analysis of –Log(P-value) of mRNAs significantly downregulated between PN and aging adult (12-15 month) time points. Orange indicates biological functions related to cell proliferation, processes shown in blue are related to cell metabolism, and green highlights focal adhesion. *p<0.05 between PN and young adult (4 months); #p<0.05 between embryonic (E14.5) and young adult (4 months); +p<0.05 between embryonic (E14.5) and PN; *p<0.05 between young adult (4 months) and aging adult (12-15 months). Supplemental Figure 1.
Figure 15. Validation of relative fold changes in gene expression between RNA-seq and qPCR expression analysis from isolated murine VECs. (A) Fold change of Itga1 and (B) Col4a2 expression level based on RPKM values from RNA-seq analysis compared to fold changes observe from independent qPCR analysis, n=3 *p=<0.05. (C) qPCR validation of highly enriched genes reported by RNA-seq (shown in Table 1), n=4, *p=<0.05.
Chapter 4: Infiltrating extra-cardiac lineages in heart valve development, maturation and aging

4.1 Introduction

As discussed in Chapter 1, the valve cell populations are heterogeneous in origin, arising from multiple cell lineages during embryogenesis. While the majority of mesenchymal valve interstitial cells (VICs) are derived from endothelial to mesenchymal transition (EMT), increasing evidence has demonstrated the contribution of non-endothelial cell lineages during development as well[74, 75]. Studies using chick-quail chimera systems and lineage tracing in mice, have shown significant contribution of epicardial-derived cells to the atrioventricular (AV) valves during early cushion stages[76, 77, 117]. Additional fate mapping studies in mice have revealed cells originating from the neural crest contribute to the outflow tract (OFT) and AV endocardial cushions[78, 118], and cells derived from the secondary heart field constitute a subpopulation of endothelial cells within the OFT cushions[79]. Although cardiac lineage contribution to the valve structures has not been reported in post-natal stages, incorporation of extra-cardiac cells, derived from the bone marrow has been observed in adult valves; however, the role of these cells is currently unknown[81, 82].
Early studies describing excised human and pig valve transplants reported chimeric cellular composition, in which both donor and host cells were present within the valve tissue[87, 88]. More recently, studies using bone marrow transplants (BMT) in adult mice have demonstrated that bone marrow-derived cells integrate into healthy valve tissue as a mechanism of normal valve homeostasis[81, 82]. BMT-derived cells within the valves are of the hematopoietic lineage as noted by their expression of the leukocyte common antigen, CD45. However, histological analysis to determine additional phenotypes of these cells has led to various results. In a study by Visconti et al, the authors conclude that the majority of transplanted cells take on a fibroblast-like phenotype (similar to VICs) due to their anatomical location and co-expression of pro collagen I[82]. Whereas, a subsequent study reported the co-expression of progenitor and immune cell markers, proposing a population of hematopoietic progenitor cells (CD133+/CD45+) and dendritic cells (CD11c+/MHC11+/CD45+)[81]. Further, some CD45+ cells are yet to be defined by co-expression studies and whether these cells are infiltrating immune cells or serve as precursors to replace the valve cell populations over time is still unknown. Thus, consensus on the phenotype and function of these populations remains incomplete and additional studies are needed to further define these cells.

To increase our understanding on this extra-cardiac population, we examined the contribution, phenotype, and heterogeneity of CD45+ cells within the valve. In addition, we investigated the effects of aging on this population based on previous reports by our lab and others, indicating VECs and VICs become increasingly dysfunctional with age[15, 44, 83], suggesting alternative mechanisms maintain valve homeostasis during aging. Our
findings show that a population of CD45+ cells are present in homeostatic murine valves beginning in embryonic stages and progressively increase in number during aging. Additionally, using bone marrow transplants, we report the incorporation of CD45+ cells into both the VEC and VIC compartments and this integration is significantly affected by the age of the transplanted cells as well as the age of the recipient tissue environment. Moreover, we show that endogenous and transplanted CD45+ cells within the valve are heterogenic for immune and valve cell markers with the majority of cells belonging to the myeloid lineage. We provide further evidence to show that the majority of CD45+, myeloid cells within the valves are not mature macrophages but rather nonclassical patrolling monocytes. Collectively, these findings clearly define the timing, contribution, and heterogeneity of extra-cardiac cells within the valves and demonstrate the effect of aging on this process.

4.2 Methods

4.2.1 Mice

Wild type C57BL/6J and C57BL/6-Tg(CAG-EGFP)1Osb/J (eGFP) were obtained from Jackson Labs. CX3CR1GFP+/− mice were a kind gift from Dr. Santiago Partida-Sanchez and CD45Cre and Ai9(RCL-tdT) reporter mice were a kind gift from Dr. Edwin Horwitz at The Research Institute at Nationwide Children’s Hospital. Male CD45Cre mice were crossed female Ai9 mice to report Cre recombinase activity in embryonic day (E)11.5, post-natal day 3 (PND3), and 6 week-old adult CD45Cre+;Ai9+/− progeny. CD45Cre+;Ai9+/− littermates were used as controls. All animal procedures were approved and performed in
accordance with IACUC and institutional guidelines provided by The Research Institute at Nationwide Children’s Hospital.

