

Dynamic and Reversible Changes of Interstitial Cell Phenotype During Remodeling of Cardiac Valves

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Background and aim of the study: The roles of cardiac valvular interstitial cells (VIC) in extracellular matrix remodeling in fetal development, adaptation and response to injury are largely unknown.

Methods: The phenotype of VIC was studied in health (normal adult human and sheep), development (fetal human and sheep), disease (human mitral valves with myxomatous degeneration), adaptation (clinical pulmonary to aortic valve autografts) and tissue-engineered heart valves matured in vitro and remodeled in vivo. Cell phenotype was assessed using expression of vimentin (V), α -smooth muscle actin (SMA, A), matrix metalloproteinase (MMP)-13/collagenase-3 (M), and SMemb (S).

Results: VIC in normal adult valves were predominantly quiescent fibroblasts immunoreactive to vimentin ($89.7 \pm 2.5\%$), but not MMP-13 or SMemb, with only $2.5 \pm 0.4\%$ of α -SMA-positive cells ('normal/quiescent' phenotype: V+/A-/M-/S-). In contrast, fetal VIC were mostly activated myofibroblasts ('developing/activated' phenotype: V+/A+/M+/S+), with $62.1 \pm 5.0\%$ of cells staining positive for α -SMA.

Heart valves are complex and dynamic tissues composed of highly specialized cells and extracellular matrix (ECM) that change in response to local mechanical forces and other environmental stimuli. However, the events and mechanisms by which valvular interstitial cells (VIC) remodel and renew the ECM and adapt to different environments are largely unknown. Moreover, key cellular processes in embryological development, growth, and adaptation to altered loading remain unknown. Information on the structural

VIC in myxomatous valves, short-term autografts and engineered valves in vitro were also activated myofibroblasts with coexpression of vimentin, α -SMA ($36.2 \pm 3.7\%$, $19.3 \pm 2.4\%$, and $60.3 \pm 9\%$ positive cells, respectively), strong MMP-13 activity indicative of collagen remodeling, and SMemb ('remodeling/activated' phenotype: V+/A+/M+/S+). In contrast, VIC in long-term pulmonary autografts and engineered valve explants had a mostly fibroblast-like phenotype, with sparse α -SMA expression ($6.0 \pm 1\%$ and $5.4 \pm 1.0\%$ positive cells) (V+/A-/M-/S-).

Conclusion: Most VIC in normal valves were quiescent with a fibroblast-like phenotype. VIC in developing, diseased, adapting and engineered valves adjust to a dynamic environment through VIC activation and secretion of proteolytic enzymes mediating extracellular matrix remodeling ('developing/remodeling/activated' phenotype), followed by a normalization of phenotype.

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plasticity and dynamic function of the native valves, especially related to cell phenotypes and ECM remodeling, may benefit the development of tissue-engineered heart valves. Efforts to elucidate the scientific concepts crucial to tissue engineering are exemplified by recent studies of heart valves, including embryological development, structure in health and disease, cell phenotypes, ECM homeostasis and tissue valve substitutes (1-7).

The authors' studies using normal, pathological and substitute valves have demonstrated that valvular ECM is the principal determinant of durability, and the quantity and quality of valvular ECM depend on the viability and function of VIC (6-8). The results of the present study show that heart valves can respond to alterations in physical signals by reversible phenotypic modulation of VIC and expression of proteolytic enzymes. Characterization of normal heart valve com-

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position, properties and plasticity in response to external stimuli is critical to the development of fundamental principles for improving engineered heart valves, including the selection of appropriate cells, replication during in vitro maturation of an environment found during normal valve function and development, and determination of the ideal end-points for their remodeling in vivo.

