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Review

The Emerging Role of Valve Interstitial Cell Phenotypes in Regulating Heart Valve Pathobiology

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The study of the cellular and molecular pathogenesis of heart valve disease is an emerging area of research made possible by the availability of cultures of valve interstitial cells (VICs) and valve endothelial cells (VECs) and by the design and use of *in vitro* and *in vivo* experimental systems that model elements of valve biological and pathobiological activity. VICs are the most common cells in the valve and are distinct from other mesenchymal cell types in other organs. We present a conceptual approach to the investigation of VICs by focusing on VIC phenotype-function relationships. Our review suggests that there are five identifiable phenotypes of VICs that define the current understanding of their cellular and molecular functions. These include embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), activated VICs (aVICs), progenitor VICs (pVICs), and osteoblastic VICs (obVICs). Although these may exhibit plasticity and may convert from one form to another, compartmentalizing VIC function into distinct phenotypes is useful in bringing clarity to our understanding of VIC pathobiology. We present a conceptual model that is useful in the design and interpretation of studies on the function of an important phenotype in disease, the activated VIC. We hope this review will inspire members of the investigative pathology community to consider valve pathobiology as an exciting new frontier exploring pathogenesis and discovering new therapeutic targets in cardiovascular diseases. ***(Am J Pathol 2007, 171:1407–1418; DOI: 10.2353/ajpath.2007.070251)***

Valve interstitial cells (VICs) are the most prevalent cells in the heart valve and are found in all three layers of the valve—the fibrosa, the spongiosa, and the ventricularis. They are thought to be responsible for maintaining the structural integrity of the valve; however, our understanding of VIC biology and pathobiology has been very poor until recently.[1–3] Pathologists relied on descriptive studies of the gross and histopathological changes in diseased human valves, discovered meticulously by cardiovascular pathologists, to provide insight into the pathogenesis of infectious, inflammatory, immunological, and degenerative valve diseases. These studies revealed that the valvular tissue response to disease is characterized by a marked accumulation of VICs associated with inflammatory cells, neovascularization, increased matrix, and eventually fibrosis and calcification.[4] These descriptive studies could not provide new discoveries on cause-and-effect relationships. These investigations did suggest two hypotheses, namely that VICs maintain normal valve structure and function and that in diseased valves, VICs become activated to regulate valve repair and remodeling.[5]

Now, new mechanistic studies on VIC regulation of valve structure and function are being performed in a new era of innovative heart valve investigation that is transforming the study of VICs from static histopathology research to dynamic mechanistic cell and molecular biology investigations.[5] This new direction was propelled forward with the development of reliable VIC culture methods,[1] similar to the establishment of the successful field of vascular biology in the 1970s that was ushered in once vascular endothelial and smooth muscle cell cultures were developed. The introduction of the concept of response to valve injury as a general paradigm has been very useful in designing appropriate studies of valve function, dysfunction, and tissue engineering.[3,5] These studies have reaffirmed the central role of VICs in repair

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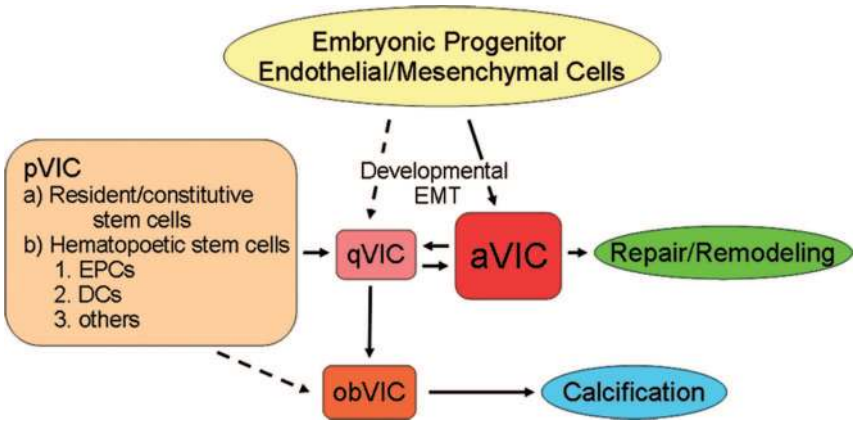

Figure 1. The current literature describes numerous VIC functions that can be conveniently organized into five phenotypes: embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), activated VICs (aVICs), stem cell-derived progenitor VICs (pVICs), and osteoblastic VICs (obVICs). These represent specific sets of VIC functions in normal valve physiology and pathophysiology. Embryonic progenitor endothelial/mesenchymal cells undergo endothelial-mesenchymal transformation in fetal development to give rise to aVICs and/or qVICs resident in the normal heart valve. The VICs undergoing the transformation possess features of aVICs, including migration, proliferation, and matrix synthesis. When the heart valve is subjected to an insult, be it abnormal hemodynamic/mechanical stress or pathological injury, qVICs become activated, giving rise to aVICs that participate in repair and remodeling of the valve. pVICs including bone marrow-derived cells, circulating cells, and resident valvular progenitor cells are another source of aVICs in the adult. The relationship between bone marrow, circulating and resident pVICs is unknown. In conditions promoting valve calcification, such as in the presence of osteogenic and chondrogenic factors, qVICs can undergo osteoblastic differentiation into obVICs. It is possible that obVICs are derived from pVICs. obVICs actively participate in the valve calcification process. Compartmentalizing VIC function into distinct phenotypes also recognizes the transient behavior of VIC phenotypes as noted (**hatched arrows** depict possible transitions for which there is currently no solid evidence).

and have shown that VICs seem to express a variety of defined phenotypes associated with remodeling and repair.