4.2.2 Histology

Whole embryos and whole hearts from embryonic, PN, and adult mice were dissected and fixed overnight in 4% PFA/1xPBS at 4°C and subsequently processed for paraffin or cryo embedding. (Adult mice underwent whole body perfusion with PBS before heart dissection and fixation). For paraffin sections, hearts were cut into 7μm sections, and subjected to immunofluorescent staining. Briefly, after deparaffinization, slides underwent antigen retrieval (Vector Laboratories H-3300) according to the manufacture’s protocol. Sections were blocked for 1 hour at room temperature followed by incubation with primary antibody diluted in 1:1 Block/1xPBS overnight (see Table 2 for antibodies and concentrations). On the next day, slides were incubated in Alexa-Fluor secondary antibodies diluted at 1:400 in PBS for 1 hour at room temperature, mounted in Vectashield containing DAPI and imaged on an Olympus BX51 microscope. Alternatively, hearts were processed and embedded for cryo and cut at 7μm sections. Slides were then blocked for 1 hour at room temperature and stained as described above. Histological quantification was performed by counting the cells of interest and total DAPI+ cells in every 18th tissue section spanning the aortic or mitral valve region of adult mice, every 9th section for PN, and every 6th section for embryonic (n=3). Results were reported at a percentage of total cells. Significance was found using the Student’s t-test between comparative time points or experimental groups.
Table 2. Antibodies and working concentrations.

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4.2.3 Cell Cycle Analysis

PND2 CD45Cre⁺;Ai9⁺⁻ mice were injected subcutaneously with 5μg/g body weight EdU (Invitrogen). 24 hours later, mice were sacrificed and hearts were harvested. Tissue was fixed in 4% PFA overnight and processed for cryo-embedding. 7μm sections were subjected to Click-it EdU (Invitrogen) detection and immunofluorescent staining for RFP (Rockland 600-401-379 1:3000) according the manufacturer’s instructions. Proliferating CD45Cre⁺;Ai9⁺⁻ cells were detected by co-expression of RFP and EdU. Every 9ᵗʰ tissue section spanning the aortic and mitral valve region was collected and used for quantification (n=3). Results were reported as a percentage of total CD45Cre⁺ cells.
4.2.4 Bone Marrow Transplants (BMT)

Preparation of Donor Cells

Femur and tibia bones from 7 week-old or >12 month-old eGFP female donors were collected, rinsed in HBSS containing 1% Pen Strep, and kept on ice. Whole bone marrow cells were isolated by flushing the bone cavity with 5 ml RPMI media containing 1% Pen Strep. Cells were strained through a 0.70μm strainer and resuspended in sterile HBSS at a concentration of 1.25x10⁶ cells/ml.

Irradiation and Bone Marrow Transplants (BMT)

7 week-old and >12 month old female C57BL/6J recipient mice received total body irradiation at 500cG followed by a second 500cG dose three hours later using an X-RAD 320 irradiator. Twenty-four hours later, recipients received 250,000 whole bone marrow cells collected from either 7 week-old or >12 month-old eGFP donors via tail vein injection. At 11 weeks post-BMT recipient mice were euthanized and subjected to whole body gravity perfusion with PBS. Organs, including the heart, were collected and fixed overnight in 4% PFA.

Peripheral blood reconstitution analysis

Peripheral blood reconstitution levels were determined at three weeks post-BMT. Briefly, recipient mice underwent submandibular bleeding. Blood was incubated in 1X RBC lysis buffer (BioLegend) and incubated for 15 minutes in the dark. Afterward, samples were washed, resuspended in HBSS containing 10% FBS, and submitted for flow
cytometric analysis for the presence of GFP. The number of GFP+ cells was reported as a percentage of total cells within the blood. Blood from homozygous eGFP mice and wild type C57BL/6J were used as positive and negative controls respectively.

4.2.5 Macrophage depletion using liposomal Clodronate

Liposomal Clodronate injections into BMT mice

Bone marrow transplantations in 7 week-old female C57BL/6J recipients were carried out with whole bone marrow from 7 week-old or >12 month-old eGFP donors (See above). At seven weeks post-BMT, recipients received intraperitoneal injections (IP) of either control empty liposomes or liposomes containing Clodronate (Encapsula NanoSciences #8901). Mice were injected every 3 days for 30 days at 40mg/kg body weight for the first dose and 20mg/kg body weight for all subsequent injections[119]. At 11 weeks post-BMT recipient mice were euthanized and subjected to whole body gravity perfusion with PBS. Organs, including the heart, were collected and fixed overnight in 4% PFA.

Assessing macrophage depletion efficiency in Clodronate treated mice

To assess the macrophage depletion efficiency of Clodronate treated mice, splenocytes and peripheral blood were isolated from mice injected with control empty liposomes and liposomes containing Clodronate at 11 weeks post-BMT. Red blood cells were lysed with 1X RBC lysis buffer and samples were blocked in Mouse BD FC Block (BD #553141). After blocking, cells were stained for F4/80 (BioLegend #123116 1:200),
GR1 (BioLegend #108433 1:400), Cd11b (BioLegend #101208 1:400) diluted in PBS containing 0.1% Sodium Azide and 1% Bovine Serum Albumin (BSA) for 20 min on ice (An isotype control against F4/80 was also prepared (BioLegend #400512)). After staining, cells were washed and resuspended in Near IR Live/Dead Cell Stain Kit (Life Technologies) for 30 minutes at room temperature. Subsequently, cells were fixed in 1% PFA for 15 minutes at room temperature and submitted for flow analysis to determine the number of cells expressing each marker of interest. Data was analyzed using FlowJo software.

4.3 Results

4.3.1 CD45+ cells in the aortic and mitral valve leaflets increase with aging.

To examine the contribution of extra cardiac cells to the murine aortic and mitral valves, histological staining for the lymphocyte common antigen, CD45, was performed in wild type mice at E14.5, PN, 4 months, and 16 months of age (Figures 16A-I). CD45+ cells were detected in the valve cushions/leaflets at each time point and the number of positive cells significantly increased between PN (aortic ~1.2%; mitral ~2.6%), adult (aortic ~8%; mitral ~6.7%), and aging time points (aortic ~10.9%; mitral ~10.5%) (Figures 16E, J). To support these findings, lineage tracing using CD45Cre mice crossed to an Ai9 reporter to generate CD45Cre*;Ai9+/− progeny at E11.5, PND3, and 6 weeks of age (Figure 17). CD45-derived cells were present in the aortic valve at each stage as marked by expression of red fluorescent protein (RFP) (Figure 17A-C), and significantly increased in number, between the PND3 (~2.3%) and 6 weeks (~10.3%) (Figure 17D). Co-staining of
CD45Cre+;Ai9+/-. With anti-CD45 revealed that the majority of CD45-derived cells, maintain endogenous CD45 expression over time (Figures 17A-C).