Materials and methods

Valve models

The following models (defined as various situations of potentially different physiology) of valves in development, health, disease, remodeling and adaptation were analyzed: (i) fetal human (n = 1) and sheep (n = 6) pulmonary valves; (ii) normal adult human pulmonary, aortic and mitral valves (n = 20) and sheep pulmonary valves (n = 5); (iii) human mitral valves with myxomatous degeneration (n = 15); (iv) clinical pulmonary (to aortic) autograft valve substitutes in place for 15 days to six years (n = 9); and (v) tissue-engineered heart valves fabricated dynamically in vitro for 14 days, implanted in lambs in the pulmonary position and explanted at 16-20 weeks (n = 10) as previously described (7,9). Normal human valves from autopsy, mitral valves with myxomatous degeneration derived from valve repair or replacement surgery (8) and clinical pulmonary autograft valves (10) were obtained according to a protocol approved by the Human Research Committee at the Brigham and Women's Hospital. Native pulmonary valves were obtained at experimental valve replacement surgery from healthy adult (2 years old) and fetal (115-140 days gestation period) sheep sacrificed for other investigations. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No.85-23, 1985).

Histological characterization of architecture and ECM components of valves

Valves were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (6 μ m) were stained with hematoxylin and eosin for general morphological analysis. ECM composition and distribution was examined by modified Movat pentachrome stain. ECM components were distinguished as proteoglycans (green staining), collagen (yellow), and elastin (black).

Determination of VIC immunophenotypic profile

VIC phenotypes were examined and smooth muscle cells (SMC) distinguished from myofibroblasts (11,12) using immunohistochemical markers for differentiation and maturation including antibodies to vimentin

(intermediate filaments; Dako), α -smooth muscle actin (α -SMA, microfilaments; Dako), and SM1, specific to a myosin heavy chain (differentiated SMC) (13). Proteolytic enzymes were detected using a mouse monoclonal antibody against human MMP-13/collagenase-3 (Calbiochem). SMemb (non-muscle myosin heavy chain) antibody was used to identify activated mesenchymal cells (14).

Immunohistochemistry was carried out using the avidin-biotin-peroxidase method after antigen retrieval when needed. Tissue sections were treated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity and incubated with primary antibodies diluted in phosphate-buffered saline supplemented with 4% of the species-appropriate normal serum. Subsequent processing was performed according to the manufacturer's recommendations (Universal Dako LSAB kit; Dako Corp.). The reaction was visualized with 3-amino-9-ethyl carbazole as substrate (AEC; Sigma Chemical Co). Sections were counterstained with Harris hematoxylin followed by 3% NH₄OH solution. Adjacent sections treated with non-immune IgG served as controls for antibody specificity.

Cytometry

The fractions of vimentin, α -SMA-, MMP-13- and SMemb-positive cells were counted, and their distribution analyzed in representative regions across the major layers of the valve cusps using an eyepiece reticle (10 mm, 1 mm division) (Fisher Scientific). The ratio of positive cells to the total number of cells was averaged over five representative high-power fields.

Statistical analysis

Statistical analysis employed a one-way analysis of variance (ANOVA) followed by Fisher's test. Data were presented as mean \pm SEM. A p-value <0.05 was considered to be statistically significant.

Results

The architecture (Fig. 1) and cell phenotype (Figs. 2 and 3) of normal adult human and sheep pulmonary valves and fetal human and sheep valves and the dynamic state of active remodeling in valves with myxomatous degeneration, clinical pulmonary autografts and tissue-engineered heart valves were compared.

Overall valve architecture

Layers in fetal human and ovine valves were indistinct, with predominant accumulation of proteoglycans, weak staining for collagen, and almost undetectable elastin (Fig. 1A, B). As previously

