

# Molecular and functional characteristics of heart-valve interstitial cells

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The cells that reside within valve cusps play an integral role in the durability and function of heart valves. There are principally two types of cells found in cusp tissue: the endothelial cells that cover the surface of the cusps and the interstitial cells (ICs) that form a network within the extracellular matrix (ECM) within the body of the cusp. Both cell types exhibit unique functions that are unlike those of other endothelial and ICs found throughout the body. The valve ICs express a complex pattern of cell-surface, cytoskeletal and muscle proteins. They are able to bind to, and communicate with, each other and the ECM. The endothelial cells on the outflow and inflow surfaces of the valve differ from one another. Their individual characteristics and functions reflect the fact that they are exposed to separate patterns of flow and pressure. In addition to providing a structural role in the valve, it is now known that the biological function of valve cells is important in maintaining the integrity of the cusps and the optimum function of the valve. In response to inappropriate stimuli, valve interstitial and endothelial cells may also participate in processes that lead to valve degeneration and calcification. Understanding the complex biology of valve interstitial and endothelial cells is an important requirement in elucidating the mechanisms that regulate valve function in health and disease, as well as setting a benchmark for the function of cells that may be used to tissue engineer a heart valve.

**Keywords:** heart valves; valve interstitial cells; cell phenotype; cell communication

## 1. BACKGROUND

Recent observations have illustrated the complexity of the biological processes that are performed by valve interstitial and endothelial cells. These mechanisms are now being recognized to an important role in the sophisticated function of heart valves. The ability of these cells to communicate with each other, with the extracellular matrix (ECM), and to respond to their environment, underpins the mechanisms that allow heart valves to function in an optimal and efficient manner. Cell types within the valve can be broadly divided into two separate phenotypes: interstitial and endothelial cells. The ICs populate the body of the valve cusp and may in fact represent a heterogeneous population of cells. The endothelial cells cover both sides of the valve cusps and act as an interface between the cusp and the blood.

## 2. VALVE-CUSP INTERSTITIAL CELLS

Valve ICs are a heterogeneous and dynamic population of specific cell types that are phenotypically different from dermal fibroblasts, and have many unique characteristics (Lester *et al.* 1988; Messier *et al.* 1994; Mulholland & Gotlieb 1996; Della *et al.* 2000; Taylor *et al.* 2000). It is probable that a family of fibroblast-like cells exists that varies its phenotype as an adaptive response to its microenvironment, as

dictated by the ECM, mechanical force and soluble factors (Komuro 1990). These cells synthesize matrix components, such as collagen, elastin, proteoglycans and glycoproteins; growth factors, cytokines and chemokines; as well as matrix remodelling enzymes, the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs; Sappino *et al.* 1990; Smith *et al.* 1997).

### (a) Phenotypic characteristics

Two cellular phenotypes have been demonstrated by electron microscopy and immunocytochemistry, both *in situ* and in cells cultured from valves. One type, 'the myofibroblast', is characterized by prominent stress fibres associated with smooth muscle  $\alpha$ -actin expression (Lester *et al.* 1988; Messier *et al.* 1994; Mulholland & Gotlieb 1996; Della *et al.* 2000; Taylor *et al.* 2000). A layer of smooth muscle  $\alpha$ -actin-positive cells has been identified in the ventricularis of both human and porcine aortic valves (Bertipaglia *et al.* 2003; Taylor *et al.* 2003). This contractile phenotype is thought to be proliferative, migratory and capable of remodelling the ECM (Arora & McCulloch 1994). The second phenotype is characterized by prominent synthetic and secretory organelles and is involved in matrix regulation and production. This phenotype is associated with the expression of prolyl 4-hydroxylase, an enzyme essential for stabilization of the collagen triple helix, which indicates that the cells are actively synthesizing collagen (Kontinen *et al.* 1989; Janin *et al.* 1990).

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Two cell morphologies are observed when valve ICs are cultured: small islands of cuboidal cells and spindle-shaped elongated cells. Once confluent, the cells exhibit a swirling pattern, characteristic of fibroblasts, and start to pile up on each other in layers (Johnson *et al.* 1987). In our experience, these cells have a slow rate of proliferation even when a variety of growth factors are added, which may reflect an inherently low turnover rate. Whether the same range of phenotypes is present in all of the valve leaflets still remains to be determined. However, our investigations have suggested that cultured valve ICs have a similar phenotypic profile, whether they are from the outflow valves (aortic and pulmonary) or the atrioventricular valves (mitral and tricuspid; Taylor *et al.* 2000). Our laboratory has recently been able to demonstrate that valve ICs in culture are capable of differentiating into other cell phenotypes including osteoblast, adipocytes and chondrocytes (Osman *et al.* 2006; N. Latif 2006, personal communication).