To further characterize the CD45+ cells in the valves at PN stages, we tested the proliferative capacity of CD45-derived cells. CD45Cre+;Ai9+/-. pups were injected with a single dose of EdU at PND2 and harvested 24 hours later. Immunohistochemistry using anti-EdU and RFP showed that the majority of CD45-derived cells in the aortic (95.1%) and mitral (91.2%) valve leaflets do not co-express EdU (Figure 18A-C). Together, these findings suggest that non-proliferative CD45-derived cells are present in the aortic and mitral valves and their presence significantly increases between PN and adult stages.

4.3.2 The majority of CD45-derived cells in the valve leaflets co-express the myeloid lineage marker, CD11b.

CD45+ cells are derived from the hematopoietic lineage and these cells have the potential to differentiate into myeloid, lymphoid, and tissue resident cell lineages[120-124]. The myeloid lineage includes circulating monocytes (CD11b+) which can subsequently differentiate into mature macrophages, marked by F4/80 expression. Anti-RFP immunofluorescent analysis of 6 week-old CD45Cre+;Ai9+/-. sections in combination with CD11b staining, revealed that ~65% of CD45-derived cells in the aortic valve are of the myeloid lineage (Figures 19A, E). However, very few CD45-derived cells were immunoreactive for F4/80 (~4%) consistent with previous reports (Figures 19B, E)[125]. To determine if CD45-derived cells differentiate into valve cell lineages, co-staining for RFP in combination with the VIC marker, Vimentin, and VEC marker, CD31 was carried
out. This analysis revealed approximately ~16% of CD45-derived cells co-localize with CD31 and ~11% co-localize with Vimentin (Figures 19C-E). This data suggests that the majority of CD45 -cells residing in the aortic valve are of the myeloid lineage and are likely monocytes due to detectable expression of CD11b and low levels of F4/80; while only a small fraction take on molecular phenotypes of resident valve cell lineages.

4.3.3 CD45+ Cells within the valve express the nonclassical, patrolling monocyte receptor CX3CR1.

As CD45-derived cells co-express CD11b+ in normal homeostatic valves (Figure 19), we hypothesized that this cell population may function as non-classical patrolling monocytes. This population of cells is characterized by expression of the homing chemokine (C-X3-C motif) receptor-like 1, CX3CR1, and recruited to tissues that express the chemokine (C-X3-C motif) ligand 1 (CX3CL1)[126]. Previously published RNA-seq data (Chapter 3) revealed expression of the CX3CL1 ligand in murine VECs at E14.5, PN, 4 months, and 12-15 month stages with increasing expression between PN and 4 month of age (Figure 20A)[83]. To determine if valvular CD45+ cells express the receptor CX3CR1, we obtained 5 month-old CX3CR1GFP+/− reporter mice. Histological analysis of these mice using antibodies against GFP and CD45, showed that a large percentage CD45+ cells in the aortic (~88%) (Figures 20B-D) and mitral valves (~77%) (Figures 20E-G) are CX3CR1GFP+. Taken together, this data suggests that the majority of the CD45+ cells detected in adult aortic and mitral valve leaflets, are likely myeloid, CX3CR1+ patrolling monocytes.
4.3.4 The contribution of extra-cardiac bone marrow derived cells to the homeostatic valve is an age-dependent process.

Previous studies have reported the contribution of extra-cardiac bone marrow derived cells (BMDCs) to the valve, yet whether circulating BMDCs are the primary source of CD45+ cells within the valve is still unknown. Further, as we detected age dependent changes in the number of CD45+ cells within the valve leaflets, we sought to determine how incorporation of the extra-cardiac bone marrow-derived cell population into the valve is affected by aging. To do this, bone marrow transplants (BMT) were carried out using 7 week-old (young), or >12-month old (aging) wild type, C57BL/6J, lethally irradiated recipient mice. Twenty-four hours after irradiation, recipients were injected with a single bolus of 250,000 whole bone marrow cells isolated from 7 week-old eGFP mice, which globally express GFP in all cells. Blood reconstitution was significantly higher at 3 weeks post-BMT in the >12 month-old recipients, consistent with previous reports (Appendix A Figure 25)[127, 128]. At 11 weeks post-BMT, hearts were harvested and analyzed for GFP+ cells within the valves using immunofluorescent staining. Approximately ~6% of total valve cells in the 7 week-old recipient and ~8% in the >12 month-old recipient were GFP+ (Figures 21A-C) and this incorporation was not due to irradiation induced injury (Appendix A Figure 26). In both recipient groups, eGFP+ cells generally localized in the distal tip (as previously described[81]) and in the ventricular mid region. To determine if transplanted cells could integrate into the both the VEC and VIC compartment, valve sections were co-stained with the endothelial marker, CD31. The number of eGFP+ cells detected in the VEC compartment (arrows) was significantly greater in the 7 week-old
recipient (~6% of total CD31+ cells) (Appendix A Figure 27) than the >12-month recipient (~3%). However, significantly more eGFP+ cells were detected within the VIC compartment in the >12 month-old recipient (~10% of total CD31- cells) when compared to the 7 week-old recipient (~6%) (Figures 21A-C).