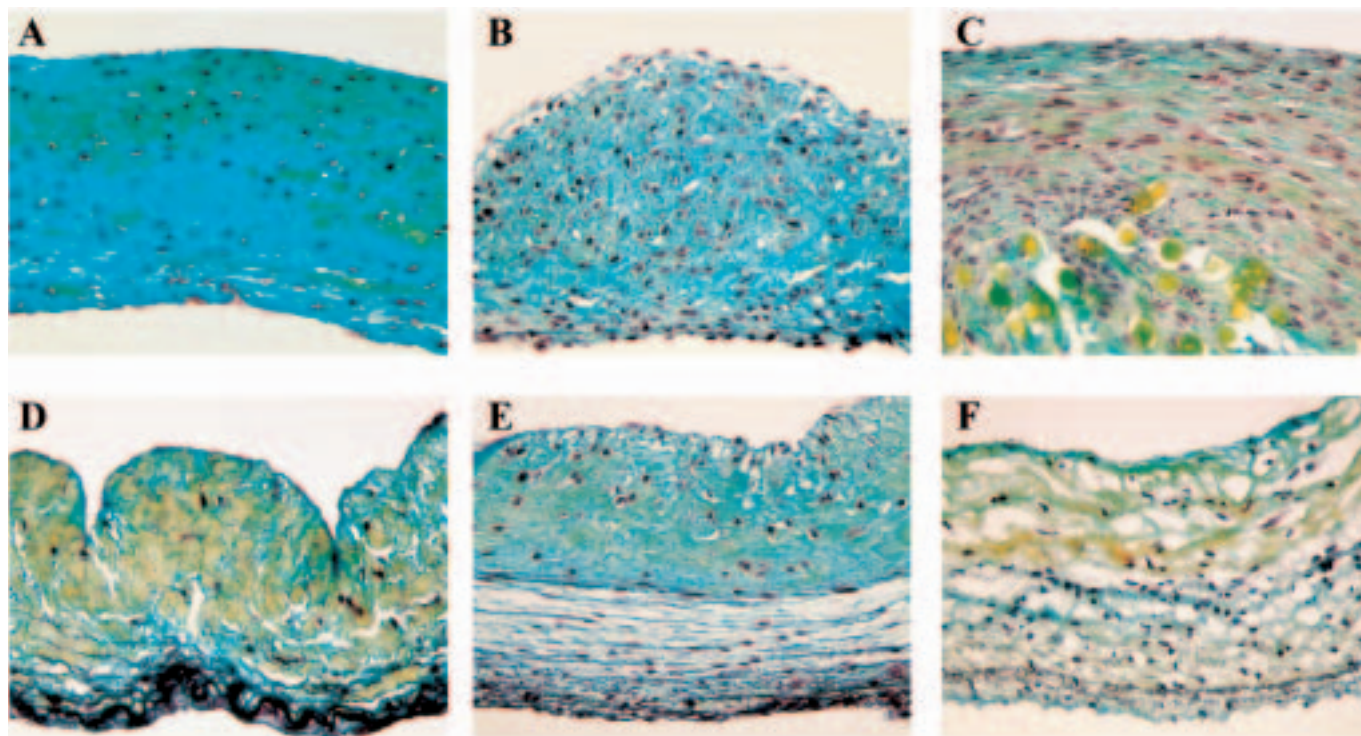


Figure 1: Extracellular matrix (ECM) composition of human and sheep fetal and adult pulmonary valves compared to tissue-engineered heart valves (TEHV) *in vitro* and *in vivo*. Layers in fetal human (A) and ovine (B) valves are indistinct, with predominant accumulation of proteoglycans, weak staining for collagen and almost undetectable elastin. C) TEHV matured *in vitro* have a predominant accumulation of proteoglycans, weak staining for collagen, and no formation of elastin, similar to native fetal valves. Adult human (D) and ovine (E) pulmonary valves have three well-defined layers, each containing the following characteristic ECM composition: the fibrosa (f) composed of collagen, the spongiosa (s) consisting mainly of proteoglycans, and the ventricularis (v) predominantly composed of elastin. F) TEHV at 20 weeks after implantation showed a layered structure with collagen in the fibrosa, proteoglycans in the central loose layer, and elastin in the ventricular side, resembling the normal native adult valve. Original magnification, $\times 200$.

described (7), engineered valves matured *in vitro* in a bioreactor have a predominant accumulation of proteoglycans, weak staining for collagen, and no formation of elastin (Fig. 1C), similar to native fetal valves.

Normal adult human and ovine pulmonary valve cusps had three well-defined layers, each containing the following characteristic ECM composition: the fibrosa composed of collagen, the spongiosa consisting mainly of proteoglycans, and the ventricularis predominantly composed of elastin (Fig. 1D, E). Myxomatous valves showed an expansion of the spongiosa by loose amorphous ECM that stained strongly positive for proteoglycans, diminished staining for collagen fibers, and fragmentation of elastin (8). Clinical pulmonary autograft valves showed near-normal architecture with a preserved tri-layered structure (10). After 20 weeks' function *in vivo*, tissue-engineered heart valves cusps showed a layered structure with collagen in the fibrosa, proteoglycans in the central loose layer, and elastin in the ventricular side, resembling the normal native adult valve (Fig. 1F).