Based on our review of the literature, we suggest that five phenotypes best represent the VIC family of cells because each of these phenotypes exhibits specific sets of cellular functions essential in normal valve physiology and in pathological processes. We refer to the phenotypes as embryonic progenitor endothelial/mesenchymal cells, quiescent VICs (qVICs), activated VICs (aVICs), progenitor VICs (pVICs), and osteoblastic VICs (obVICs) (Figure 1). The embryonic progenitor endothelial/mesenchymal cells undergo endothelial-to-mesenchymal transformation (EMT) that initiates the process of valve formation in the embryo. The qVICs are the cells that are at rest in the adult valve and maintain normal valve physiology. The aVICs are the cells that regulate the pathobiological responses of the valve in disease and injury. The pVICs are the least well defined and consist of a heterogeneous population of progenitor cells that may be important in repair. The obVICs regulate chondrogenesis and osteogenesis (Table 1). Although these phenotypes may exhibit plasticity and convert from one form to another (Figure 1), compartmentalizing VIC function into distinct phenotypes should bring some clarity to our understanding of the complex VIC biology and pathobiology by focusing investigations on the interaction of each specific VIC phenotype with both the tissue and systemic environment in which it resides. Further, although the term "valve fibroblasts" is still used in the literature, it should be abandoned. The term "valve interstitial cells" (VICs) should be used because these cells do have specific features likely determined at the time of embryonic EMT and are then regulated by environmental factors throughout life to maintain day-to-day physiological activities. As investigations continue into the future, it is likely that our

Table 1. Classification of VIC Markers and Functions into Five Phenotypes

Cell type	Location	Function
Embryonic progenitor endothelial/mesenchymal cells	Embryonic cardiac cushions	Give rise to resident qVICs, possibly through an activated stage. EMT can be detected by the loss of endothelial and the gain of mesenchymal markers
qVICs	Heart valve leaflet	Maintain physiologic valve structure and function and inhibit angiogenesis in the leaflets
pVICs	Bone marrow, circulation, and/or heart valve leaflet	Enter valve or are resident in valve to provide aVICs to repair the heart valve, may be CD34-, CD133-, and/or S100-positive
aVICs	Heart valve leaflet	α-SMA-containing VICs with activated cellular repair processes including proliferation, migration, and matrix remodeling. Respond to valve injury attributable to pathological conditions and abnormal hemodynamic/mechanical forces
obVICs	Heart valve leaflet	Calcification, chondrogenesis, and osteogenesis in the heart valve. Secrete alkaline phosphatase, osteocalcin, osteopontin, bone sialoprotein

understanding of the nature of these phenotypes will grow and that new phenotypes will be discovered as well.

The VIC Family

Embryonic Progenitor Endothelial/Mesenchymal Cells

During the earliest stage of valve development, a complex process occurs in which a subset of endothelial cells overlying the endocardial cushion undergoes EMT. Individual endothelial cells delaminate from the single layer of endothelium and invade into the cardiac jelly of the endocardial cushion.[6] This cellular migration and subsequent proliferation results in the transformation of the endothelial cells into VICs, and the cushion undergoes matrix remodeling to develop into heart valves. Transforming growth factor (TGF)-β superfamily members, such as TGF-β and bone morphogenetic proteins (BMPs), as well as Notch and vascular endothelial growth factor (VEGF) have profound regulatory effects on EMT.[6–8] VEGF expression in development is well reviewed by Armstrong and Bischoff.[6] They suggest that VEGF expression is tightly controlled during valve development. Thus, with decreased VEGF, endothelial cells overlying the cardiac cushion do not proliferate sufficiently for prominent EMT to occur. With increased VEGF, endothelial cells overlying the cushion maintain their endothelial phenotype, preventing EMT and cushion formation. The observations at the extremes led Armstrong and Bischoff[6] to conclude that VEGF establishes an equilibrium between proliferation and differentiation to facilitate valve formation. However, EMT regulation is likely to be complex and involve several factors. Rivera-Feliciano and Tabin[9] recently published an extensive developmental study that led them to conclude that the presence of BMP-2 is essential in conferring progenitor cell status to endothelial cells in the atrioventricular canal.

The Notch signaling pathway is also implicated in development of endocardial cushions. *Notch1* germline mutations disrupt normal development of the aortic valve, and occasionally of the mitral valve, often leading to a bicuspid aortic valve that may calcify in adulthood.[10] Notch and its ligand Jagged1 occur specifically in the ventricular outflow tract during endocardial cushion formation in which Jagged1, Notch1, and Notch4 are coexpressed by endothelial cells.[8] Recently, Kluppel and Wrana[11] suggested in their review that there is strong evidence that direct functional links exist between the Notch and TGF-β pathways and that Notch signaling interacting with members of the TGF-β superfamily activates Notch-responsive genes.

The value in characterizing embryonic progenitor endothelial/mesenchymal cells is that this area of developmental research is likely to be important in understanding molecular pathogenesis in adult responses to disease. All these regulatory proteins and their associated signal transduction pathways expressed during development are of interest because the EMT paradigm has been thought to reappear in adult valves in response to injury and disease. In fact, the endothelial cells overlying the cushion have properties of heart valve progenitor cells and as discussed later may play a role in giving rise to VICs that participate in repair in the injured adult valve.[12,13] More investigations of the EMT phenomenon in adult disease are required to confirm the presence of EMT-like processes in the injured adult valve.

qVICs

qVICs are thought to maintain physiological valve structure and function. In the normal valve, VICs are considered to be quiescent, although the actual *in vivo* mitotic indices are not known for human valves. One study in the rat reported a cumulative labeling fraction throughout 7 days of 9.4 $\pm$ 2.6% in the mitral valve.[14]

The qVICs, both *in vivo* and *in vitro*, show two types of intercellular junctional complexes, gap junctions and adhesion junctions.[1] Connexin-26 and -45 are transmembrane gap junction proteins weakly expressed by VICs.[1,15] These junctions are likely used by VICs to communicate with adjacent VICs, as shown when small molecules microinjected into a VIC *in vitro* are transferred to an adjacent VIC.[16] The other junctional complex in VICs is the cell-cell adhesion junctions, present especially between the long processes seen to extend from adjacent cells. N-Cadherin and desmoglein are detected in minute amounts in these junctions.[15] These adhesion junctions may be important in keeping VICs in close contact to allow for gap junction function. Another interesting possibility, which remains unproven, is that the VICs may form a cellular structural network that facilitates valve function, perhaps by sensing physical forces and then rapidly transmitting the information across the cell network. However, at present the physiological significance of these junctions remains unknown and is definitely worth studying.