To further understand the effect of aging on BMDC incorporation into heart valves, we next tested whether BMDCs isolated from aging mice could incorporate into young and aging valvular tissue. To do this, we performed bone marrow transplants in 7 week-old and >12 month-old recipients using whole bone marrow harvested from >12 month-old donors. Blood reconstitution levels at 3 weeks post-BMT were found to be significantly higher in 7 week-old recipients as previously reported (Appendix A Figure 25)[128]. Interestingly, eGFP+ cells incorporated at a rate of ~7% in 7-week-old recipients but only at about ~2.4% in >12 month-old recipients (Figures 21D-F). Additionally, significantly more eGFP+ cells were detected in the VIC compartment compared to the VEC compartment in both the 7 week-old recipient (VIC ~7.9%; VEC ~2.2%) and >12 month old recipient (VIC ~2.7%; VEC ~1.04%). Localization of eGFP+ cells in the 7 week-old recipient was similar to that of recipients reconstituted with BMDCs derived from 7 week-old donors. Collectively, these data suggest that incorporation of transplanted BMDCs into the valve is dependent on the age of the cells as well as the age of the valvular tissue environment.

4.3.5 The heterogeneity of transplanted BMDCs within the valve is dependent on age.

In order to determine if bone-marrow derived cells express immune and resident valve cell markers, and how this expression is affected by age, co-expression of eGFP+
cells with immune and valve cell markers within the aortic valve leaflets of 7 week-old recipients transplanted with bone marrow from a 7 week-old donor showed that the majority of eGFP+ cells were CD11b+ (~46.2%), similar to the CD45Cre-derived cells described earlier (Figure 19) and ~5.6% of eGFP+ cells were F4/80+ (Figures 22A-B, E). Interestingly, in aortic leaflets of >12 month-old recipients, the number of GFP+ cells co-expressing CD11b+ was drastically reduced (~10.4%) and only ~1.3% of eGFP+ cells were F4/80+ (Figures 22F-G, J). Histological analysis for valve cell markers in 7 week-old recipients transplanted with bone marrow from a 7 week-old donor showed eGFP+ cells co-expressing CD31 (~8.1%) (Appendix A Figure 27) and Vimentin (~8%) (Figures 22C-E). Further, in >12 month-old recipient valves, the distribution of eGFP+ cells co-expressing valve cell markers was skewed with ~2.7% expressing CD31 and ~13% expressing Vimentin (Figures 22H-J).

Co-expression analysis in 7 week-old recipient mice that received >12 month-old bone marrow showed that eGFP+ cells co-localize with CD11b (~26.7%), CD31 (~5.6%), and Vimentin (~12.1%) (Figures 23A, C-E); whereas eGFP+ cells in 12 month-old recipients co-expressed CD11b (~33.3%), and Vimentin (12.2%) with no eGFP+/CD31+ cells detected (Figures 23F, H-J). Additionally, no F4/80+ cells were detected in either group (Figures 23B, G). Moreover, >90% of eGFP+ cells in all BMT groups co-localized with CD45 (Appendix A Fig. 28). Taken together this data implies that both the age of the native tissue environment and the age of the transplanted cells impacts the molecular phenotype of integrated BMDCs.
4.3.6 Transplanted BMDCs within the aortic valve are not mature macrophages.

To confirm F4/80 co-expression findings, we used liposomal Clodronate to deplete macrophages in BMT recipients. To do this, 7 week-old recipient mice underwent bone marrow transplants in which they received whole bone marrow from either 7 week-old or >12 month-old eGFP donors. At 7 weeks post-BMT, mice were treated with the macrophage depletion drug, liposomal Clodronate, every 3 days for 30 days in order to deplete macrophages within adult murine tissue[119]. Alternatively, transplanted mice received empty (vehicle) liposomes as control. At 11 weeks post-BMT, macrophage depletion, assessed by flow cytometry, demonstrated a ~90.4% reduction in splenic macrophages in recipients who received 7 week-old bone marrow and a ~95.7% reduction in those treated with >12-month old bone marrow (Figure 24A). Macrophage depletion was also observed in the liver of Clodronate treated mice (data not shown). Immunofluorescence for eGFP in heart valve sections of clodronate and control treated mice showed no significant difference in the number of infiltrating eGFP+ cells within the aortic valve (Figures 24B-F). These results further confirm that transplanted BMDCs integrating into the heart valves are not mature macrophages.

4.4 Discussion

In this study, we describe the contribution and molecular phenotype of CD45+ cells within murine heart valves, and how the contribution of extra-cardiac cells is affected by aging. Through lineage tracing and histological analysis, we demonstrate a progressive
increase in the number of CD45+ cells in aortic and mitral valves throughout maturation and aging. Phenotypic analysis of these cells shows that only ~16% co-express the resident VEC marker, CD31, and ~11% co-express the VIC marker, Vimentin; whereas ~65% belong to the myeloid lineage and are likely non-classical, patrolling monocytes. Additionally, bone marrow transplant experiments reveal that the contribution of transplanted CD45+ cells to the valves is affected by age of donor cells, and age of the recipient tissue; and further, phenotype of transplanted cells recapitulates that of endogenous CD45+ cells. Thus, this study has significantly impacted our understanding of extra-cardiac cells within the valve and how their contribution and phenotype is affected during maturation and aging.