Interstitial cell phenotype

Two distinct phenotypes of VIC were identified in the present study: the 'normal/quiescent' phenotype, characterized by the expression of vimentin (V), but not α -SMA (A), MMP-13/collagenase-3 (M) or SMemb (S), classified as fibroblast-like cells; and the 'developing/remodeling/activated' phenotypes, characterized by co-expression of vimentin (V), α -SMA (A), MMP-13/collagenase-3 (M), and SMemb (S), classified as activated myofibroblast-like cells.

Interstitial cells of fetal valves are activated myofibroblasts

A large fraction of VIC in fetal human and sheep valves were activated myofibroblasts. Most fetal human and ovine VIC stained positively for vimentin (V: $90 \pm 2.5\%$ and $78 \pm 5.1\%$, respectively) and α -SMA (A: $62.1 \pm 5.0\%$ and $36.8 \pm 6.4\%$), with strong expression of MMP-13/collagenase-3 (M: $83 \pm 5.2\%$ and $70 \pm 3.2\%$) and SMemb (S: $78 \pm 8.7\%$ and $62.5 \pm 3.4\%$) ('developing/activated' phenotype: V+/A+/M+/S+

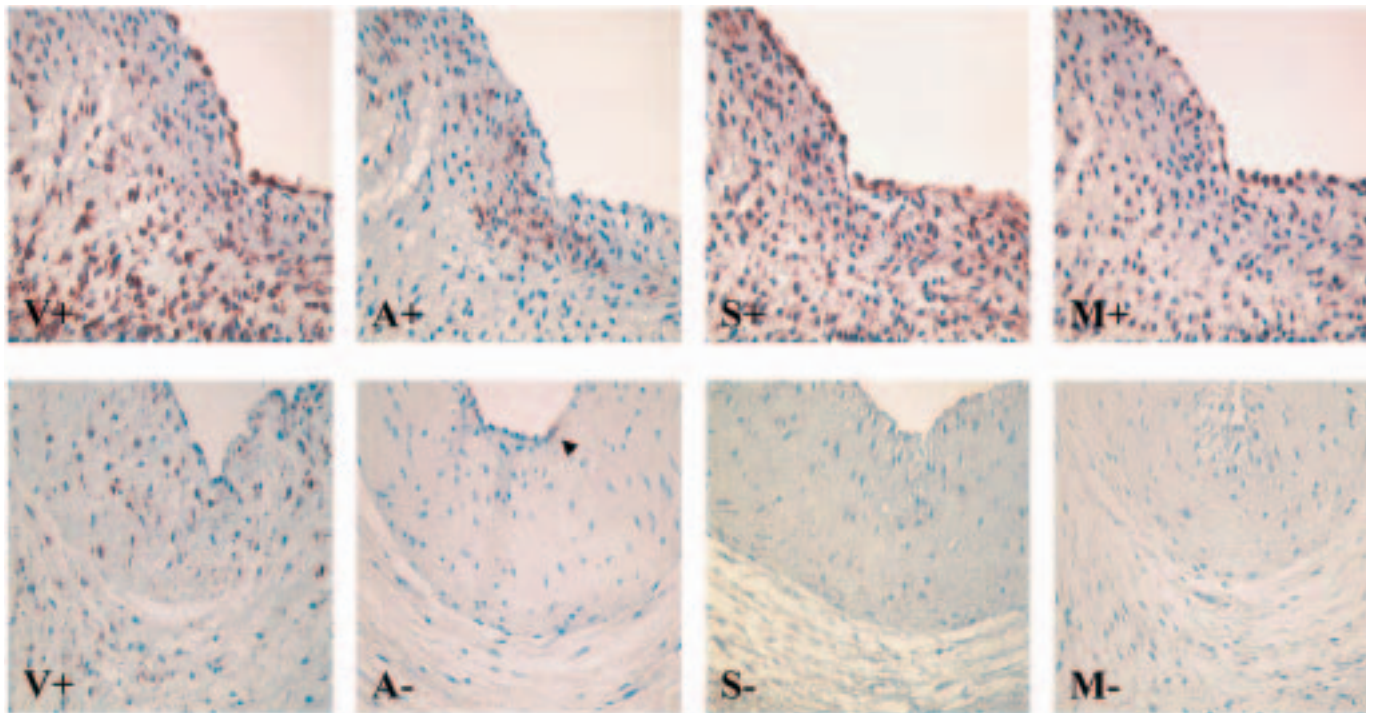


Figure 2: Immunophenotype of valvular interstitial cells (VIC) in fetal and adult sheep valves. VIC in fetal sheep valves are activated myofibroblasts stained positively for vimentin (V) and α -SMA (A), with strong expression of MMP-13/collagenase-3 (M) and SMemb (S) ('developing/activated' phenotype: V+/A+/M+/S+) original magnification, $\times 400$. VIC in healthy adult ovine pulmonary valves are mostly fibroblasts-like cells immunoreactive to vimentin, but not α -SMA, MMP-13, or SMemb ('normal/quiescent' phenotype V+/A-/M-/S-). Only a very small population of VIC located subendothelially expressed α -SMA in adult valves (arrowhead).

(Figs. 2 and 3A-C). Interestingly, a progressive decrease was found in α -SMA expression in fetal sheep valves during gestation: 47% at 115 days, 40% at 130 days, and 27% at 140 days.

