

### **Formation of cartilage in aortic valves of Syrian hamsters.**

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#### **SUMMARY**

The formation of cartilage in aortic valves of Syrian hamsters was studied using histological, histochemical and immunohistochemical techniques. The sample consisted of 281 specimens aged 0-363 days, all of which had a normal (tricuspid) aortic valve. The first sign of valvular chondrogenesis is the presence of small groups of cells embedded in a type II collagen-positive matrix. These groups of cells, which can appear as early as one day after birth, increase in size and differentiate into hyaline cartilage or fibrocartilage. From the fourth day of life, all hamsters examined displayed cartilaginous foci in the aortic valve. They were located along the fibrous attachments of the valve leaflets to their respective sinuses, including the valve commissures. A considerable proportion (76%) of cartilages formed within the first 40 days of life, that is during the period of time in which the histogenesis of the valve takes place. The present observations are consistent with the assumption that in mammals, the precursors of the aortic valve chondrocytes are neural crest-derived cells. Results of a statistical analysis substantiate that the incidence is significantly higher in (1) the territory that comprises the collagenous condensation of the ventral commissure and the ventro-lateral and proximal fibrous attachments of the right leaflet to its sinus, and (2) the proximal fibrous attachment of dorsal leaflet to its sinus. These findings together with data in the literature concerning the distribution of stress in each leaflet-sinus assembly of the valve during the cardiac cycle, suggest that mechanical action might play an inductive role in the formation of the cartilaginous tissue in the aortic valve of mammals. In addition, they point to the possibility that locally intense mechanical stimulation is responsible for the differentiation of the anticipated cartilaginous tissue into hyaline cartilage.

**Key words:** Heart, Aortic valve, Cartilage, Syrian hamster, Mammals

#### **INTRODUCTION**

The presence of cardiac cartilage has been reported in several reptilian, avian and mammalian species (see Matumoto (1938), Kashyap (1959), White (1956, 1959), Kelsall and Visci (1970), Webb (1979), Young (1994) and Sans-Coma et al. (1994) for reviews of the literature). Much of this work is concerned with the incidence, location, size, shape, histological features, and function of the cartilaginous loci occurring in adult animals. However, information about the morphogenesis of the loci is still scarce. Up to now, this process has been studied in the aorticopulmonary septum of the Spanish terrapin (López et al. 2003), in aortic and pulmonary valves of chick and quail

(Stiefel 1926; Matumoto 1938; Tsusaki et al. 1956; Sumida et al. 1989; López et al. 2000), and in pulmonary valves of Syrian hamsters (López et al. 2001).

In the Syrian hamster, the presence of cartilage in the aortic valve is a regular event (Kelsall and Visci 1970; Sans-Coma et al. 1994), whereas occurrence of cartilaginous deposits in the pulmonary valve is less frequent (López et al. 2001). In the pulmonary valve, the formation of cartilaginous foci can start as early as one day after birth (López et al. 2001). In contrast, Sans-Coma et al. (1994) detected no cartilaginous deposit in aortic valves from Syrian hamsters aged less than 40 days using histological techniques. They claimed, however, that further investigations should be done to decide when chondrogenesis starts in the aortic valve of this rodent species.

On this basis, we conducted a study to illustrate the ontogeny of the cartilaginous tissue that develops in aortic valves of Syrian hamsters. Our ultimate goals were: 1) to gather new data on the appearance, differentiation, and structural features of the cartilaginous foci with regard to their location in the valve, and 2) to gain insight into the factor or factors implicated in the formation of cardiac cartilage. The study was carried out in young and adult hamsters, which were examined using histological, histochemical and immunohistochemical techniques.

## **MATERIAL AND METHODS**

### **Animals**

The Syrian hamsters examined belonged to a family subjected to systematic inbreeding by crossing siblings. As described elsewhere (Sans-Coma et al. 1994; Fernández et al. 2000), the incidence of anomalous aortic valves is relatively high in this inbred family. However, the present study concerned 281 specimens (132 male; 149 female), aged 0-363 days, possessing normal (tricuspid) aortic valves. They were taken at random from a single substrain in which the incidence of cartilaginous tissue in aortic valves of adult specimens was 100%.