There are several important questions that remain unanswered concerning qVIC structure and function. How cellular organization is arranged into the three distinct layers of the valve is not known. qVICs are also thought to regulate very low-grade matrix synthesis and degradation; however, this has been difficult to confirm because *in vivo* measurements are difficult to do reliably. It is not known if the qVICs are metabolically active in other ways. It may be that these cells actively keep the valve avascular by inhibiting angiogenesis, as discussed later. Another question that deserves study is whether there is heterogeneity of qVICs both within and across the valve layers. There are some data suggesting that VICs located at the atrial and ventricular sides of the mitral valve respond differently to valvular endothelial cell (VEC) injury.[17]

To perform cell biology investigations, qVICs have been successfully grown in culture.[1,16] Much evidence suggests that over several passages the cultured cells are activated and become aVICs; however, more knowledge is required to assess the role of culture conditions including passage number and density of culture on phenotype transformation. VICs in monolayer cultures

show elongated (spindle-shaped) morphology and form an orthogonal pattern of overgrowth at postconfluence that resembles fibroblasts (Figure 2). In ~20% of long-term cultures, VICs spontaneously form cobblestone-type contact-inhibited monolayers.[3] This phenotype has been difficult to study because it seems to be unstable, often reverting to the elongated morphology.

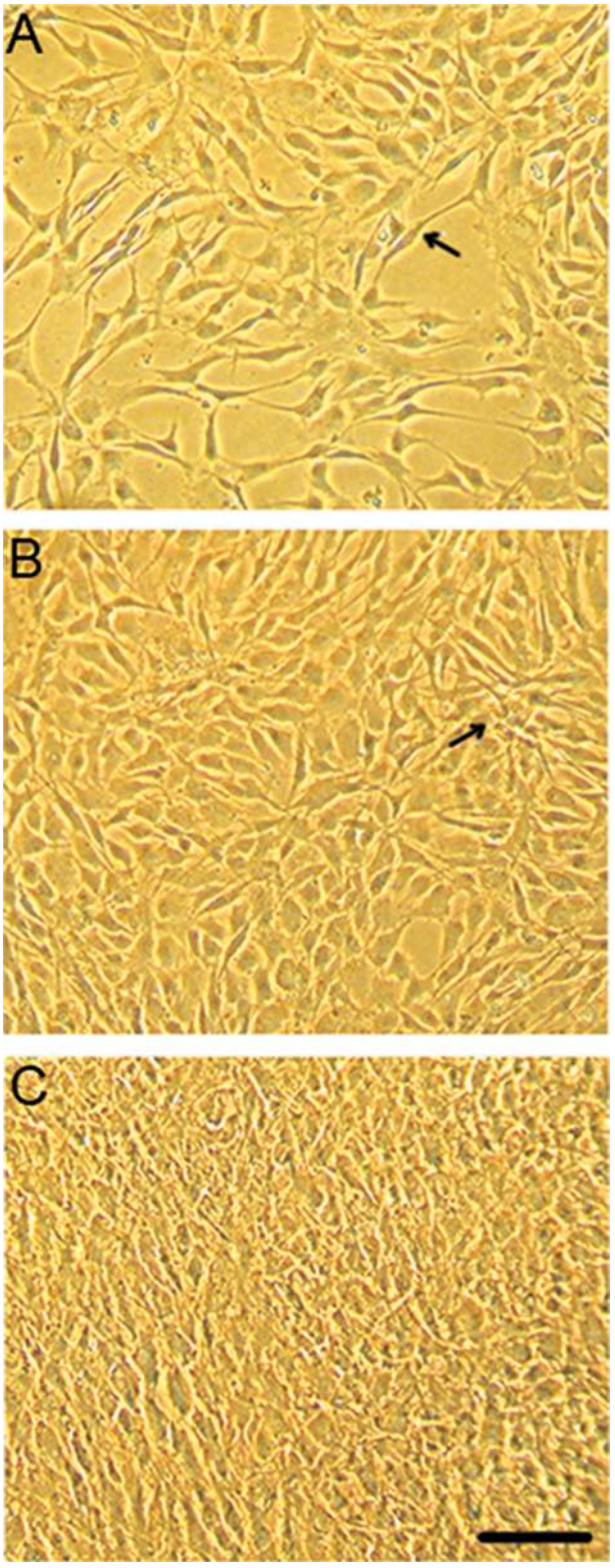

Figure 2. Phase contrast photomicrographs of VICs in monolayer culture at moderate (**A**), confluent (**B**), and superconfluent (**C**) densities. Note the elongated morphology in **A** and the overlapping growth pattern in **B** as indicated by **arrows**. Scale bar = 20 μm. Original magnifications, ×200.

VICs have unique structural and functional features compared with other mesenchymal and vascular cells. In monolayer culture, the absence of a hill and valley pattern in postconfluent cultures and differences in adhesion to and spreading on cell substratum distinguish spindle-shaped VICs from cultured vascular smooth muscle cells.[18] Furthermore, differences exist between VICs and both pericardial interstitial cells and skin fibroblasts.[19] The notion that valve cells are distinct from cells in other organs is supported by two elegant studies on VECs. VECs respond to hemodynamic shear stress with a different transcriptional profile when compared with vascular endothelial cells.[20] In culture, VECs align perpendicularly in response to hemodynamic shear stress, whereas vascular endothelial cells align parallel to flow.[21] The reasons for these differences are unknown, but they emphasize the need to study valve cells directly. Thus, it is now becoming clear that to study VIC structure, function, and dysfunction, it is necessary to study VICs directly and not extrapolate information from other cell types, as was done previously.