Embryonic valve precursor cells are constituted from multiple cell lineages during development. Although cells of extra-cardiac origin have been previously reported in adult valves[81, 82, 87], we demonstrate for the first time that this population is present as early as E11.5 and significantly expands in cell number between PN and young adult stages. Using a single dose of EdU, we show that these cells do not undergo significant proliferation at the PN stage, and therefore the expansion of this population is likely due to increased infiltration from the circulation. Through immunofluorescent co-expression analyses, we detected rare endogenous and transplanted extra-cardiac cells co-expressing VEC (CD31) and VIC (Vimentin) markers; however, these cells do not appear to fully take on the typical valve cell phenotype as mainly noted by their continuous expression of CD45. Previous studies using the BMT model, reported a subset of transplanted cells take on a fibroblast-like phenotype as indicated by their morphology and expression of pro-
collagen 1[82] and that all transplanted cells express Vimentin[81]. This discrepancy could be explained by the population of bone marrow derived cells used for transplantation in which our study utilized whole bone marrow isolations compared to the isolated hematopoietic stem cells (HSCs) employed in previous studies. Also, variation in the age of the mouse at the time of BMT and time of sacrifice, also likely contribute to differential results. Conversely, we detected the majority of transplanted cells to co-express CD11b. CD11b+ cells mark the myeloid lineage, including circulating monocytes, which are divided into two major categories. Classical, inflammatory monocytes are recruited to sites of injury via their expression of the chemokine receptor CCR2 and subsequently differentiate into inflammatory macrophages once inside the injured tissue[126]. Whereas, nonclassical, patrolling monocytes express high levels of CX3CR1 and continually patrol homeostatic tissues, removing debris and serving as a ‘watch-dog’ for sites of injury. Analysis of CCR2−/− knockout mice revealed no difference in the number of CD45+ cells within the aortic valves compared to age matched C57BL/6J controls, indicating that the majority CD45+ cells within the valves are not inflammatory monocytes (data not shown). Further, F4/80 co-localization and macrophage depletion experiments indicated very few mature macrophages in the valves of wild type and BMT mice, further corroborating our findings that most CD45+ cells within the homeostatic valves are likely nonclassical, patrolling CX3CR1+ monocytes (Figures 22-24). Patrolling monocytes are known to produce high levels of anti-inflammatory and wound healing factors[126, 129]. Thus, these cells may aid in promoting tissue homeostasis throughout life, in which the concomitant increase in patrolling monocytes during aging is necessary to maintain tissue health as the
exposure to circulatory risk factors and mechanical stress increases. Additionally, the loss of these cells likely promotes the progression of disease states by increasing the susceptibility of the valvular tissue to inflammation-induced tissue damage.

Through BMT studies, significantly more transplanted cells were found to incorporate into the valves of >12 month-old recipients. Although we cannot directly compare endogenous CD45+ cells to transplant models, we speculate that the mechanisms underlying age-dependent increases in cell infiltration are likely conserved. We previously demonstrated age-dependent changes in VEC distribution, in which VECs become more disperse, correlating with compromised endothelial barrier function (Chapter 3)[83]. Enhanced permeability of the valve endothelium could potentially lead to increased infiltration of circulating cells. Additionally, we detected a significant increase in CX3CL1 chemokine expression during adult stages and showed that the majority of extra-cardiac cells within the valves express the corresponding homing receptor, CX3CR1. Thus, increased expression of homing molecules, in conjunction with decreased barrier function could help explain the increases in CD45+ cells within adult and aging homeostatic valves. Contradictory to these results, we observed significantly fewer cells within the valves of >12 month old recipients given bone marrow from >12 month old donors. Although we cannot directly explain this result, we predict that this is likely due to the decreased ability of old/compromised donor cells to effectively establish a functional niche within the bone marrow following transplantation (reflected in the peripheral blood reconstitution percentage Appendix Figure 25), a phenomenon that has been well described in the literature[127, 128].
In summary, our findings demonstrate that extra-cardiac CD45+ cells within in valve increase in number throughout maturation and aging and are heterogeneous in phenotype, with the majority of these cells expressing nonclassical, patrolling monocyte markers. We also provide new evidence that aging significantly impacts the incorporation and phenotype of these cells. A significant influx in the number of CD45+ cells within the valves has been reported during disease states [130, 131]. While this study focused on extra-cardiac cells in homeostatic and aging valves, future studies are needed to determine the role of CD45+ cells in disease states.
Figure 16. Percentage of CD45+ cells in aortic and mitral valve leaflets increases with age. (A-I) Immunofluorescent staining for CD45 (red) and DAPI (blue), for total nucleated cells, in aortic (A-D) and mitral valves (F-I) from wild type E14.5 (A, F), PN (B, G), 4 month-old (C, H) and 16 month-old (D, I) mice. (E, J) Quantification of CD45+ cells represented as a percentage of total cells at each time point in aortic (E) and mitral (J) valves. n=3 Statistical significance based on *p<0.05.
Figure 17. Percentage of CD45-derived cells in the aortic valve increases with age. Immunofluorescent staining of CD45Cre⁺;Ai9⁺/⁻ mice at E11.5 (A), PND3 (B), and 6 weeks (C) of age against RFP (red), to detect CD45-derived cells, and against CD45, to detect endogenous CD45 expression (green). (D) Quantification of the number of CD45-derived (RFP+) cells out of total number of cells at each time point. n=3. *p<0.05.
Figure 18. The majority of CD45-derived cells are non-proliferative at the postnatal stage. (A-B) Representative aortic (A) and mitral (B) valves from CD\textsuperscript{45}Cre\textsuperscript{+};Ai9\textsuperscript{+/−} mice labeled with EdU (green) and RFP (red) from a single 24 hour injection at PND2. Arrows indicate EdU+ CD45-derived cells. (C) Quantification of EdU+ CD45-derived cells in aortic and mitral valve leaflets. n=3. No significant difference was found in the number of proliferating CD45-derived cells between aortic and mitral valves.
Figure 19. CD45-derived cells are heterogeneous but commonly express the myeloid lineage marker, CD11b. (A-D) Immunofluorescent staining of 6 week-old CD45Cre⁺;Ai9⁺⁺ aortic valves for immune (Cd11b and F4/80) (A-B), and valve cell (CD31 and Vimentin) markers (green) (C-D). Costaining with RFP denotes CD45-derived cells in red. Arrows mark double positive cells. (E) Quantification of the number of CD45-derived cells expressing CD11b, CD31, Vimentin, and F4/80. n=3 Statistical significance based on *p<0.05 between each group.
Figure 20. The nonclassical monocyte recruitment signaling axis, CX3CL1/CX3CR1 is expressed in aortic and mitral valves. (A) Average RPKM values for CX3CL1 mRNA expression in VECs at E14.5, PN, 4 months, and 12-15 months of age, obtained from RNA-seq data on isolated murine VECs (REF, See Chapter 3). (B-G) Immunofluorescence to detect CX3CR1-GFP and CD45 in aortic (B-D) and mitral (E-G) valve leaflets of 5-month old CX3CR1GFP+/− reporter mice. n=3 Statistical analysis based on *p<0.05 between RPKM values at each time point.
Figure 21. Contribution of transplanted bone marrow-derived cells to the aortic valve is an age dependent process. (A-B) Immunofluorescent analysis for eGFP (green) and CD31 (red) in aortic valves from 7 week-old (A, D) and >12 month-old (B, E) BMT recipients treated with bone marrow from a 7 week-old donor (A-B) or >12 month-old donor (D-E) to show integration of eGFP+ cells into the VIC and VEC (arrows) compartments. (C, F) Quantification of total eGFP+ cells, eGFP+ CD31+ double positive (VEC compartment) and eGFP+ CD31 negative (VIC compartment) cells in BMT recipients given 7 week-old bone marrow (C) or >12 month-old bone marrow (F). n=3 p*<0.05 in >12 month old recipients compared to 7 week-old recipients.
Figure 22. Heterogeneity of transplanted 7 week-old BMDCs in the aortic valve is affected by the aging tissue environment. (A-D) Immunofluorescent staining of aortic valves from 7 week-old (A-E) and >12 month old (F-J) BMT recipients who received bone marrow from a 7 week-old donor, against immune (Cd11b and F4/80) (A-B, F-G) and valve cell (CD31 and Vimentin) markers (green) (C-D, H-I). Costaining with eGFP denotes transplanted cells in green. (E) Quantification of the number of eGFP+ cells expressing CD11b, CD31, Vimentin, and F4/80 in 7 week-old recipients (E) and >12 month-old recipients (J). Arrows mark double positive cells. n=3 Statistical significance based on p<0.05 between CD11b+. CD31+, Vimentin+, F4/80+ cells.
Figure 23. Molecular phenotypes of transplanted BMDCs within aortic valves is influenced by age and native tissue environment. (A-D) Immunofluorescent staining of aortic valves from 7 week-old (A-E) and >12 month old (F-J) BMT recipients who received bone marrow from a >12 month-old donor, against immune (Cd11b and F4/80) (A-B, F-G) and valve cell (CD31 and Vimentin) markers (green) (C-D, H-I). Costaining with eGFP denotes transplanted cells in green. (E) Quantification of the number of eGFP+ cells expressing CD11b, CD31, Vimentin, and F4/80 in 7 week-old recipients (E) and >12 month-old recipients (J). Arrows mark double positive cells.
Figure 24. Transplanted BMDCs within the aortic valve do not differentiate into mature macrophages. (A) Representative flow cytometry plots of isolated splenocytes from wild type, control (empty liposome), and liposomal Clodronate treated 7 week-old recipient BMT mice, stained for CD11b-PE and F4/80-APC. The gated (circled) area shows the depleted macrophage population in clodronate treated mice. (B-E) Immunofluorescent staining of control (B, D) or Clodronate (D, E) treated 7 week-old
Figure 24 Continued