All hamsters were handled in compliance with the international policies for animal care and welfare. They were housed in polypropylene cages in a room in which both the temperature and photoperiod were controlled. Commercial mouse food (UAR/Panlab s. 1. A.04) and water were given as required, starting at weaning. There was no known exposure of the animals to teratogenic agents.

The hamsters were sacrificed by overdosing with chloroform or with carbon dioxide at a concentration of 75%, delivered into a chamber. One hundred forty-six hearts were examined using histological, histochemical and immunohistochemical techniques for light microscopy. In the remaining 135 specimens, a wholemount immunostaining technique was used for the detection of type II collagen.

### **Histological and histochemical techniques or light microscopy**

Hearts were removed after perfusion with 0.02 M phosphate buffered saline (pH 7.3), fixed by immersion in Bouin's solution (ratio of fixative to tissue volume = 80:1), and embedded in Paraplast (Sigma Chemical Co., England). Serial sections,

transversely, longitudinally, or sagittally cut at 10 µm, were stained with haematoxylin-eosin and Mallory's trichrome stains for a general assessment of the histological features of the valve components, with Weigert-van Gieson stain for the detection of elastin, and with 0.05% alcian blue 8GX in 0.05 M acetate buffer (pH 5.8) plus 0.65 M magnesium to identify sulphated glycosaminoglycans (see: Scott and Dorling 1965). Another procedure applied was picrosirius for the detection of collagen (Junqueira et al. 1979).

### **Immunohistochemical technique for the detection of type II collagen**

This technique was used considering that synthesis of type II collagen is a cartilage-characteristic event (Miller and Matukas 1969; Miller 1976; Kosher 1983; Hall and Miyake 1992, 1995), even though type II collagen is also produced by several nonchondrogenic cell types (see Kosher (1983) and Swiderski et al. (1994) for extensive reviews of the literature).

The removed hearts were washed in phosphate buffered saline and fixed by immersion in Bouin (ratio of fixative to tissue volume = 80:1). Then, the specimens were embedded in Paraplast, and transversely cut at 10 µm. Sections were dewaxed in xylene, hydrated in an ethanolic series, and washed in Tris-phosphate buffered saline (TPBS, pH 7.8). Thereafter, the tissues were digested for 30 min-1 h at 37°C with 0.5% papain in phosphate buffer (pH 4.7). Endogenous peroxidase activity was quenched by incubation with 3% hydrogen peroxide in TPBS for 30 min. After washing in TPBS, nonspecific binding sites were saturated for 30 min with 10% sheep serum and 1% bovine serum albumin in TPBS (SB). Sections were then incubated overnight at 4°C in the monoclonal antibody ClC1 (Developmental Studies Hybridoma Bank, University of Iowa), which is specific for type II collagen, diluted in SB. Control slides were incubated in SB or in nonimmune rabbit serum diluted 1:200.

After incubation, the sections were washed in TPBS (3 x 5 min), incubated for 1 h at room temperature in biotin-conjugated anti-mouse IgG (Sigma) diluted 1:100 in TPBS, washed again, and incubated for 1 h in ExtrAvidin®-peroxidase conjugate (Sigma) diluted 1:150 in TPBS. Peroxidase activity was developed with Sigma Fast® 3,3'-diaminobenzidine tablets, according to the instructions of the supplier. In several cases, the sections were counterstained with haematoxylin.

### **Type II collagen whole mount immunostaining technique**

Removed aortic valves were transferred to Cornwell™ centrifuge tubes, fixed by immersion in Bouin's solution, washed with TPBS, and permeated in acetone. The tissues were then digested in proteinase K and papain. Finally, the specimens were processed following the protocol used for the detection of type II collagen in tissue sections, starting from the incubation with the primary antibody. A more detailed description of this procedure is given in López et al. (2001).

### **Statistical method**

The Student t-test and the  $\chi^2$ -test were used. In both cases, a probability of 0.05 or less was required as evidence of a significant difference.

## **Nomenclature**

The nomenclature used for aortic valve components is that of Angelini et al. (1989), McKay et al. (1992), Sans-Coma et al. (1996), and Hokken et al. (1997). The terms proximal and distal are used to describe the location of these components with regard to the ventricles.

