

## Evidence of Woven Bone Formation in Heart Valve Disease

Masoud Mirzaie, MD,<sup>1</sup> Michael Schultz, MD, PhD,<sup>2</sup> Peter Schwartz, PhD,<sup>2</sup>  
Marlon Coulibaly, MD,<sup>1</sup> and Friedrich Schöndube, MD<sup>1</sup>

**The pathogenesis of acquired cardiac valve disease still remains a matter of controversy. In this work, scanning electron and polarised light microscopic investigations in addition to energy dispersive X-ray microanalyses (EDAX) were carried out on explanted human aortic and mitral valves to determine the morphology and element composition of calcified areas in valvular lesions. Biopsies were taken from aortic valves removed from 28 male patients (average age, 75±1 years) and 46 females (68±3 years) and from mitral valves obtained from 18 male patients (72±3 years) and 8 females (71±6 years). By means of scanning electron microscopy, multiple foci of calcified areas were identified. Endothelial cells in these areas appeared swollen and displayed reduced cell-cell contacts. The calcium deposits were separated from the adjacent tissue by layers of collagen fibers. Often a layer of woven bone tissue separated intravalvular inclusions from hyperplastic collagen fibers. Using EDAX analysis, calcium and phosphorus were detected in these valvular lesions. The major finding of our study is the presence of woven bone tissue in explanted cardiac valves, which may result from pathological strains or mechanical overloading of the collagen fibers. (Ann Thorac Cardiovasc Surg 2003; 9: 163–9)**

**Key words:** woven bone, human heart valves

### Introduction

Dystrophic calcification is the most common pathological finding in surgically explanted heart valves. Recently, several reports described bone formation in surgically excised heart valves and suggested an unexpected process of tissue repair.<sup>1-3</sup> The exact pathomechanisms of acquired cardiac valve disease are still unknown, although the calcification process of biological valves has been well investigated. Mechanical stress-induced factors are regarded to induce the calcification of biological valves.<sup>4-6</sup> In this report, we describe the morphology of degenerative changes in explanted aortic and mitral valves by us-

ing polarised light microscopy in addition to conventional electron microscopical techniques. We were especially interested in identifying bony structures mimicking valve calcification.

### Materials and Methods

#### Tissue biopsies

Tissue samples from human aortic and mitral valves were taken from patients undergoing routine valve replacement operations at the Department of Thoracic, Heart and Vascular Surgery. From each valve one leaflet was removed for further study. Biopsies were taken from 74 aortic and 26 mitral valves. The aortic valves were removed from 28 male patients (average age, 75±1 years) and 46 females (68±3 years) and the mitral valves were taken from 18 males (72±3 years) and 8 females (71±6 years). The study population included patients suffering from aortic stenosis (n=38, 51.4%) and aortic insufficiency (n=36, 48.6%). There were 17 cases of mitral insufficiency (65.4%) and 9 cases (34.6%) of mitral stenosis. Table 1

From <sup>1</sup>Department of Thoracic, Heart and Vascular Surgery, The University Clinics Göttingen, and <sup>2</sup>Institute for Anatomy, University of Göttingen, Germany

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Address reprint requests to Masoud Mirzaie, MD: Department of Thoracic, Heart and Vascular Surgery, The University Clinics Göttingen, Robert-Koch-Str. 40, D-37075 Göttingen, Germany.

**Table 1. Baseline characteristics of the study population**

	Aortic valve disease (n=74)	Mitral valve disease (n=26)
Valve pathology		
Stenosis exclusively	38 (51.3%)	9 (34.6%)
Insufficiency/mixed	36 (48.6%)	17 (65.4%)
Male	n=28	n=18
Female	n=46	n=8
Age (mean±SD)		
Gender		
Male	75±11	72±9
Female	68±8	71±8
NYHA		
I/II	13 (17.5%)	5 (19.2%)
III/IV	61 (82.4%)	21 (80.7%)
Concomitant diseases		
CHD	42 (56.7%)	14 (53.8%)
Syncope	13 (17.5%)	0
Embolism	2 (2.7%)	1 (3.8%)
Heart failure	19 (25.6%)	9 (34.6%)
Hypertonia	53 (71.6%)	12 (46.1%)
Endocarditis	4 (5.4%)	0
Rheumatic fever	2 (2.7%)	0
Concomitant CABG		
Isolated AVR	32	Isolated MVR 12
AVR+CABG	42	MVR+CABG 14
AVR+MVR	9	

Continuous variables are presented as mean ± standard deviation. Categorical variables are presented as an absolute percentage. NYHA, New York Heart Association; CHD, coronary heart disease; CABG, coronary artery bypass grafting; AVR, aortic valve replacement; MVR, mitral valve replacement

summarises the basic data of the study patients.