recipients who received bone marrow from 7 week-old donors (B-C) or >12 month-old (D-E) donors stained for eGFP (green), to show transplanted cells, and CD31 (red), to mark the VEC compartment. (F) Quantification of the number of eGFP+ cells in the aortic valves of control and Clodronate treated BMT recipients. n=3 *p<0.05 between Control and Clodronate groups and between recipients who received either 7 week-old and >12 month-old donor cells.
Chapter 5: Concluding remarks and future directions

It is well appreciated that heart valves require a delicate balance of molecular cues and structural components to maintain homeostasis throughout life; and disruptions in these factors commonly result in valvular dysfunction. This has been widely described by the observed VIC activation and changes in ECM composition leading to disorganization and altered biomechanics. However, much less is known about how age-related changes in the VEC population contribute to the progression of degeneration. The current work presented here, provides detailed descriptive analyses of how changes in the phenotype and function of VECs, occurs throughout maturation and aging, identifying key cellular processes that significantly decline over time. Additionally, we investigate an extra-cardiac cell population within the valves and describe the molecular phenotype of these cells as well how aging affects their incorporation into the valve structure. Together, our data shows that both the resident and extra-cardiac valve cell populations are highly sensitive to age-related changes which could have large implications for valvular degeneration and disease. Through this work, we also provide novel tools for the field of heart valve biology in our development of a novel method for the isolation of murine VECs, as well as the generation of a temporal, transcriptional map of VECs throughout aging. These tools will inevitably allow for previously unfeasible experiments to be performed, generating new insight into valvular biology.
5.1 A novel method for the isolation of murine VECs

Until now, experiments aimed at understanding the role VECs have been limited to a narrow range of tools. Although VEC isolations had been previously reported using large adult animal models, limited genetic and biomolecular tools in these species restricted the experimental applications available. As an expansive set of these tools are available in mice, the development of a murine VEC isolation method, was a critical step in an effort to expand the set of experimental designs for heart valve research. Therefore, work in Chapter 2, described the development of a novel method to isolate murine VECs. This protocol utilized careful dissection of the valvular regions of hearts, isolated from Tie2GFP reporter mice, followed by fluorescence-activated cell sorting (FACS) to collect GFP+ valvular endothelial cells. Notably, our method was not only the first to report VEC isolation in mice, but was also the first method designed to isolate VECs from embryonic stages. Although our method combines VECs from all four valves, this protocol can be easily adapted to collect VECs from each valve independently. Most importantly, by crossing the Tie2GFP model to mouse models of valve disease, we can now pursue previously unfeasible experiments to understand the specific roles of VECs during disease.