## **RESULTS**

**Anatomical findings.** All hamsters examined possessed a normal aortic valve. It had three aortic sinuses, right, left and dorsal, three leaflets, and a fibrous interleaflet triangle between each adjacent leaflet, so that overall three interleaflet triangles were present in the subaortic outflow tract. In 81 (29%) of the 281 specimens, the ventral commissure, between the right and left leaflets, was slightly fused. Yet, in the Syrian hamster, this valvular arrangement is considered to be within the spectrum of anatomical normality (Sans-Coma et al. 1996).

Cartilaginous deposits occurred at different sites of the fibrous attachments of the valve leaflets to their respective aortic sinuses. For a clear presentation of these findings, we made a diagram in which 12 topographic regions of the aortic valve are considered (Fig. 1). The following description of these topographic regions refers to the adult condition of the aortic valve.

Regions VC, RDC and LDC are the three collagenous condensations of the aortic wall which constitute the most distal extensions of the valvular commissures and which, more proximally, where the leaflets emerge from the arterial wall, bulge into the arterial lumen. VC corresponds to the collagenous condensation of the ventral commissure, RDC to that of the right-dorsal commissure, and LDC to that of the left-dorsal commissure.

Regions R1, R2, L1, L2, D1 and D2 are the lateral fibrous attachments of the leaflets to the aortic sinuses. R1 and R2 correspond, respectively, to the ventral and dorsal lateral attachments of the right leaflet, L1 and L2 to the dorsal and ventral lateral attachments of the left leaflet, and D1 and D2 to the right and left lateral attachments of the dorsal leaflet.

Regions R3, L3 and D3 are the proximal attachments of the leaflets to their respective sinuses. R3 corresponds to the proximal attachment of the right leaflet; it consists of fibrous tissue inserted into the myocardium of the ventricular septum. L3 corresponds to the proximal attachment of the left leaflet; it is composed of fibrous tissue that merges with that of the left fibrous trigone and the myocardium of the left ventricle free wall. D3 corresponds to the proximal attachment of the dorsal leaflet; it is made of fibrous tissue which is continuous with that of the right fibrous trigone.

In the present hamsters, no differences related to sex were observed with regard to the occurrence of cartilage in the aortic valve. Therefore, male and female data have been pooled.

The first event which could be related to cartilage formation was the appearance of small groups of cells embedded in a type II collagen-positive extracellular matrix (Fig.

2A). This occurred in 6 (21.4%) of the 28 hamsters aged 1 day (Table 1). In each specimen there was a single type II collagen-positive cellular group. It was situated in the ventral commissure (region VC) in 3 cases. In another case, it was located in the ventro-lateral attachment of the right leaflet to its supporting sinus (region R1). In the remaining 2 cases, the group occupied two regions, VC and R1, and was computed in each of them (Table 1).

Type II collagen-positive cellular groups of similar or slightly larger size (Fig. 2B) were present in 13 (50.0%) of the 26 animals aged 2 days and in 14 (56.0%) of the 25 animals aged 3 days (Table 1). In 21 (77.8%) of these 27 hamsters having cardiac cartilages there was a single type II collagen-positive cellular group. In the remaining 6 (22.2%) cases there were two groups.

The 25 hamsters aged 4 to 40 days possessed cartilaginous deposits in the aortic valve (Table 1). The cartilaginous tissue appeared in all topographic regions, except for the dorso-lateral attachment of the left leaflet to its supporting sinus (region L1). In the animals aged 4 to 9 days, most cartilaginous deposits were larger than in the preceding stages. However, the deposits could only be assessed using immunohistochemical techniques (Figs. 2C, D). Several type II collagen-positive cellular groups were embedded in a remarkably developed fibrous cellular matrix, a fact which hindered their detection when using conventional histological procedures. In the animals aged 10 to 40 days, the cartilaginous loci varied in size and histological condition. Some of them consisted of a small group of cells surrounded by a type II collagen-positive matrix. Other deposits were composed of a high amount of chondrocytes embedded in an extracellular matrix that contained a considerable amount of glycosaminoglycans (Fig. 2E). The remaining deposits were made up of small rows of cells surrounded by a type II collagen-positive matrix and were contained within a meshwork of collagen fibres (Fig. 2 F).