In comparison, postmortal tissue samples from 3 male patients (average age, 78.5 years) and 3 female patients (average age, 73.3 years) who died primarily from non-cardiac causes, were explanted and investigated.

### Scanning electron microscopy

In order to reveal the surface morphology of the explanted valves, scanning electron microscopy (SEM) was performed. Specimens from valve leaflets were fixed for 6 h in a solution containing 2.5% glutaraldehyde and 0.2 M cacodylate. Samples were then dehydrated in an ascending series of alcohol. After critical point drying, all samples were sputtered with gold-palladium. Samples were visualised using the digital scanning microscope DSM 960 (Carl Zeiss, Oberkochen, Germany).

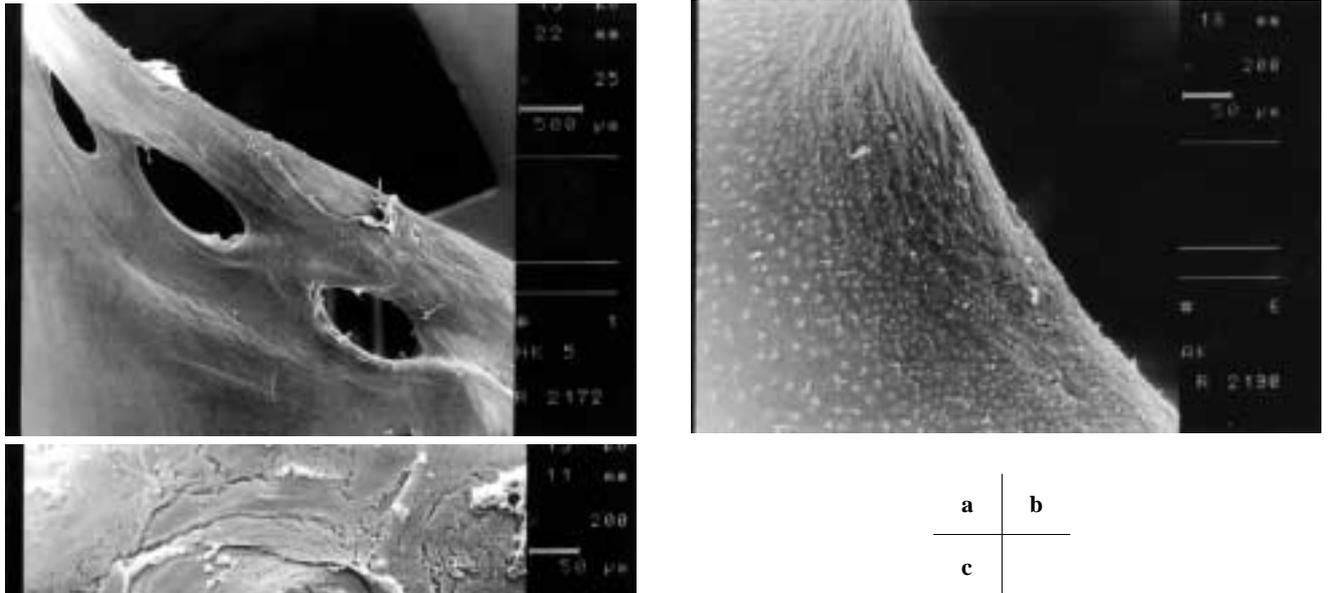
### Polarised light microscopy

To evaluate the presence of woven bone tissue in calcified areas of pathologically altered aortic and mitral

valves, polarised light microscopy was performed. To prepare thin ground sections from undecalcified materials for polarised light microscopy, a special technique was established based upon the method of plastination developed by Hagens et al.<sup>7)</sup> and modified for histological purposes by Schultz and Drommer.<sup>8)</sup> Samples were dehydrated using ascending concentration steps of alcohol, put into methyl chloride as an intermediate solution for the exchange of substances and embedded in epoxy resin Biodur®. Unstained thin ground sections (30, 50 and 70 µm) were prepared and viewed in transmitted plane and in polarised light using a quartz plate red first order, equipped with photo documentation.<sup>9,10)</sup>