5.2 Age-dependent changes in VECs

VECs are in direct contact with the hemodynamic environment and therefore exposed to circulating risk factors, inflammatory cells, and mechanical stress[20, 21]. In addition to providing a physical barrier around the underlying VICs and ECM proteins, VECs are known to molecularly communicate with VICs to regulate their phenotype and
function[31, 34, 35]. In contrast, degenerative valves have been associated with disruptions in the VEC layer indicating that integrity and function of the valve endothelium is imperative to maintain valve homeostasis[5, 6, 50, 97]. Similarly, in other cardiovascular systems, a decline in endothelial cell integrity and function play a major role in promoting pathological changes. For example, work from the vascular field has shown that decline in vascular endothelial functions, including nitric oxide bioavailability, metabolism, and barrier function underlies cardiovascular age-associated disease states such as atherosclerosis and hypertension[65]. While similar mechanisms have been speculated in the valves, studies to support this are lacking.

In Chapter 3, we identify key cellular processes and pathways within VECs that significantly decline with aging including nitric oxide bioavailability, metabolism, endothelial-to-mesenchymal potential, membrane self-repair and proliferation. In addition, we noted that the density of VEC distribution along the endothelium decreases from embryonic to aging stages and this is associated with changes in morphology, decreased cell-cell interactions, and increased permeability; suggesting that the aging valve is more susceptible to permeation of circulatory risk factors and immune cells. As the important role of endothelial barrier function has been established in other systems and several anti-permeability factors have been identified for use as potential therapeutic candidates[132], further understanding of age-associated VEC permeability is an attractive target for age-associated valve degeneration research.

Although decline in the pathways and processes identified in this study do not seem to independently lead to disease states, it is likely that the compromised functionality of
VECs in combination with other influences, including increased exposure to risk factors and genetic susceptibility, sets the stage for the onset of age-associated disease. For example, mouse models of hypocholesteremia develop aortic valve stenosis when compounded with old age. Further examination of the valve leaflets in these mice, reveals increased numbers of circulating immune cells as well as increased oxidative stress[59, 70]. These noted alterations could be related to the age-related changes in VEC cellular processes reported here, including decreased metabolism, increased oxidative stress, and decreased barrier function leaving the valve more susceptible to the hypercholesteremic environment. However, more work is needed to determine how the complexities of genetic alterations and the hemodynamic environment contribute to VEC dysfunction in mouse models of human valve disease.

5.3 Contribution, phenotype and effect of aging on CD45+ cells within the valve

Bone marrow derived cells include cells of the hematopoietic lineage which are identified by their expression of the lymphocyte common antigen, CD45. Hematopoietic cells give rise to circulating myeloid and lymphoid cell lineages that ultimately differentiate into the various blood and immune cell types[126]. Additionally, hematopoietic cells have been shown to integrate into peripheral tissues and differentiate into resident cell lineages[120-124]. Previous studies have reported the presence of CD45+ cells within the leaflets of porcine[88], murine[81, 82], and human[87] homeostatic adult valves, and although there is some evidence that these cells differentiate into fibroblast and dendritic cell fates, the results are inconclusive and thus the phenotype and function of
these cells remains highly controversial. Further, although these cells have been identified in adult valves, the timing and contribution during embryonic and post-natal stages remains unexplored.

In Chapter 4, we conducted lineage tracing analysis to show that CD45 derived cells are present in the valve structures starting at embryonic stages and are increasingly detected within the valve throughout maturation and aging. Through immunofluorescent co-expression and macrophage depletion studies, we demonstrate that the majority of CD45+ cells within the adult valve leaflets are CD11b/CX3CR1 positive and do not differentiate into mature macrophages, indicating they are likely patrolling monocytes. Patrolling monocytes are known to circulate throughout the body, entering peripheral tissues and surveying for sites of damage or infection[126]. Therefore, the majority of CD45+ cells within the valve leaflets are most likely transient, continuously moving in and out of the valve tissue. Although transient, these cells likely play a significant role in maintaining valve homeostasis. As patrolling monocytes express high levels of anti-inflammatory and wound healing factors it is possible that these cells function to promote tissue homeostasis throughout life, expanding in number as tissues become increasingly damaged and inflamed throughout life[126, 129]. In addition, loss of this cell population may leave the valve tissue more susceptible to damaging stimuli thereby accelerating tissue degeneration. Thus, future work will focus on the role of this cell population within healthy and degenerating valves.

Through bone marrow transplant (BMT) experiments we traced a CD45+ population within the valve that recapitulated the phenotype and distribution observed with
CD45Cre fate mapping. To explore the effects of aging on these cells and their integration into the valve, we performed BMTs using both young (7 week-old) and old (>12 month-old) donors, and young and old recipients and revealed that the incorporation and phenotype of CD45+ transplanted cells was dependent on both the age of the cell and the age of the tissue environment. Although we did not address the long term effects or functions of transplanted BMDCs within the aging valve, these studies support the possibility that transplantation of young BMDCs could have the potential to serve as a therapeutic for age-related valvular degeneration. Evidence from other systems also supports this notion. For example, it has been shown that subsets of BMDCs derived from young donors can home into the vasculature and promote cardiac angiogenesis in old recipients, improving vascular function[133]. Similarly, another study examining the renal system, showed that young BMDCs transplanted into old recipients attenuated renal aging[134]. Mechanisms underlying this effect include paracrine effects of young BMDCs on the surrounding host cells as well as increased maintenance of the anti-aging protein, Klotho[134]. Our studies show that transplanted BMDCs from young donors can integrate into the aging recipient valve at similar efficiency to that of young recipients; however, future studies are required to determine if these same mechanisms shown in other systems are at play in the valve.