It is well documented that ECM and cytokines are important in fibroblast differentiation and function (Sappino *et al.* 1990; Juliano & Haskill 1993; Arora & McCulloch 1994; Desmouliere & Gabbiani 1994). Fibroblasts synthesize collagen, elastin, proteoglycans, glycoproteins, growth factors, cytokines and chemokines, as well as MMPs and TIMPs (Sappino *et al.* 1990; Smith *et al.* 1997). The ability of valve ICs to secrete growth factors, or stimulate their release from the ECM via the action of MMPs (Segura *et al.* 1998), may play a vital role in regulation of cell phenotype within the cusp. It has been shown that in several valve pathologies, there is increased expression/activity of matrix remodelling enzymes and secretion of cytokines (Rabkin *et al.* 2001; Kaden *et al.* 2003, 2004b; Guilherme *et al.* 2004; Fondard *et al.* 2005).

Valve ICs have been shown to express a range of skeletal and non-muscle cell markers. Molecular expression of  $\beta$ -myosin heavy chain ( $\beta$ MHC) has been reported, which is associated with the slower contractile isoform of myocytes due to its lower ATPase activity compared with  $\alpha$ MHC (Brand *et al.* 2006). In addition to cardiac muscle markers, valve ICs express skeletal muscle components of the troponin complex. The expression of these skeletal muscle proteins may be regulated by the transcription factor myogenin, which is also expressed by valve ICs (Brand *et al.* 2006). The expression of these muscle cell markers suggests that valve cusps express the required machinery for contractile responses. These cells also express genes that encode components of the *wnt*/ $\beta$ -catenin signalling pathway. Members of the Frizzled family of *wnt* receptors have also been detected in cells from all four heart valves (Brand *et al.* 2006). Believed to play a role in the embryonic development of the valves (Butcher & Markwald (2007) review developmental signalling in this issue), the components of *wnt* growth factor signalling have been implicated in pathological mechanisms in mature valves (Hurlstone *et al.* 2003; Rajamannan *et al.* 2005).

### (b) Cell communication

Communication between valve ICs themselves, and between the ECM and ICs, is a feature that is important to the regulation of valve function.

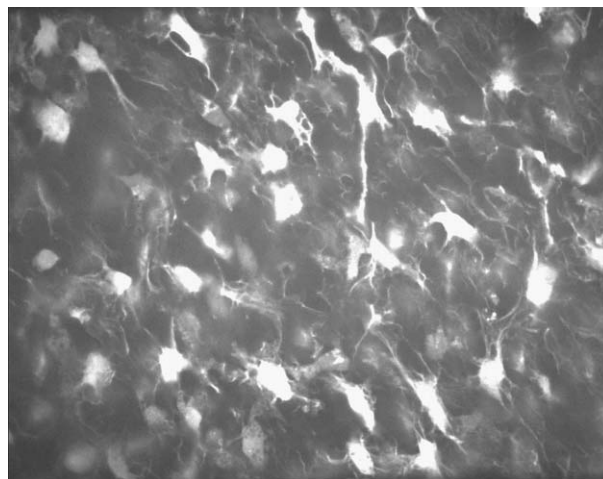


Figure 1. Photomicrographs of a porcine aortic valve leaflet stained with calcein AM. The panel shows a confocal slice at  $z=200\ \mu\text{m}$  from ventricular surface showing ICs.

Recently, we identified a range of the molecules involved in communication between valve ICs (Latif *et al.* 2005b). These markers include components of cell-to-cell junctions, such as cadherens (*N*-cadherin), desmosomal junctions (desmoglein) and some expression of gap-junction proteins (Connexin-26 and 45), but an absence of proteins associated with tight junctions. The cells that populate valve cusps reside within, and make connections to, the ECM as well as connections to each other (figure 1). This enables them to sense and respond to the mechanical forces experienced by the valve. It has been shown that cells in intact valves and those maintained in culture express a range of integrin molecules, including  $\alpha 1$ ,  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$  and, to a lesser extent,  $\alpha 6$  and  $\alpha V$ . Expression of  $\beta 1$ , but not  $\beta 3$  or  $\beta 4$ , integrins has also been reported (Latif *et al.* 2005a). A number of studies have demonstrated the importance of integrins in the regulation of cell behaviour due to their ability to act as sensors and transducers of signals in a bidirectional manner.