pVICs

The increased number of VICs observed in response to injury has been thought to occur through proliferation of VICs at the site of injury and possibly by decreased apoptosis during remodeling. Recent findings suggest that other sources of VICs may exist. Valvular stem cells, which we refer to as progenitor VICs (pVICs), are derived from various origins including bone marrow-derived cells, circulating cells, and resident valvular progenitor cells. Whether pVICs can become directly activated or require transformation through a qVIC stage is not known at present. Two such cell types are the endothelial progenitor cell (EPC) and dendritic cell (DC). EPCs are characterized by stem cell markers, ie, CD133 and CD34, a high proliferative capacity, and the ability to form blood vessels.[22] DCs are identified by S100, an intracellular calcium-binding protein. EPCs and DCs have been found in degenerated porcine bioprostheses and calcified stenotic aortic valves. Six percent of cells in degenerative valves are positive for EPC markers, and in severely diseased native cusps, 6% of cells are DCs.[23–25] The co-localization of EPCs and DCs at the aortoluminal border of degenerative valves may suggest a common circulating progenitor cell, possibly the $CD34^+$ hematopoietic stem cell.[23] An elegant study using sex-mismatched murine chimeras and multilineage hematopoietic stem cell engraftments has shown that hematopoietic stem cells engrafted into recipient heart valves express mRNA for procollagen α1 and differentiate into cells that are morphologically similar to native VICs in the recipient.[26]

Other attempts at identifying potential pVICs have been performed recently. Progenitor cells from the pulmonary valve have been isolated, and they were shown

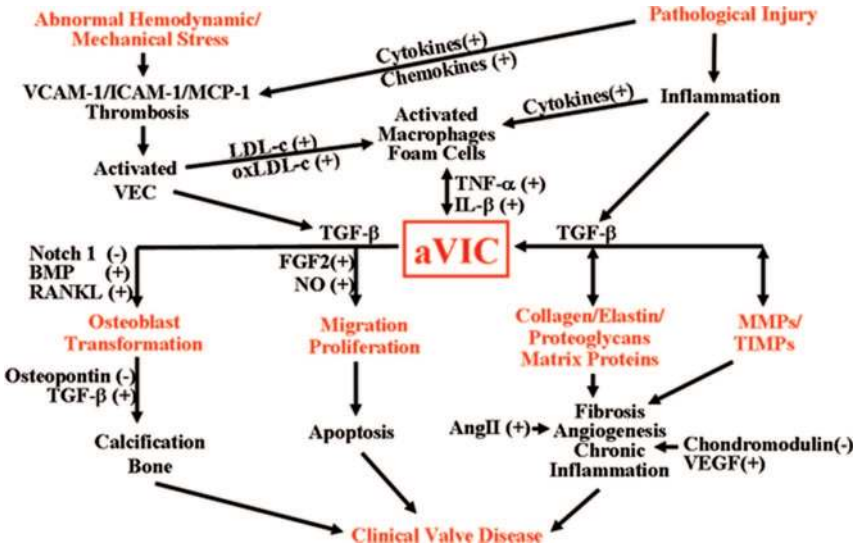

Figure 3. The normal adult heart valve is well adapted to its physiological environment, able to withstand the unique hemodynamic/mechanical stresses under normal conditions. Under conditions of pathological injury or abnormal hemodynamic/mechanical stresses, VICs become activated through activation of VECs and by inflammation and associated cytokine and chemokine signals. Macrophages will also be activated. aVICs increase matrix synthesis, upregulate expression of matrix-remodeling enzymes, migrate, proliferate and undergo apoptosis, and undergo osteoblast transformation. These processes are regulated by a variety of factors, several secreted by the aVIC. If the aVICs continue to promote these cellular processes, angiogenesis, chronic inflammation, fibrosis, and calcification result, leading to progressive clinical valve disease.

to undergo transition to VIC-like cells in response to TGF-β2. It is interesting that these progenitor cells exhibit markers of both endothelial and mesenchymal cells and are responsive to both TGF-β and VEGF, the former favoring mesenchymal transformation and the latter giving rise to endothelium.[13] This plasticity reinforces the need to focus on discovering the regulatory factors that promote differentiation of progenitor cells.

The study of pVICs is in its infancy, and there is a need for innovative hypothesis-driven research to discover how pVICs participate in repair. A simple paradigm to begin hypothesis-driven mechanistic studies may focus on identifying and characterizing soluble signal molecules arising from tissue injury and/or necrosis in the diseased heart valve, which may then travel through the circulation and recruit stem cells from the bone marrow. Further studies are required to determine whether and how these bone marrow pVICs then traffic to the injured heart valve, perhaps by recognizing specific ligands or adhesion molecules expressed at the site of injury. Another possible pathway is one in which pVICs reach the valve through microvessels at the base of the leaflets or at sites of inflammation in the diseased valve. Alternatively, there may already be existing precursor cells in the circulation that are attracted to sites of valve injury.[27,28] There is some *in vitro* evidence suggesting that the microenvironment of the valve also influences the function of human bone marrow-derived pVICs once they are present within the valve.[29] In addition, the heart valve itself may be the source of pVICs either constitutively present in very small numbers or through an EMT-like mechanism originating from adult VECs.