Interstitial cells of adult valves are quiescent fibroblasts

Few VIC in healthy adult human and sheep pulmonary valves expressed α -SMA ($2.5 \pm 0.4\%$ and $4.9 \pm 0.7\%$ of cells α -SMA-positive, respectively). Thus, VIC in normal adult human and sheep valves were fibroblast-like cells immunoreactive to vimentin ($76.5 \pm 6.0\%$ and $62.4 \pm 4.6\%$), with minimal expression of α -SMA, MMP-13 ($12.5 \pm 2.7\%$), or SMemb ($11.6 \pm 1.2\%$) ('normal/quiescent' phenotype: V+/A-/M-/S-) (Figs. 2 and 3).

Many interstitial cells during valve remodeling are myofibroblasts

Cells in valves with myxomatous degeneration and short-term pulmonary autograft explants were mostly myofibroblasts of the vimentin/ α -SMA-positive phenotype, and showed strong MMP expression indicative of collagen remodeling ('remodeling/activated'

phenotype: V+/A+/M+/S+). The present results demonstrated that valves during disease and adaptation adjust to different environmental stimuli through VIC activation and increased α -SMA and MMP expression: $36.2 \pm 3.7\%$ and $19.3 \pm 2.4\%$ of cells α -SMA-positive, and $90.1 \pm 6.8\%$ and $42.6 \pm 5.0\%$ MMP-13-positive in myxomatous and pulmonary autograft valves, respectively. The number of activated myofibroblasts in myxomatous valves and short-term pulmonary autografts characterized as α -SMA/MMP-13-positive cells was significantly increased compared to normal valves ($p < 0.005$) (Fig. 3B and C).

Interstitial cells in engineered valves matured in vitro are activated

As previously reported (7), and demonstrated quantitatively in the present study, cells in tissue-engineered constructs grown in vitro for 14 days were activated myofibroblasts identified by strong coexpression of vimentin ($63.7 \pm 3.6\%$), α -SMA ($60.3 \pm 8.9\%$), MMP-13 ($86.2 \pm 7.5\%$), and SMemb ($97.8 \pm 3.3\%$): ('remodeling/activated' phenotype: V+/A+/M+/S+ (Fig. 3A-C).