The ability of valve ICs to form a network *in vitro* is demonstrated by their ability to contract collagen gels in an efficient and unique manner (Butcher *et al.* 2004). They have been shown to have contractile properties and synthesize matrix components. Unlike some fibroblast-type cells and vascular smooth-muscle cells, valve ICs are able to induce contraction of collagen gels, the magnitude of which was inversely proportional to the number of cells seeded into the gel (Smith *et al.* in press). Morphologically, the valve ICs within the collagen gels generated force initially by spreading, elongation and finally sending out extension processes. The maintenance and gradual increase in force appeared to be due to continued and extensive formation of cellular processes that created a network of cells within the collagen gel. Given that these cells normally reside in a highly dynamic mechanical environment, the intensive direct cell-to-cell and cell-to-matrix communication may reflect a mechanism that contributes to the maintenance of tension within the valve cusp during systole. Examining the response of cells in collagen gels may be a useful gauge of the

similarity of different cells to valve ICs, and therefore aids in the choice of cells with which to tissue engineer a heart valve.

#### (c) *Contractile capacity*

The presence of contractile cell phenotypes within porcine and human aortic valves has been demonstrated via molecular and histological studies (Bairati & DeBiasi 1981; Taylor *et al.* 2000). In addition, aortic cusp tissue has been shown to exhibit contractile responses to a range of different vasoactive agents, a number of which are also capable of increasing levels of intracellular calcium (Chester *et al.* 2000; Taylor *et al.* 2000). Specific receptor systems have been demonstrated to mediate these contractile responses (Wassenaar *et al.* 1997; Chester *et al.* 2000, 2001; Misfeld *et al.* 2002). These include responses to commonly encountered vasoconstrictors such as catecholamines, histamine, 5-hydroxytryptamine and endothelin. In addition to the endothelium-derived vasoconstrictor endothelin, it has also been demonstrated that cusp tissue can relax in response to the release of nitric oxide from the endothelium (Pompilio *et al.* 1998). The responses to vasoactive mediators of cusp tissue are most probably mediated by contraction of a band of smooth-muscle  $\alpha$ -actin-positive cells that can be localized along the ventricular surface of aortic valve cusps (figure 2). The role of these cells, and whether they represent an 'activated' cell phenotype, is still a matter of debate. The percentage of cells that are smooth-muscle  $\alpha$ -actin-positive increases in cells which are involved with development of the valve or under diseased conditions, compared to normal undiseased adult cells (Rabkin-Aikawa *et al.* 2004). The pattern of expression of smooth-muscle  $\alpha$ -actin in foetal tissue has been shown to represent activated/immature cells (with high expression) that decrease with age as the cells become quiescent, suggesting a remodelling role for these cells as the valve responds to the post-natal haemodynamic environment (Aikawa *et al.* 2006). It has been demonstrated that valve ICs respond to local tissue stress by increasing their stiffness via changes in their smooth-muscle  $\alpha$ -actin content (Merryman *et al.* 2006), indicating that the contractile machinery in valve cells may assist in tissue homeostasis. The role and relevance of contractile mechanisms in valve tissue remains unclear. Investigation is required to elucidate if certain subpopulations of valve cells mediate specific responses of the valve, such as those to mechanical force, autocrine or paracrine mediators, or neuronal responses (Hafizi *et al.* 2000; Xing *et al.* 2004a,b; Ku *et al.* 2006). Indeed, if certain cells do become activated as part of a pathological response, this suggests that such cells go on to alter their phenotype further and participate in the disease process.

#### (d) *Secretory properties*

Valve ICs have the capacity to synthesize and release mediators that are involved in ECM synthesis, remodelling of the matrix and mediation of inflammatory responses. We have previously shown the expression of MMPs and TIMPs in valve cusps (Dreger *et al.* 2002). The valve ICs constitutively express these enzymes, indicating their key role in the

regulation of the ECM. In addition, valve ICs in culture can secrete collagen in response to stimulation by vasoactive mediators, such as 5-hydroxytryptamine and angiotensin II, and in response to mechanical force (Hafizi *et al.* 2000; Xing *et al.* 2004b). The ability of valve ICs to regulate and remodel the ECM is important to maintain the strength and durability of valve cusps. It will be important for any cell type that is used to tissue engineer a heart valve to possess similar properties.