### Energy dispersive X-ray microanalysis (EDAX)

In order to determine the element content in explanted human aortic and mitral valves, the specimens were examined by means of EDAX. The samples were fixed for 6 h in a solution containing 2.5% glutaraldehyde and 200 mM cacodylate, dehydrated in an ascending series of al-



**Fig. 1.** Scanning electron micrographs of explanted aortic and mitral valves.

Multiple tears in the leaflet tissue parallel to the edge of the leaflet (a, aortic valve, male patient, 74 years old). The tears showed an endothelial cover (b, aortic valve from the same patient as in Fig. 1a). Calcified material predominantly accumulated in the center of the leaflet and along its attachments to the aortic and mitral rings (c, mitral valve, female patient, 68 years old).

cohol and dried using a critical point drier. The specimens were then sputtered with carbon and irradiated with electrons in a digital scanning microscope (DSM 960, Carl Zeiss). The X-rays emitted from the samples were measured by energy dispersion using an EDS system (Chromeritz, Leipzig, Germany) coupled to the microscope.

## Results

### Scanning electron microscopical findings

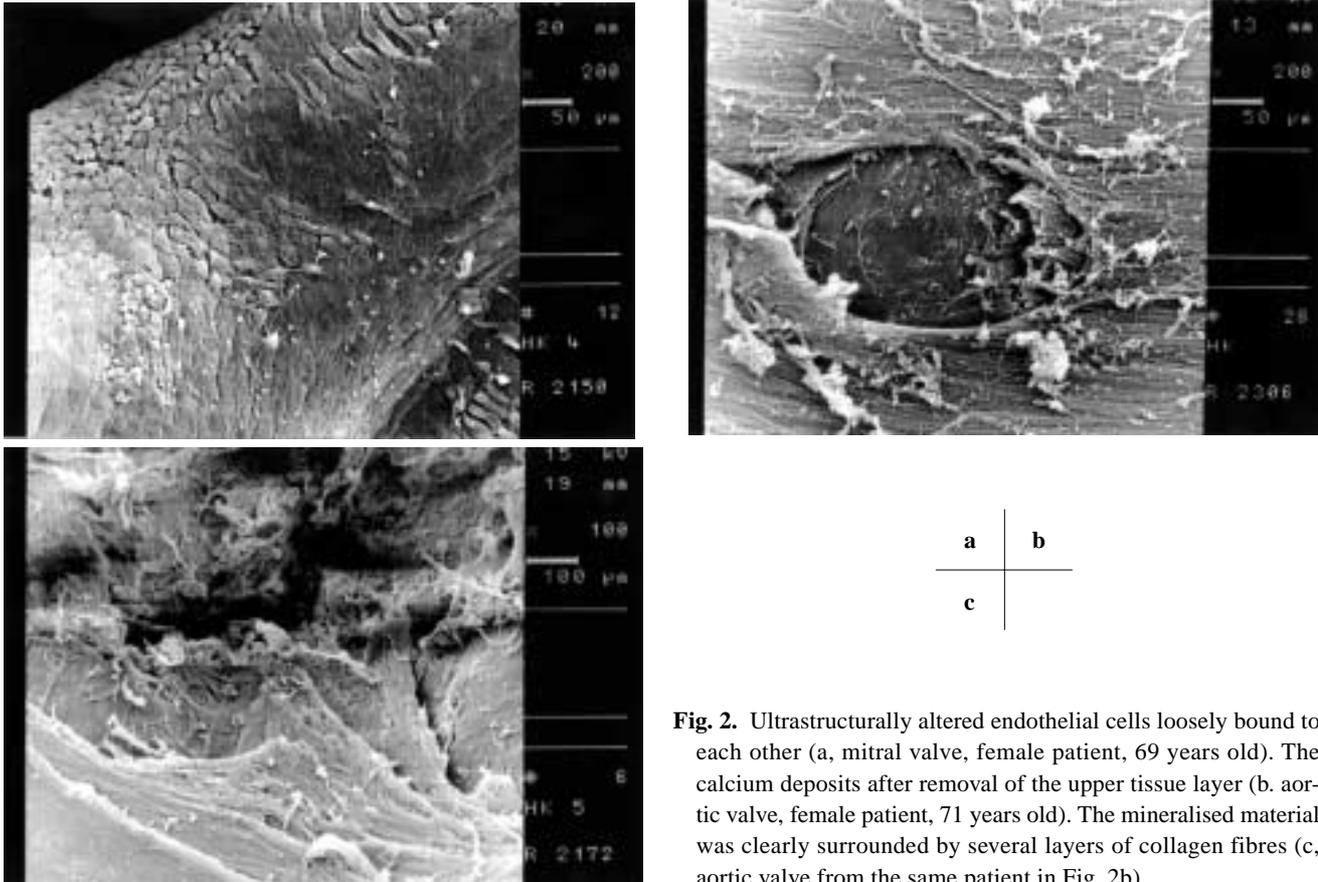
The tissue biopsies from the human aortic and mitral valves examined showed various types of endothelial lesions which have already been described in detail.<sup>11)</sup> In all of the explanted valves, we observed multiple tears in the leaflet tissue oriented parallel to the edge of the leaflet (Fig. 1a). At higher magnification, these areas usually exhibited an endothelial cover (Fig. 1b). Calcified material was predominantly accumulated in the center of the leaflet and along its attachments to the aortic and mitral rings (Fig. 1c). The endothelial cells in these regions were ultrastructurally altered and, furthermore, loosely bound to each other (Fig. 2a). The mineralised