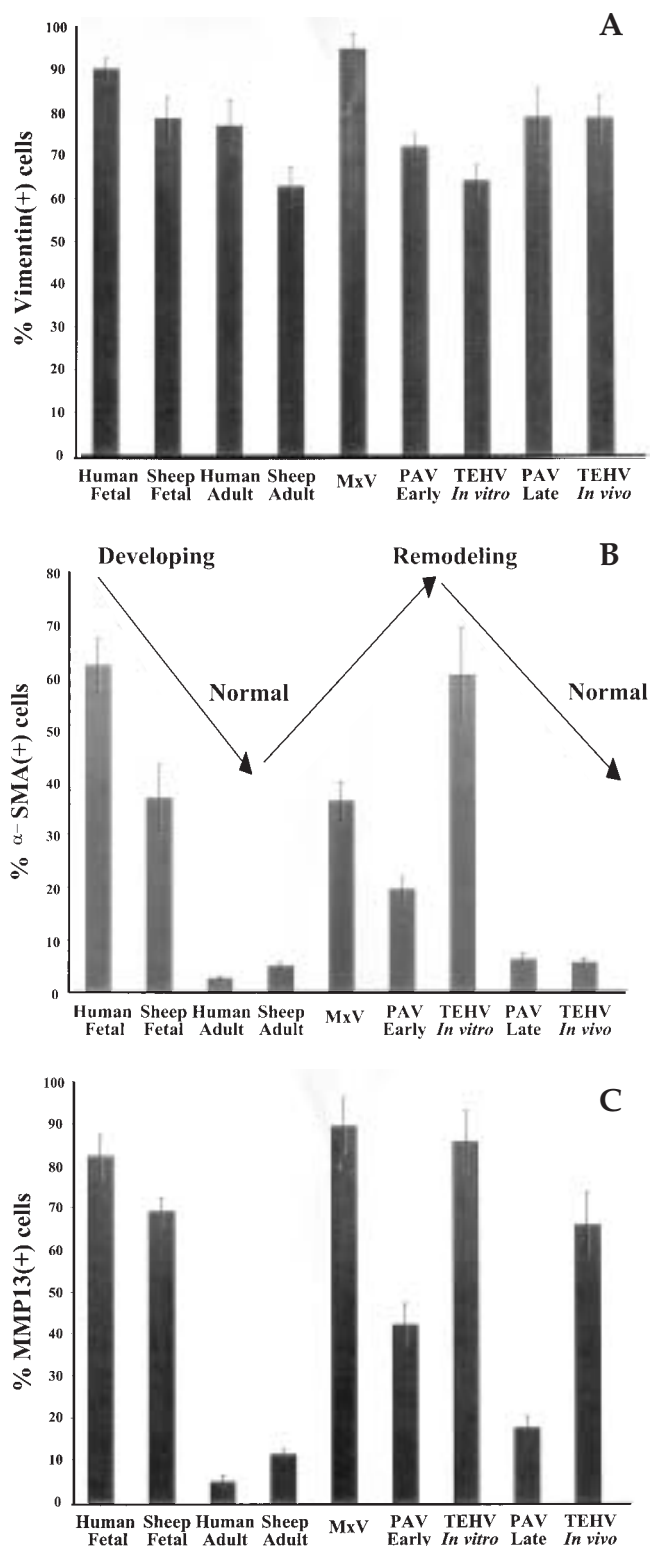


Figure 3: Valvular interstitial cell (VIC) plasticity was demonstrated as dynamic and reversible through changes in phenotype shown as fraction of (A) vimentin-, (B) α -SMA- and (C) MP₁₃-positive cells in studied valve models. MxV: Valves with myxomatous degeneration; PAV: Pulmonary (to aortic) autograft valves; TEHV: Tissue-engineered heart valves.