The role of the valve ICs in orchestrating inflammatory responses in valve cusps has not been addressed. There are a number of non-immunological cell types that are thought to play a role in mediating inflammatory reactions due to their capacity to release cytokines and/or chemokines; these include vascular and airway smooth-muscle cells, vascular endothelial cells and lung epithelial cells (Bouma *et al.* 1996; Saunders *et al.* 1997; Stanford *et al.* 2002). It would appear that valve ICs also have the capacity to secrete a range of pro- and anti-inflammatory cytokines under basal conditions. We have obtained evidence that human valve ICs can release significant levels of IL-6 and IL-8 under resting conditions. IL-12, IL-10 and TNF- $\alpha$  were also detectable; in contrast, there was no release of IL-1. The levels of these cytokines increased when the cells were stimulated with lipopolysaccharide (figure 3). Localized disruptions in the balance between the release of anti- and pro-inflammatory cytokines and chemokines may be induced by stimuli associated with valve-disease risk factors.

It has been proposed that valve calcification is an inflammatory disease (Durbin & Gotlieb 2002). The ability of valve ICs to participate in the initiation or progression of inflammatory reactions may represent important mechanisms through which these cells are involved in the disease process, and identifies them as potential targets for pharmacological intervention.

#### (e) *Signalling pathways involved in valve calcification*

The presence of osteoblast-like phenotypes has been described in calcified aortic valves. This observation suggests that valve ICs are capable of undergoing cell differentiation and expressing osteoblast markers such as osteopontin, bone sialoprotein, osteocalcin and osteoblast-specific transcription factors (O'Brien *et al.* 1995; Rajamannan *et al.* 2003). Bone formation in cardiac valves has been associated with expression of bone morphogenetic proteins 2 and 4, as well as infiltration of B- and T-lymphocytes, in areas of ossification (Mohler *et al.* 2001). The expression of bone markers in degenerating aortic valves and the formation of cartilage in diseased mitral valves have been shown to involve activation of the lipoprotein-receptor-related protein 5 (Lrp5; Caira *et al.* 2006). Lrp5 is a co-receptor that binds to the secreted glycoprotein *wnt* and activates  $\beta$ -catenin, a pathway involved in bone formation.

Calcified valves have differential expression of RANKL (ligand for the receptor activator of NF- $\kappa$ B, RANK) and osteoprotegerin (OPG), both of which are members of the cytokine system that regulate bone turnover (Kaden *et al.* 2004a). Via the stimulation of

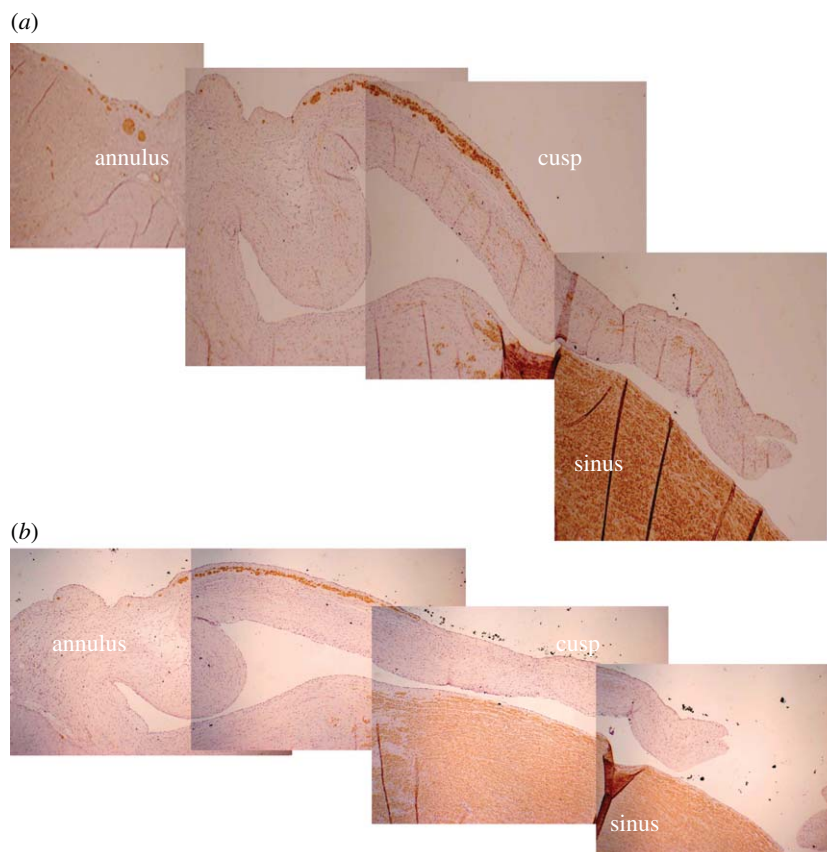


Figure 2. Composite photomicrograph of sections of porcine aortic roots shown staining (brown colour) for antibodies against (a) smooth muscle cell  $\alpha$ -actin and (b) smooth muscle cell myosin.