material was frequently surrounded by a layer of collagen fibres. When the calcium deposits were removed, it was seen that the collagen fibres were arranged in several layers (Fig. 2b, c).

### Polarised light microscopical findings

Recent techniques employing polarised light microscopy are potentially useful to evaluate the presence of woven bone tissue. Tissue samples from normal aortic valves revealed no pathological alterations. Even though bundles of collagen fibres were present, no morphological changes were detected (Fig. 3).

When unstained thin ground sections of pathologically altered samples were viewed in transmitted plane light, the intravalvular localised inhomogeneous inclusions represented as a secondary substance of yellowish color (Fig. 4a). In regular light, the character of this substance could be described as fibrous resembling natural woven bone. By high magnification ( $\times 620$ ), bundles of collagen fibres were detected (Fig. 4b). Using the quartz plate red first order as a compensator, the bundle of collagen fibres were clearly identified by their blue colors (Fig. 4c).



**Fig. 2.** Ultrastructurally altered endothelial cells loosely bound to each other (a, mitral valve, female patient, 69 years old). The calcium deposits after removal of the upper tissue layer (b, aortic valve, female patient, 71 years old). The mineralised material was clearly surrounded by several layers of collagen fibres (c, aortic valve from the same patient in Fig. 2b).

### Results of the energy dispersive X-ray microanalysis

Energy dispersive X-ray microanalysis (EDAX) was carried out to determine the element content in various endothelial lesions of the explanted aortic and mitral valves. With the exception of degenerative lesions, no calcium signals were detected in the extracellular matrix of valvular alterations. However, mineralised deposits were frequently observed in areas resembling bone-like tissue features. These areas contained calcium and phosphorus as demonstrated by characteristic EDAX patterns (Fig. 5).