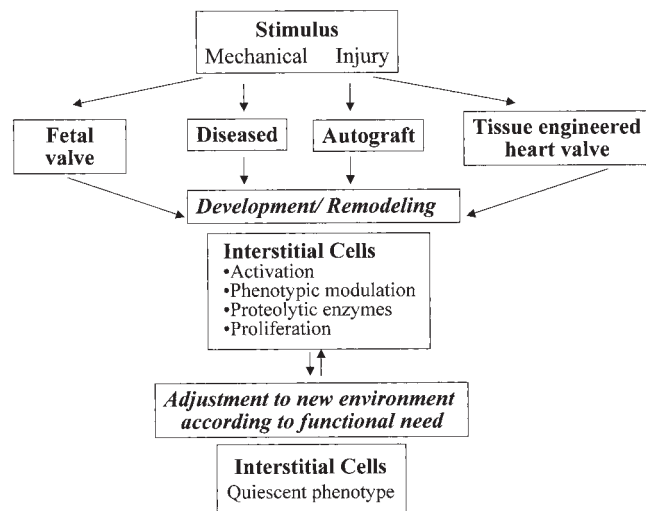


Figure 4: Common pathway for environment-mediated remodeling of valvular structure. In response to different environmental stimuli (altered mechanical loading, injury or genetic disorder), interstitial valvular cells during development, adaptation, disease and in tissue-engineered valves undergo activation, phenotypic modulation, and secrete proteolytic enzymes that mediate ECM remodeling according to functional need. When equilibrium is restored, the cells return to the quiescent state.

Interstitial cell plasticity is reversible

Long-term pulmonary autografts and tissue-engineered explants in vivo at 20 weeks mainly expressed vimentin and resembled the fibroblast-like cells of normal pulmonary valve ('normal/quiescent' phenotype: V+/A-/M-/S-). Reversible changes of VIC phenotype were demonstrated in long-term pulmonary autografts and tissue-engineered valve explants as a reduction of α -SMA expression, suggesting normalization of the phenotype. The number of α -SMA-positive cells significantly decreased from $19.3 \pm 2.4\%$ in the short term to $6.0 \pm 1.1\%$ in long-term pulmonary autografts, and from $60.3 \pm 8.9\%$ to $5.4 \pm 1.0\%$ in engineered valves ($p < 0.005$) (Fig. 3B). The number of MMP-13-positive cells also significantly decreased from $42.6 \pm 5.0\%$ in the short term to $17.8 \pm 2.8\%$ in long-term pulmonary autografts, and from $86.2 \pm 7.5\%$ to $66.4 \pm 7.8\%$ in engineered valves ($p < 0.005$) (Fig. 3C). The number of SMemb-positive cells in engineered valves, however, did not decrease and did not reach the baseline levels observed in normal valves. It is not clear what effect longer periods of implantation would have on SMemb expression in engineered valves.

Discussion

Cardiac valves are complex tissues composed of multiple layers of ECM of markedly different density, composition and function, which is maintained,

renewed and remodeled by highly specialized cells (1). Valvular interstitial cells are dynamic populations of resident/structural cells of multiple sources and phenotypes. Fibroblast-like cells form the majority of the interstitial cell population in normal cardiac valves, yet their behavior in development, disease and adaptation is largely unknown.

The system developed in the present study allows for the identification and categorization of VIC that will be a useful tool for clinical and research applications. Using this system, it could be demonstrated that fetal valves and tissue-engineered valves matured *in vitro* have a similar ECM composition, while adult valves and engineered valves remodeled *in vivo* have a comparable tri-layered structure. Furthermore, it was shown that interstitial cell plasticity is dynamic and reversible, by demonstrating that interstitial cells of fetal valves are predominantly activated myofibroblasts ('developing/activated' phenotype: V+/A+/M+/S+), interstitial cells of adult valves are quiescent fibroblast-like cells ('normal/quiescent' phenotype: V+/A-/M-/S-), and many interstitial cells during valve remodeling and in engineered valves matured *in vitro* are activated myofibroblasts ('remodeling/activated' phenotype: V+/A+/M+/S+). The results of the present study showed that, *in vivo*, both healthy adult human and sheep pulmonary valves are populated predominantly with quiescent fibroblasts, characterized as cells that do not express α -SMA. Developing and diseased valves, however, contain numerous myofibroblast-like VIC expressing α -SMA, suggesting a role for myofibroblasts in the active remodeling of connective tissue. Previous *in vitro* studies using isolated myofibroblasts from heart valves showed that 50-90% of the cells were α -SMA-positive, suggesting that culture conditions might stimulate VIC activation (2,4,15,16).