All of the 151 hamsters aged 41 days and older had cartilaginous loci in the aortic valve. The distribution of such foci, according to the topographic regions, is given in Table 1. The cartilaginous tissue was detected by means of the type II collagen whole mount immunostaining technique in 135 specimens. In the remaining 16, it was observed in tissue sections.

Histologically, two types of deposits could be recognized, namely, hyaline cartilages and fibrocartilages. The hyaline cartilages were composed of chondrocytes embedded in a type II collagen positive-matrix that stained metachromatically with haematoxylin. They displayed a thin perichondrium composed of collagen fibres that ran in a circumferential direction and contained one or two layers of flattened cells. The hyaline cartilages were mainly located in the valve commissures and in the proximal attachments of the leaflets to their respective sinuses (regions R3, L3 and D3. Fig. 2 G). They also occurred in the right and left lateral attachments of the dorsal leaflet to its sinus (regions D1 and D2), although in a very limited number of specimens. Presence of hyaline cartilage in any other topographic region must be regarded as an uncommon event. Most hyaline deposits displayed a nodular shape, varying in size.

Those located in the proximal part of the dorsal sinus wall (region D3) often appeared as C-shaped bars.

The fibrocartilages consisted of a few rows of cells embedded in a type II collagen-positive matrix (Fig. 2H) and were contained within a meshwork of collagen fibres and a few elastic fibres. The arrangement of these deposits widely varied, ranging from an isolated, small deposit to a series of concatenated loci which extended along a variable number of adjacent topographic regions. In the latter cases, the loci were computed as a single focus for statistical purposes. The fibrocartilages were prevalent in the lateral attachments of the leaflets to their respective sinuses (regions R1, R2, L1, L2, D1 and D2); they rarely occurred in other valvular regions.

**Statistical analysis.** In the specimens aged 4 to 40 days, the number of cartilaginous foci occurring in the aortic valve ranged between 1 and 4, while in those aged 41 days or older, it ranged from 1 to 5. The mean values and standard deviations were  $2.16 \pm 0.80$  for the first group of specimens and  $2.84 \pm 1.13$  for the second group. This difference between means is statistically significant at  $p < 0.001$  (Student-t test). From these computed mean values (2.16 versus 2.84) it can be inferred that about 76% of the valvular cartilaginous foci formed within the first 40 days of life.

To test for any association between the incidence of cartilaginous foci and their location in the aortic valve  $\chi^2$  contingency test was performed under the null hypothesis that they are independent events. The test was carried out for the specimens aged 41 days and older (Table 2). The computed value of the  $\chi^2$  statistics is 183.15, with 11 degrees of freedom. Therefore, the null hypothesis is rejected at  $p < 0.001$ . As is also given in Table 2, the major departures from homogeneity are: (1) the relatively large fraction of specimens with cartilaginous foci in the ventral commissure (VC), in the ventro-lateral (R1) and proximal (R3) attachments of the right leaflet to its sinus, and in the proximal attachment of the dorsal leaflet to its sinus (D3); (2) the relatively small proportion of foci in the right-dorsal commissure (RDC), in the right lateral attachment of the dorsal leaflet to its sinus (D1) and in the dorso-lateral (L1) and proximal (L3) attachments of the left leaflet to its sinus. These results are shown diagrammatically in Figures 3 A and 3 B.

## DISCUSSION

Matumoto (1938) stated that the cartilages occurring in aortic valves of mammals develop after birth. This assumption relies on the fact that he detected no cartilage in embryonic hearts from laboratory rats. Hollander (1968) reported that in R-Amsterdam rats, cartilaginous foci appear in the proximal wall of the dorsal aortic sinus from the second week of life.

The present findings prove that in the aortic valve of the Syrian hamster, the formation of cartilaginous tissue can start from the first day of life, as is also the case in the pulmonary valve of this species (López et al. 2001). The earliest sign of chondrogenesis in the aortic valve is the appearance of small groups of cells

embedded in a type II collagen-positive extracellular matrix. Thereafter, these groups of cells increase in size and differentiate into hyaline cartilage or into fibrocartilage.

Sans-Coma et al. (1994) detected no cartilaginous loci in Syrian hamsters aged less than 41 days. In contrast, the present findings indicate that a considerable percentage (76%) of loci appear within the first 40 days of life, that is during the period in which the histogenesis of the valve takes place (Sans-Coma et al. 1994). Nonetheless, the number of cartilaginous loci was significantly higher in the hamsters aged 41 days or older than in those aged 4 to 40 days, denoting that formation of new cartilages in the aortic valve continues after the sixth week of life.