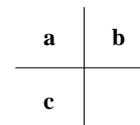
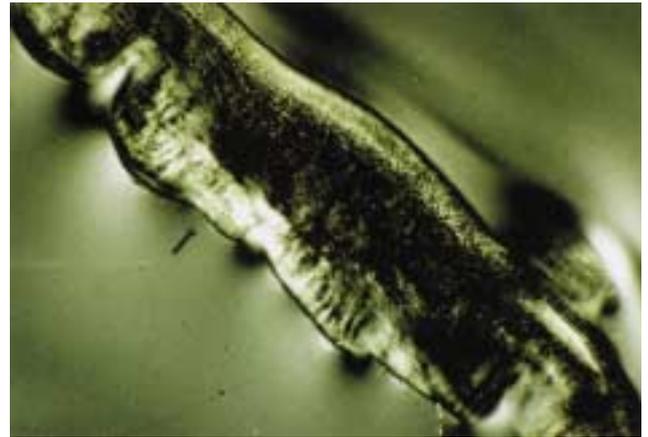
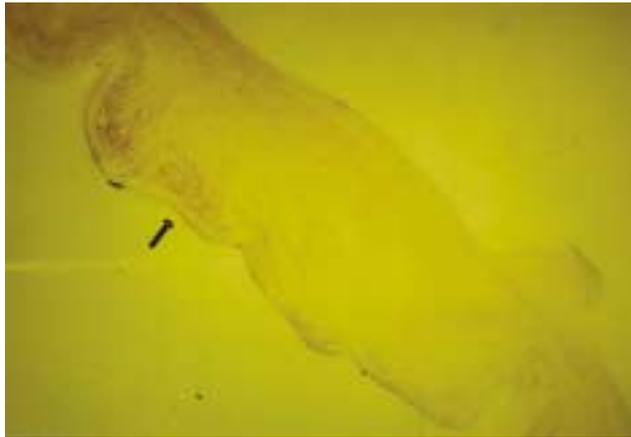
### Discussion

In this paper we report on our systematic SEM and polarised light microscopical investigations as well as on the EDAX data on explanted human aortic and mitral valves. In all the explanted valves, we detected uniform changes in the endothelium and the basement membrane. The endothelial cells often showed hyperplasia with loose binding to each other. Rarely, an endothelial layer was completed. The loss of endothelial cells may expose the extracellular matrix, which obviously sets various patho-

logical processes in motion.<sup>12-15)</sup> The increased activation of matrix metalloproteinases in pathologically altered human cardiac valves emphasises the crucial role of the extracellular matrix in the development of this disease.<sup>15)</sup>

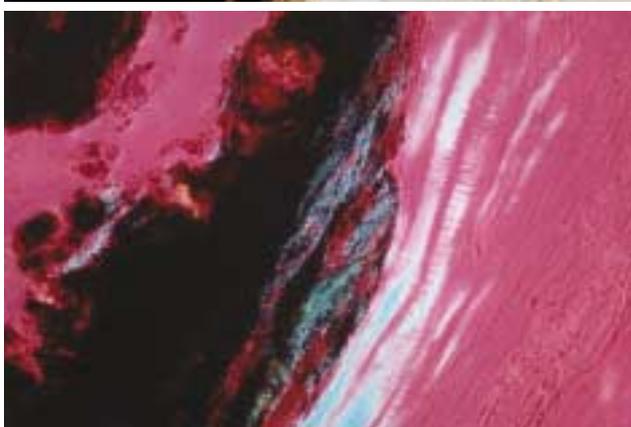
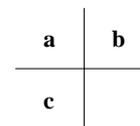
The longitudinal tearing which we frequently found parallel to the edge of the leaflet suggests that mechanical factors regulate the integrity of the endothelial coverage in human aortic and mitral valves. Similar tearing was already discovered by Ishihara et al. in explanted biological valves prostheses and in native valves in the pulse duplicator and appeared as a result of inadequate strain with damaged collagen fibres.<sup>16)</sup> In explanted human aortic and mitral valves, we identified predominantly longitudinal tearing, which was regarded to result from inadequate strain on the leaflet. There was no significant correlation with regard to duration of the disease, age or sex in the study population. In a consecutive study on pathological altered aortic and mitral valves we intend to evaluate if there is a correlation between the aforementioned factors and collagen malformation.

The causes of acquired cardiac valve disease are still largely unknown. In addition to hemodynamic parameters,



**Fig. 3.** Polarised light microscopical findings of explanted aortic and mitral valves showing woven bone formation (aortic valve, female patient, 72 years old).

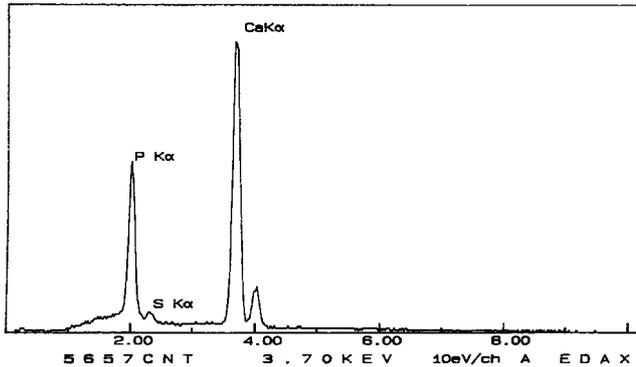
In normal light view, bundles of collagen fibres could be detected as a yellow layer (a, arrow). In polarised light, the white layer consisted of collagen fibres (b, arrow). Using quartz plate red first order, the collagen fibres were seen as light blue lines, no morphological changes were detected (c, arrow).