At present, our knowledge of pVICs is still very limited, although this area of research offers great potential for prevention and treatment of heart valve diseases. We require better progenitor markers, especially those that are specific for pVICs. The function of pVICs during their recruitment and transdifferentiation to aVICs needs to be carefully studied. Heterogeneity among different sources of pVICs requires characterization as well. Finally, it is important to recognize that at present the role and the impact of these pVICs in valve repair has not been studied directly, and thus their importance in human valve disease is unknown. It is premature to begin to use these cells as therapeutic agents or for tissue engineering without having a much better understanding of their biology and pathobiology than we currently have.

aVICs—The Master Cells

In injured or diseased valves, cellular signals promote qVICs to become activated VICs (aVICs) (Figure 3). aVICs take on the features of myofibroblasts showing increased contraction, prominent stress fibers, and other contractile proteins, such as the striated-muscle isoform of myosin heavy chain.[30] The aVICs are not smooth muscle cells because smooth muscle cells have intact basement membranes whereas VICs show incomplete basement membranes.[1] In tissue culture, comparison of porcine VICs with porcine aortic smooth muscle cells showed differences in growth pattern, proliferation, and cell adhesion properties.[18] The marker for aVIC is α-smooth muscle actin (α-SMA), a cytoskeletal isoform of actin, which is normally not found in qVIC. In primary cultures, with increasing passage, many VICs are positive for α-SMA (Figure 4). This has been used to enrich aVICs in culture by exploiting the fact that aVICs expressing high levels of α-SMA adhere more firmly to the substratum than those with less α-SMA.[31] In a detailed study of motility, single VICs in moderately dense culture express α-SMA yet exhibit a number of different morphologies. Round and rhomboid aVICs are found to have more α-SMA-containing stress fibers and are less motile whereas tailed and spindle-shaped (elongated) aVICs are more proliferative and show higher motility.[32] Thus, although all these cells are activated, they express heterogeneity in motility, suggesting that regulation of transitions in function needs to be studied in this very versatile cell.

qVICs become aVICs under conditions of pathological injury or abnormal hemodynamic/mechanical stress (see

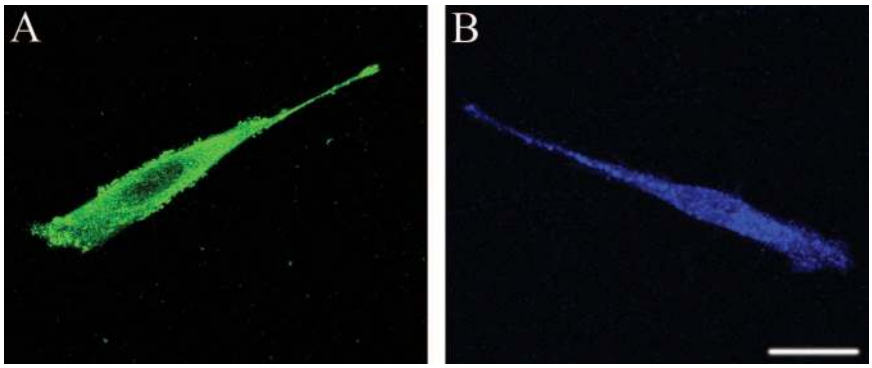

Figure 4. Immunofluorescent photomicrographs of single porcine mitral valve aVICs in culture showing a migrating aVIC. aVICs express prominent amounts of α-SMA (**A**, green), a widely accepted marker for VIC activation, as well as TGF-β (**B**, blue), an important factor that regulates this activation as well as several aVIC functions, and is secreted by aVICs in turn. Scale bar = 10 μm.

below) in which activated VECs and macrophages/foam cells arise, and a number of chemokines and growth factors stimulate qVIC activation. Activation of VICs is associated with increased extracellular matrix (ECM) secretion and degradation, expression of matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs (TIMPs), as well as increased proliferation and migration, which are all important features of the wound repair process[5] and may be regulated by FGF-2 and NO.[33,34] Furthermore, aVICs also increase secretion of cytokines, one of the most important being TGF-β, which has important autocrine functions (Figure 4). After completion of remodeling, many aVICs are eliminated by apoptosis.[35] When dysregulation of this process occurs, aVICs persist with continued force generation. Dysfunction of apoptosis, in combination with abnormal ECM production and remodeling, may result in pathological fibrosis, angiogenesis, chronic inflammation, and calcification,[35,36] giving rise to clinical valve diseases (Figure 3). Thus, aVICs are promising therapeutic targets to treat and prevent heart valve disease.

obVICs

obVICs refer to VICs that undergo osteoblastic differentiation and promote calcification as seen when VICs are cultured in osteogenic culture medium. At present, there seems to be no evidence that obVICs are a separate cell population (Figure 1). Descriptive studies using state of the art histomicroscopy and immunomicroscopy have shown many associations between chondrogenic and osteogenic proteins. Cartilaginous nodules and mature lamellar bone have been observed in surgically explanted degenerated human heart valves,[37,38] and proteins associated with chondrogenesis and osteogenesis, such as osteopontin, bone sialoprotein, alkaline phosphatase, and bone morphogenetic protein (BMP)-2 and -4, have been identified.[37–40]

Cultured VICs do not normally promote calcification spontaneously. When growth medium is supplemented with organic phosphate, VICs undergo osteoblastic differentiation, expressing chondrogenic and osteogenic markers and forming calcific nodules. The calcification process depends on up-regulation of alkaline phosphatase activity because its inhibition prevented *in vitro* calcification.[41] The calcified nodules in culture showed the presence of hydroxyapatite. The addition of TGF-β, 25-hydroxycholesterol, and BMP-2 increased the rate of nodule formation, suggesting a role for these molecules in regulating valve calcification.[42] TGF-β promoted migration, aggregation, and formation of apoptotic alkaline phosphatase-enriched nodules, followed by calcification of these nodules.[36,43] The addition of an anti-apoptotic agent, ZVAD-FMK, inhibits obVIC calcification and apoptosis induced by TGF-β although it has no effect on nodule formation.[36] The actin-depolymerizing agent cytochalasin D inhibits nodule formation by preventing VIC migration in culture, but it does not prevent calcification. Thus, TGF-β mediates the calcification of VICs in culture through mechanisms involving apoptosis.[36]

VICs also undergo osteoblastic differentiation in the presence of BMPs and TGF-β.[44] This calcification can be reversed by addition of adenosine to the obVIC cultures.[43] Extracellular adenosine triphosphate and its P2Y receptor are important regulators of osteoblast differentiation and bone remodeling, whereas its breakdown product, adenosine, is known to have anti-inflammatory properties.[45]