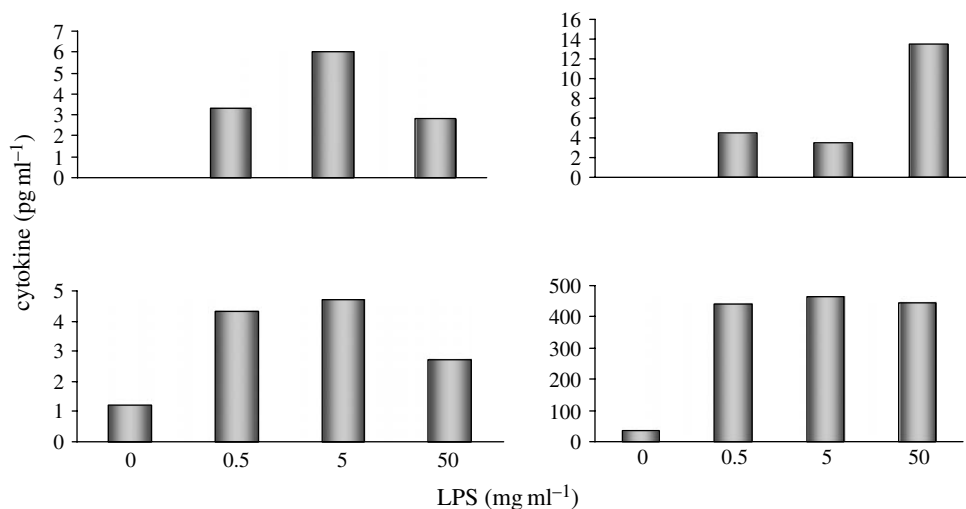


Figure 3. Graphs showing the release of IL12, IL10, TNF- $\alpha$  and IL-6 from cultured valve ICs in response to increasing concentration of lipopolysaccharide over a 24 h period.

RANK, RANKL induces osteoclastogenesis and has been shown to increase the DNA binding of osteoblast transcription factor *cbfa-1*, which is essential for osteoblast differentiation (Kaden *et al.* 2004a). Importantly, in human valve cells grown in osteogenic media, RANK/RANKL was shown to induce expression of *cbfa-1*. Interestingly, RANK, RANKL knockout mice and mice deficient in the p50 and p52 subunits of NF- $\kappa$ B all develop severe osteopetrosis (Franzoso *et al.* 1997; Dougall *et al.* 1999; Kong *et al.* 1999). The RANK/RANKL pathway is inhibited by expression of OPG, which serves as a soluble decoy receptor for

RANKL to limit activation of RANK. Deletion of the OPG gene results in severe calcification of the vasculature and expression of RANK/RANKL in calcified areas. It has been shown that TNF- $\alpha$  is capable of increasing the responsiveness of RANKL, via induction of RANK (Nanes 2003). In addition, IL-1, IL-4, IL-6, TGF- $\beta$ 1, GM-CSF and IFN- $\gamma$  have all been shown to affect the regulation of bone synthesis and reabsorption (Riancho *et al.* 1995; Deyama *et al.* 2001; Yeh *et al.* 2002; Postiglione *et al.* 2003).

There appear to be a number of different potential mediators that are involved in valve function and/or

dysfunction. The reason why those mediators that are involved in valve development (BMPs, endothelin) are then implicated in valve disease in a later stage of life, or how multiple mediators can initiate the calcification process, is undefined. It is probable that there are a number of independent signalling pathways which either act in a cooperative manner or merely converge to induce phenotypic changes in valve cells. The precise molecular and cellular mechanisms, that either regulate valve development and function or valve dysfunction, are the subject of intense investigation by a number of groups.

### 3. SUMMARY

The cells that reside in or on heart valve exhibit a host of unique characteristics and functions. These properties allow them to regulate the complex function of the heart valve by serving as a source of structural proteins or biological mediators. Valve cells also act as a target for biological mediators and enzymes, via the activation of complex signalling pathways. These signalling pathways can mediate both physiological and pathophysiological mechanisms. As our understanding of the properties and function of valve ICs and endothelial cell increases, we are becoming increasingly aware of the challenge that is presented by the need to tissue engineer a heart valve. Replication of valve function will be a fundamental requirement for the successful production of a tissue-engineered heart valve.

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