**Fig. 4.** Aortic valve, male patient, 74 years old.

Inhomogeneous inclusions located in the interior of the valve were covered by a yellow layer as determined in normal light view (a). In polarised light, amorphous masses (arrow) surround the yellow layer, which turned out to resemble characteristic features of woven bone tissue. The white layer adjoining bony structures consisted of collagen fibres (b). Using quartz plate red first order, the bundle bone tissue appeared as a blue-green layer and the collagen fibres were seen as light blue lines (c).

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**Fig. 5.** Results of the energy dispersive X-ray microanalysis. Mineralised deposits containing calcium and phosphorus as demonstrated by characteristic EDAX patterns were frequently observed in areas resembling the bone-like tissue lesions.

altered expression of matrix proteins, increased calcium metabolism in dialysis patients, inherited malformation of the aortic valve (e.g. the bicuspid aortic valve) and unphysiological mechanical strains have all been addressed as pathophysiologically relevant factors.<sup>17-20)</sup>

The major finding of our study is the presence of woven bone tissue in explanted cardiac valves. Dystrophic calcification first described by Mönckeberg is the most common pathological finding in surgically explanted valves.<sup>21)</sup> Virchow recognised already that the mineralisation of the walls of arteries in atherosclerosis is a process of ossification and not only a process of calcification.<sup>22)</sup> Several case reports and clinical studies have identified bone proteins in ossified areas. In particular, the extracellular bone morphogenetic proteins BMP2 and BMP4 were detected in diseased heart valves.<sup>1-3,21-24)</sup> Mohler et al. demonstrated in a study on 324 consecutive explanted aortic and mitral valves the presence of dystrophic calcification.<sup>25)</sup> Dystrophic calcification is a passive process in degenerating connective tissue, whereas heterotopic ossification is an active process of abnormal tissue repair.

The exact pathophysiologic mechanisms of heterotopic ossification and the origin of bone cells in ossified valves are unknown. The studies on the pathophysiology of heterotopic enchondral ossification in atherosclerotic plaque of arterial walls showed that osteoprogenitor cells resemble microvascular pericytes.<sup>26,27)</sup> Myofibroblast-like cells, situated throughout the fibrosal layer of cardiac valves and cultured in vitro, are capable of phenotypic

differentiation into osteoblast-like cells.<sup>25,28)</sup> Many authors therefore suggest the existence of a population of ossifying cells in both aorta and cardiac valves.<sup>25,26,28-30)</sup> Taking into consideration the fact that in recent studies enchondral ossification was detected in its early stages, we identified the presence of woven bone tissue in the explanted cardiac valves.

During the process of desmal ossification, collagen is produced which can easily be diagnosed by light microscopic examination using polarised light. The newly formed primitive woven bone separates intravalvular inclusions from surrounding collagen fibres.<sup>10)</sup>

Our study also demonstrated the utility of EDAX analysis to identify crystals of calcium and phosphorus. The detection of woven bone tissue suggests that inadequate strain favours the mineralisation of valve tissue. The altered mechanical environment and the associated abnormal hemodynamic flow conditions may induce proliferative stimuli for the endothelium resulting in a combination of degenerative and hyperplastic responses. Pathological strains on the leaflet finally result in the development of primary bundle bone-like tissue with mineralised deposits containing calcium and phosphorus. Thus, the use of polarised light microscopy in combination with EDAX analysis permits a reliable and sensitive technique to identify areas of desmal ossification within highly affected valves. The findings of present and prior studies document the complex nature regarding the ossification of acquired cardiac valve disease. Further studies employing biochemical and biophysical techniques may reveal the pathological basis of the underlying bony development and will help to understand the contribution of calcification in the context of acquired cardiac valve disease.

**Conclusions**

In degenerative valve disease, calcified areas showed characteristic morphological features of woven bone formation. Pathologically altered heart valves appear to exhibit distinct stages of desmal osteogenesis.

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