MMPs are thought to play important roles in the regulation of calcification and ECM degradation. How MMPs affect obVIC function, possibly through a feedback loop, is not understood. A number of MMPs that degrade various components of the ECM are found in aortic valve lesions. However, more data are needed to clarify whether the levels of natural inhibitors of MMPs, TIMPs, are increased or unchanged in these lesions.[46–48] Tenascin, an ECM glycoprotein, may promote MMP-2 mRNA expression and gelatinolytic activity leading to calcific aortic stenosis.[49] Another bone matrix protein, osteopontin, detected in calcified human aortic and mitral valves,[50] may be an important inhibitor of valvular calcification. Studies show that calcification was dramatically increased after subcutaneous implantation of glutaraldehyde-fixed porcine aortic valve leaflets into osteopontin-deficient mice.[51] Furthermore, VIC calcification may be associated with early growth response-1 (Egr-1), a transcription factor that is increased in calcified heart valves and regulates proteins associated with calcification including tenascin-C and osteopontin.[52]

The well-known atherogenic lipoproteins low-density lipoprotein and Lp(a) are found to be deposited in human aortic valve lesions, and aortic valve cholesterol

content is increased.[53–55] Similar to atherosclerosis, lipoprotein deposition in aortic lesions is likely mediated by accumulated ECM proteoglycans such as biglycan and decorin.[39] Oxidized lipids have been detected in aortic valve lesions, particularly in areas of developing calcification. *In vitro* studies have shown that oxidized cholesterol stimulates calcified nodule formation by VICs and that calcified nodule formation by these cells is inhibited by simvastatin, a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor that may act independently of lipid reduction.[56,57]

O'Brien[58] reviews the evidence that atherogenic risk factors including oxidized lipids, tumor necrosis factor-α, and hyperglycemia may all mediate osteoblast differentiation and subsequent valvular calcification through pathways activated by BMP2. BMP2 is present in human aortic valve lesions and stimulates calcified nodule formation by VICs *in vitro*.[42,47,59] Mechanistic studies suggest that BMP2 can up-regulate both an osteogenic pathway involving the transcription factor Msx2, which activates Wnt signaling, and a chondro-osteogenic pathway involving the transcription factor Runx2/Cbfa1,[60] which is increased in aortic valves of hypercholesterolemic rabbits.[38,54] A mechanism that implicated Runx2/Cfba1 involves mutations in the Notch1 gene resulting in failure to repress Hairy-related family of transcription factors (Hrt), which then promote Runx2/Cfba1-regulated osteoblast differentiation.

Using microarray technology, VECs have been shown to differentially express 584 genes on the aortic side versus the ventricular side of normal adult pig aortic valves.[61] Several of these observed differences could help explain the vulnerability of the aortic side of the valve cusp to calcification in diseases such as calcific aortic stenosis. However, because calcification occurs within the valve tissue, it is likely that VECs may be playing more of a transducing role, regulating aVIC or obVIC function.

Regulation of VIC Activation

The Role of TGF-β

TGF-β superfamily of proteins is a family of peptide growth factors that regulate biological functions in many systems. It has profound effects on VIC differentiation,[62,63] increasing the expression of α-SMA, smooth muscle myosin, and calponin.[64] On the cell surface, TGF-β binds to TGF-β receptor type I and II leading to signaling through the Smad proteins, which interact with transcription factors including FoxH1, c-Jun, c-fos, and Gli-3. Other pathways may also be initiated, including the mitogen-activated protein kinase (MAPK) family pathways. These pathways converge to regulate cell cycle, proliferation, migration, cytokine secretion, and ECM synthesis and degradation, processes that are important in EMT and in adult valve remodeling and repair.[65]

The bioavailability of TGF-β is tightly regulated by secretion with a precursor sequence as well as through binding to a larger latent TGF-β-binding protein (LTBP). Molecules such as thrombospondin-1 and plasmin are involved in release of TGF-β from inactive complexes.[66] The specific cellular responses to TGF-β are highly context-dependent, varying with cell type and physiological state.[65] When VICs are cultured with exogenously added TGF-β, there is a dose-dependent increase in α-SMA expression, VIC contractility, and fibronectin remodeling.[62]

Matrix components heparin and fibronectin play important roles in regulating the effect of TGF-β on VICs.[66] For example, TGF-β contains heparin-binding motifs, comprised of basic residues posed to interact noncovalently with polyanions.[67] Heparin induces α-SMA expression in VICs by binding and increasing the stability of TGF-β in the pericellular microenvironment.[63] TGF-β is a fibronectin-associated growth factor that binds directly to the active form, as well as through interactions with latent TGF-β-binding protein.[66] VICs express and actively remodel fibronectin, a major component of the insoluble ECM and a soluble constituent of plasma. Fibronectin's ability to sequester TGF-β also allows it to activate VICs.[63] Seeding VICs on heparin and/or fibronectin-enriched matrices leads to the retention of TGF-β in the matrix and induction of VIC activation. Heparin further induces *de novo* production of TGF-β by VICs, synergistically increasing VIC activation.[63]

VIC activation by TGF-β includes dramatic augmentation of stress fiber formation and alignment leading to enhanced contractility and increased mechanical stress. These contractile VICs exert tension on the ECM resulting in a striking realignment of extracellular fibronectin fibrils.[62] This may link VIC contractility with pathological valve matrix remodeling in which activation of contractility by TGF-β may be a first step in promoting alterations to the valve matrix architecture evident in valve disease.

aVICs in mitral valve prolapse express excessive levels of catabolic enzymes.[68] Clinical disorders associated with increased serotonin 5-hydroxytryptamine (5-HT) levels, such as carcinoid syndrome, and the use of serotonin agonists, such as fenfluramine, have been associated with hyperplastic valvular endocardial lesions with increased ECM. Serotonin up-regulates TGF-β1 in aortic valve VICs,[69] and in carcinoid heart disease TGF-β is associated with VIC formation of fibrotic lesions.[70] Studies of the effects of 5-HT on TGF-β1 expression and activity in VICs show that 5-HT also increased collagen biosynthesis in VICs, whereas the addition of TGF-β1 increased the production of sulfated glycans and hyaluronic acid. These studies suggest that increased exposure of VICs to 5-HT may result in increased TGF-β1 expression and activity. This may be attributable to serotonin receptor-mediated signal transduction and activation of GΥq G-protein signal transduction with subsequent up-regulation of phospholipase C.[69,71]