Altered mechanical forces and injury are associated with phenotypic changes of valvular cells. The present results suggested that VIC undergo phenotypic modulation from resting fibroblast-like cells in normal valves to activated myofibroblasts in myxomatous valves, short-term pulmonary autograft valve explants, and tissue-engineered heart valves *in vitro*. Collectively, this and previous data suggest a general paradigm by which cardiac valvular tissue is dynamically and reversibly responsive to environmental conditions - particularly mechanical loading - and demonstrate that similar changes occur in valves during development, adaptation, pathology and substitution. Under equilibrium conditions, valvular cells are quiescent and the extracellular matrix is well adapted. When stimulated by mechanical loading, the interstitial cells become activated and mediate connective tissue remodeling to restore the normal stress profile in the tissue. When equilibrium is restored, the cells return to the quiescent state. Moreover, a similar state

of activation occurs physiologically during fetal development. The present data, which are summarized schematically in Figure 4, imply the existence of a common pathway associated with physiological/adaptive and pathological remodeling of valvular tissue. This pathway involves a potentially reversible and likely highly regulated phenotypic modulation of VIC that mediates ECM remodeling by the secretion of proteolytic enzymes. Dysregulation of this pathway may contribute to a variety of valve diseases, including myxomatous degeneration. The dynamic nature and reversibility of VIC phenotype (fibroblast \rightarrow myofibroblast \rightarrow fibroblast) in valves may reflect the ability of viable tissue to repair and remodel *in vivo* according to dynamic functional need, and, ultimately, to recapitulate the architectural features of normal valves.

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Meeting discussion

DR. ROBERT NEREM (USA): You showed a comparison of 14 days in vitro versus 20 days in vivo for cell growth. Once you implant, how long does it take for the changes to occur? For example, if you looked at cells after 10 days in vivo, would they appear more like the in p 847

vitro cells, or the 20 day in vivo cells?

DR. ELENA RABKIN (Boston, Massachusetts, USA): In early explants, the cells were activated and still expressed alpha-actin and other markers that we suggest using for cell phenotyping. But after about 16

weeks they became quiescent and lost alpha-actin, SMemb and MMP-13 expression.

DR. S. HOERSTRUP (Switzerland): My concern from a tissue engineering perspective is that the activated myofibroblasts are found in a tissue-engineered construct in a very pathological situation. Is it really the same cells that are simply changed in phenotype, or might it be a different cell population?

DR. RABKIN: Both possibilities exist, but I think that in our model the cells are changing their phenotype. A tissue-engineered valve in vitro actually recapitulates the stages of valve development. We found exactly the same expression of markers as were involved during development and maturation in vitro. After implantation, the direction is similar to a native valve, towards a reduction in cell activation.

DR. HOERSTRUP: My concern is if you compare this directly to the pathological situation - myxoma, for example - and it is the same cell type, we must be very careful what we are producing in the bioreactor.

SIR MAGDI YACOUB (UK): Do you think that the origin of the cell as well as the origin of the phenotype can have a bearing on your findings? For example, Dr. Taylor has shown that valvular interstitial cells have a very specific type of phenotype that is unlike that of any other cell. We know that these cells originate from the neural crest, which is a very specific embryological origin. In the tissue engineering, what was the source of the cells, and would that have any bearing on the findings? Were they smooth muscle cells from the arterial wall, or skin fibroblasts? They are already committed cells.

DR. RABKIN: We used a vascular-derived model of tissue engineered valves, which we compared to valves during adaptive remodelling as pulmonary autograft valves. What is interesting is that tissue engineered valve development and maturation actually show a similar trend in cell phenotype changes toward those of native valves.

MR. YACOUB: But does the origin of the cells make a difference?

DR. RABKIN: It is very difficult to say. Basically, we need to have a phenotype that can eventually recapitulate the phenotypes of normal valve cells.

MR. YACOUB: So any type of cell can do anything?

DR. RABKIN: Recently we have worked on tissue-engineered valves from bone marrow cells, and were able to show similar results.

MR. YACOUB: If you have progenitor cells, perhaps they recapitulate in an embryologic program, but is this the case for committed cells like fibroblasts or smooth muscle cells from the arterial wall?

DR. RABKIN: In our study we were able to demonstrate reversible plasticity of fibroblast-like interstitial valvular cells.