Two major categories of circulating monocytes have been described, the nonclassical patrolling monocytes described above, which continually patrol homeostatic tissues, serving as ‘watch dogs’ for sites of damage and the classical inflammatory monocytes that are recruited to injured sites where they subsequently differentiate into
macrophages, eliciting an immune response[126]. Recent studies have reported an influx in the number of CD45+ cells detected within diseased valves. In these examples, infiltrating CD45+ cells appear to mainly promote disease progression. For example, in myxomatous valves, hematopoietic cells are shown to contribute to matrix disruption through secretion of ECM remodeling proteins[131]; and in stenotic aortic valves of ApoE−/− mice, CD45+ cells give rise to osteoblast-like smooth muscle cells which are localized to calcific regions[59]. Interestingly, sheep with ischemic mitral valve regurgitation following myocardial infarction display CD45+ VECs that subsequently undergo EMT; however, in this example it is unclear as whether this EMT event is reparative or damaging[130]. As the presence of patrolling monocytes within the tissues is a transient event, it is likely that the population of monocytes we describe in homeostatic valves is independent of the that reported in disease states. We predict that during homeostasis, patrolling monocytes primarily account for the majority of CD45+ cells within the valve; however, upon disease onset, classical monocytes home into the tissue, essentially shifting the CD45+ population from patrolling to inflammatory. Thus, further studies are needed to determine the transient nature of these cells and the role of both classical and nonclassical monocytes in homeostasis and disease.

5.4 Clinical perspectives

Despite the increasing burden of heart valve disease there are currently no pharmacological agents available to cure valve disease. Drugs such as statins, Angiotensin-converting enzyme (ACE) inhibitors, vasodilators, and β-blockers have shown promising
trends in their ability to partially alleviate the effects of either valve stenosis or regurgitation, but solidifying evidence of improved clinical outcomes from these treatments alone is lacking[135]. Therefore, identifying additional mechanisms underlying valve disease and degeneration is critical for the development of novel therapeutic strategies. The work presented here provides insight into the mechanisms underlying age-related changes in the valve cell populations. While previous work has described the age-associated changes in VICs and ECM components, this study has provided the first comprehensive view of the VEC population, demonstrating their previously unappreciated temporal heterogeneity while establishing the essential processes affected during aging in wild type mice. The temporal transcriptomes reported here provide a ‘map’ outlining both homeostatic and degenerative processes that can be used to identity and target endothelial dysfunction. As a progressively dysfunctional VEC layer likely contributes to disease onset, developing combinatorial therapeutic strategies to target changes in age associated VEC functions, including increased oxidative stress and permeability will likely lead to improved outcomes. Additionally, our work clearly defines the increasing contribution of extra cardiac cells to the valve structures throughout life, and identifies the previously unreported patrolling monocyte phenotype which likely promotes valve homeostasis. Using this knowledge, future studies will aim at stimulating endogenous valve cell functions while enhancing extra cardiac cell infiltration and function in order to prevent disease onset.

At present, tissue engineered heart valves (TEHV) are the most promising replacement application due to many factors including their long-term structural integrity
and decreased immunogenicity[136, 137]. Although the application of seeding bone marrow-derived stem cells onto TEHV scaffolds before implantation has shown encouraging results both in vivo and vitro[136], current TEHV designs are highly susceptible to degenerative processes including calcification and thrombus formation, requiring additional intervention. As patrolling monocytes are known to have anti-inflammatory and wound healing functions our findings suggest that seeding TEHV scaffolds with the appropriate BMDC molecular phenotype and concentrations could improve the permanence of TEHVs. In addition, we hypothesize that during the onset of degeneration, classical monocytes are recruited to the valve, essentially shifting population from patrolling to inflammatory. Thus we propose that directly boosting the patrolling monocyte integration after implantation (through BMTs for example) could promote reparative mechanisms necessary to promote longevity. In addition, we demonstrate the sensitivity of these cells to intrinsic and extrinsic age-related changes in which young BMDCs incorporate at higher frequency and phenocopy endogenous extra-cardiac populations. Therefore, seeding intrinsically young BMDCs into TEHV scaffolds could also lead to improved outcomes.

In closing, the multifactorial nature of aging, combined with the experimental limitations available for valve cell research has limited the progress of understanding age-associated valve degeneration. The work presented here reveals new insight into the mechanisms underlying age-related changes in valve cell populations that can be harnessed to develop new therapeutic strategies. Moreover, through this work we provide novel tools
for the cardiovascular community will push the field forward by allowing for previously unfeasible studies to be performed.
Appendix A: Chapter 4 supplementary material
Figure 25. Peripheral blood recantation in mice receiving bone marrow transplants. (A) Representative flow cytometry plots of peripheral blood samples collected from C57BL/6J (GFP negative control), eGFP+/− (GFP+ control), and BMT mice 3 weeks post-BMT to show GFP+ cells within the bloodstream. (B) Percent of peripheral blood cells expressing GFP is plotted for each mouse. *p<0.05 compared to 7 week-old recipients.
Figure 26. **CD45+ cell infiltration is not due to irradiation induced injury.** 7 week old and >12 month-old mice were given a split dose of 500cG irradiation, 3 hours apart. 24 hours later mice hearts were harvested for histochemical analysis. (A-C) Immunofluorescence for the apoptotic marker, Cleaved Caspase 3 in 7 week old mice (A) and > 12 month old mice (B) reveals no irradiation induced apoptosis. Tissue sections of non-irradiated E14.5 limb buds were used as a positive control for Cleaved Caspase 3 (C).
Figure 27. Transplanted cells incorporate into the VEC layer. Immunofluorescent staining for eGFP (green) and CD31 (red) reveals the incorporation of transplanted BMDCs into the VEC compartment of aortic (A) and mitral (B) valves from 7 week-old recipient mice transplanted with bone marrow from >12 month-old donors at 11 weeks post-BMT.
Figure 28. The majority of transplanted cells within the valve leaflets express CD45.

(A-D) Representative immunofluorescent images of aortic valves from 7 week-old recipients (A, C) and >12 month-old recipients (B, D) who received a BMT from either 7 week-old (A-B) or >12 month-old (C-D) donors stained for CD45 (red) and eGFP (green).

(E) Quantification of immunofluorescent images to show the percent of eGFP+/CD45+ cells out of total transplanted (eGFP+) cells for each experimental group. n=3
References


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