The Role of Matrix

VICs reside in a three-dimensional ECM composed primarily of collagen, elastin, proteoglycans, and glycosaminoglycans, which provide external signals to regulate VIC function. It is likely that VICs produce, secrete, and degrade most of the surrounding ECM to maintain a

physiological matrix and to remodel the valve tissue during repair.[72] The overlapping compartments of the trilaminar aortic valve have distinct ECM content. The fibrosa contains mainly collagen, the spongiosa mainly proteoglycans, and the ventricularis predominantly elastin. Collagen III is ubiquitously expressed in the valve whereas collagen I is expressed predominantly in the outflow layer.[73] Different glycosaminoglycans have distinct distribution. Keratin sulfate and decorin are found throughout the leaflet, whereas the chondroitin sulfates are more strongly expressed in the outer layers.[74]

The trilaminar compartmentalization of the ECM is a fascinating biological phenomenon that is not well understood; however, it seems to be very important in the physiological function of the adult heart valve and is indeed disrupted in diseased valves. Careful studies of normal valve development by Hinton and colleagues[75] show that ECM organization is characterized by spatiotemporal coordination. The distinct ECM stratification first appears during cusp and leaflet remodeling, in the chicken at E16 and the mouse at E18.5. As the ECM begins to stratify into layers, variations in the density of VIC distribution occur, and the fibrosa and ventricularis layers show a higher VIC density than in the spongiosa.[75] Aikawa and colleagues[76] performed studies of human semilunar heart valves to determine the content of collagen and elastin during fetal development. They found that collagen, although unchanged in the postnatal period, showed a progressive increase during fetal development. Elastin, however, showed a major increase in the postnatal period suggesting that its synthesis is regulated to a larger extent by hemodynamic forces than that of collagen.[76] A careful histological analysis of the fetal valves show that there is a homogeneous distribution of glycosaminoglycans at 14 weeks gestation without compartmentalization. Compartmentalization occurs at late gestation; however, a very interesting observation is that between 20 and 39 weeks the valves have a bilaminar structure. How this remodeling of the valve into compartments occurs is not known. It is clear that physical forces do play some role because the compartmentalization seen in the adult architecture is not complete until early childhood.[76] VICs are likely to play a major role in this compartmentalization because the embryonic progenitor endothelial/mesenchymal cell-derived VICs are activated, show significant proliferation, and express MMPs. How the aVICs become segregated to establish the compartments, first bilaminar and then trilaminar, is a mystery. One possibility is that the VICs at the interface of the fibrosa and ventricularis adapt to the physical forces imposed at birth by secreting new matrix to establish the middle spongiosa layer, which is an important structure to support cusp flexibility and cushion the physical forces to which the valve is exposed.

VICs interact with ECM by focal and fibrillar adhesions. Cultured VICs express α1, -2, -3, -4, and -5 and β1 integrins to varying degrees.[73] VIC integrin $\alpha 9\beta 1$ forms focal contacts with osteopontin, but no stimulation of migration or proliferation is observed. It is likely that $\alpha 9\beta 1$ integrin is predominantly involved in controlling anchorage of the VIC to the ECM rather than movement or proliferation.[77] Fibronectin is secreted by VICs and interacts with $\alpha 5\beta 1$ integrin in migrating cells during wound repair, linking the actin cytoskeleton to fibronectin via tensin and $\alpha 5\beta 1$ integrin.[78]

Valves contain molecules that regulate the degradation of matrix especially during remodeling of ECM. Human heart valves have a specific pattern of expression of MMPs and TIMPs that vary among different valve types. One study showed that MMP-1 is found in all four types of heart valves, but MMP-2 is found in only aortic and pulmonary valves. MMP-3 and MMP-9 are not expressed in heart valves whereas TIMP-1 and TIMP-2 are expressed in all valve leaflets. Furthermore, TIMP-3 is present only in tricuspid valve leaflets.[79] In human bicuspid aortic valves, MMP-1, -2, and -9 as well as their inhibitors TIMP-1 and -2 are detected. The up-regulation of MMP-2 and -9 may correspond to a decrease in elastin and collagen content in diseased valves and may contribute to valve dysfunction.[80] MMP-3 and -9 as well as TIMP-1, which are thought to be secreted by aVICs, are significantly increased in stenotic and regurgitant aortic valves.[81]

In culture, VICs synthesize TIMP-1, TIMP-2, and MMP-2.[81] It seems that under most conditions, MMP-2 is the predominant ECM-degrading enzyme in VICs. It is interesting that this same MMP is important in the initiation of abdominal aortic aneurysms.[82] VICs seeded onto a three-dimensional type I collagen matrix show increased mRNA and protein expression of collagenases, stromelysins, and membrane-type MMPs suggesting that VICs have the intrinsic capacity to remodel ECM.[81] Other proteolytic enzymes that VICs express include cathepsin D, other collagenases, and other MMPs.[48,71,81,83–85]

Stimulation of VICs with RANK-L increases MMP-1 and MMP-2 expression, as well as cell proliferation.[86] MMP expression is also stimulated by cytokines, including interleukin-β and tumor necrosis factor-α, in cultured aVICs.[46,85] The cardiac T-box factor Tbx20 seems to be a regulator of ECM gene expression. Overexpression of Tbx20 in cells of developing endocardial cushions induces increased expression of MMP-9 and -13 and decreased expression of chondroitin sulfate proteoglycans, including aggrecan and versican.[48]

In tissue engineering applications, it has been found that fibronectin enrichment of collagen- and laminin-coated surfaces facilitate VIC adhesion and proliferation. Studies using crosslinked hyaluronan scaffolds as a biologically active carrier for VICs show that low-molecular weight degradation products of hyaluronan gels significantly increase VIC proliferation, total matrix production, and elastin production.[87]

The Role of Mechanical Forces

VICs reside in a complex three-dimensional environment within the valve matrix where they are exposed to mechanical forces exerted on valve tissue including shear, pressure, and tension (stretch).[88] Pulsatile hemodynamic shear stress affects VICs through VEC mechanotransduction and/or through bulk matrix shear stress, which

induces tensile forces within the valve matrix, as suggested by Weston and Yoganathan.[89] Cyclic flexure and bending occurs during the opening and closing of the valves. Compressive strains and tension (stretch) is exerted on the closed valve because of a pressure gradient across the valve. VICs are found to respond to local tissue force or stress by altering their cellular stiffness and biosynthesis of matrix components. For example, VICs from the left side of the heart, where the transvalvular pressure impose larger tissue stress on VICs, show increased α-SMA content and collagen synthesis.[88]

The amount of collagen synthesis is found to be dependent on the degree and duration of stretch.[90] Studies of collagen gene expressions in VICs subjected to different levels of stretching forces show that stretch increases collagen III gene expression but not collagen I gene expression. Mechanical stretch in VICs also results in an increase in the expression of collagen-processing enzymes such as lysyl oxidase and prolyl 4-hydroxylase, which are responsible for modification, aggregation, and covalent cross-linking of secreted procollagen.[90]

An exciting observation that supports the importance of physical forces in determining valve architecture and matrix organization is that tissue engineered heart valve constructs placed in the *in vivo* circulation change throughout time from a diffuse arrangement of cells within the matrix to a trilaminar structure similar but not identical to that of a native valve.[91] This observation could be refined into a model to study the formation of the trilaminar valve structure.

In addition, physical forces affect the components of ECM differently. In studies modeling circumferential cyclic stretch, a 15% strain applied for a period of 48 hours in an *ex vivo* bioreactor showed no significant change in elastin content, a significant increase in leaflet collagen content, and a reduction in sulfated glycosaminoglycans, all associated with the presence of aVICs.[92]

Mechanical stresses are transduced by VECs to regulate VICs. Some identified factors released by VECs include nitric oxide (NO), vasodilatory prostanoids,[93] and endothelin-1.[94] These have the potential to affect VIC function through paracrine signaling. In three-dimensional aortic valve leaflet models comprised of VECs and VICs to which luminal fluid flow is imposed, VECs are able to align perpendicularly to flow. VICs cultured independently of VECs under flow stress express vimentin strongly and α-SMA to a lesser extent compared with VIC and VEC co-cultures. VECs also stimulate a decrease in VIC proliferation, an increase in protein synthesis with shear stress, and reduce the loss of glycosaminoglycans with flow.[95] These VEC relationships with VICs are intrinsic to the valvular response to hemodynamic injury and to maintaining valvular tissue integrity.

The major challenge in understanding the effects of mechanical forces on valve structure and function is the ability to design experimental models that apply measurable mechanical forces and allow for quantitative analysis of specific VIC functions. The areas that have generated some controversy in experimental design include the use of static versus pulsatile mechanical forces, the application of forces to planar cell monolayers versus three-dimensional matrix models, the use of cell cultures versus organ cultures, and the study of the interactions between various forces that are applied to the valve *in vivo*. Thus, the next few years will require a stronger interaction between engineering and biology to produce model systems that are as close as possible to the human valve.

Angiogenesis

Normal adult heart valves are avascular; however, in all pathological conditions there are numerous blood vessels present. This neovascularization is attributable to an imbalance in angiogenic factors, such as VEGF,[96] and anti-angiogenic factors. Chondromodulin-I has been shown to be a potent anti-angiogenic factor in the heart valve. It is abundantly expressed in normal heart valves, being detected in the fibrosa, spongiosa, and ventricularis layers but not in endothelial cells, whereas VEGF is absent in all cell layers. In regions of new vessel formation, chondromodulin-I is markedly down-regulated and VEGF is up-regulated. Chondromodulin-I knockout mice show increased VEGF expression, angiogenesis, lipid deposition and calcification, and eventually aortic stenosis. In culture, VICs secret chondromodulin, which strongly inhibits angiogenesis by inhibiting mobilization of endothelial cells, inducing their apoptosis and preventing tube formation.[97] Endostatin and SPARC are also implicated as anti-angiogenic factors in valvular diseases.[98,99]

Inflammation

Inflammatory cells including variable numbers of macrophages, foam cells, lymphocytes, and foreign body giant cells are present in diseased heart valves. Very little is known about the pathophysiology of these cells within the diseased valve. It is likely that these cells are recruited to the valve by up-regulation of endothelial-leukocyte adhesion molecules[76] and, once in the valve, secrete cytokines, chemokines, growth factors, and proteolytic enzymes, which all participate in remodeling. Recently, the renin-angiotensin system, particularly angiotensin-converting enzyme, angiotensin II, and the angiotensin II type I receptor, has been implicated in stimulating inflammation in the heart valve.[55] Angiotensin II is generated from angiotensin I by angiotensin-converting enzyme and has a number of potential angiotensin II type I receptor-mediated, lesion-promoting effects in aortic stenosis. These include promoting macrophage cholesterol accumulation, increasing oxidative stress, and stimulating expression of the lipoprotein-retaining proteoglycan, biglycan, all of which may contribute to inflammation in valvular diseases.

Summary

VICs are distinct from other mesenchymal cell types, and their investigation will result in new discoveries that will add to our understanding of human biology and pathobiology.

The valve is also an excellent tissue in which to study the impact of physical forces on tissues and cells. Based on the data reviewed, we present a conceptual approach to the investigation of VICs by focusing on VIC phenotype-function relationships. We also present a conceptual model that should be useful in the design and interpretation of future studies on the function of the aVIC—the pivotal cell that controls valve structure and function in health and disease. We hope that this review will inspire members of the investigative pathology community to consider valve biology and pathobiology as an exciting new frontier to explore pathogenesis and discover new therapeutic targets in cardiovascular diseases.

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