The cartilaginous loci occurring in the cardiac semilunar valves of birds originate from neural crest-derived cells (Sumida et al. 1989; Bachnou et al. 1996) of nonmuscular nature (López et al. 2000) which invade the cardiac outflow tract during its septation (Hiruma and Hirakow 1992; Yablonka-Reuveni et al. 1995, 1998; Bergwerff et al. 1996, 1998; Waldo et al. 1998, 1999). Cells from the neural crest have been adduced as possible precursors of the chondrocytes occurring in the aortic and pulmonary valves of the Syrian hamster (López et al. 2001). The present findings are consistent with this hypothesis in showing that in the aortic valve, the cartilages develop along the attachments of the leaflets to their respective sinuses, where, in mammals, neural crest cells are present during both the embryonic (Waldo et al. 1999; Jiang et al. 2000) and postnatal life (Jiang et al. 2000).

In the cardiac semilunar valves of birds (López et al. 2000), chondrogenesis starts during embryonic life with the formation of the so-called prechondrogenic condensations. They consist of a considerable number of loosely packed mesenchymal cells, embedded in a type II collagen- positive extracellular matrix, and can be well recognized in tissue sections using histological techniques. The cellular condensations become converted into hyaline cartilage or fibrocartilage (López et al. 2000). We were unable to detect any conspicuous prechondrogenic condensation in the aortic valves of the present Syrian hamsters. This is also the case for the pulmonary valve of this species, where cartilage formation does not involve the aggregation of large numbers of cells prior to their differentiation into chondrocytes (López et al. 2001).

The present findings show that in the aortic valve of the Syrian hamster, the cartilaginous foci are not confined to the ventral wall of the valve and the dorsal aortic sinus, as reported in previous papers (Kelsall and Visci 1970; Sans-Coma et al. 1994). The cartilaginous tissue can develop in any part of the sinus boundaries. These are the valvular portions to which are transmitted the large stresses generated by the valve leaflets along the cardiac cycle (Broom 1988). The stress is distributed in each leaflet-sinus assembly as if a circumferential continuity exists between the leaflet and the sinus (Thubrikar 1990). Hence, mechanical action appears to be a factor that might induce the formation of cartilage in the aortic valve. This agrees with the viewpoints of Hueper (1939), Hollander (1968) and Sans-Coma et al. (1994), and contradicts our former speculation (López et al. 2001), based on the study of a limited number of

cases, that the cartilages occurring in cardiac semilunar valves of Syrian hamsters, i.e. pulmonary valves, are not primarily due to mechanical tensions, since these tensions would have operated over a too short period of time when the cartilage begins to develop.

Most of the cartilaginous foci that form in the lateral attachments of the leaflets to their respective sinuses differentiate into fibrocartilages, whereas the hyaline cartilages usually occur in the valvular commissures and proximal attachments of the leaflets. In this regard, it should be noted that during ventricular systole, each aortic sinus is under the combined influence of two mechanical factors that produce opposite effects. Increased aortic pressure increases the diameter of the sinus at the level of the commissures, while contraction of the left ventricle reduces the diameter at the level of the sinus bottom (Thubrikar 1990). In contrast, during ventricular diastole, the shape of the sinuses adapts so that the sinus and the leaflet together become a self-sustained unit to contain the pressure within, thereby preventing the concentration at any given point (Thubrikar 1990). These facts suggest that the relationship between the condition of the cartilaginous tissue and the aortic valve topography relies on the large stresses generated by the ventricular systole in the commissures and sinus bottoms. These stresses might induce the differentiation of the developing cartilaginous tissue into hyaline cartilage.

The number of hamsters aged 1 to 40 days reported herein was too small to apply any statistical analysis in order to seek any association between the occurrence of cartilaginous tissue and its location in the aortic valve. Nonetheless, the data given in Table i already indicate that chondrogenesis takes place earlier in the ventral wall of the valve, and especially in the ventral commissure, than in other valvular topographic regions. In addition, they prove that the ventral commissure and the proximal portion of the dorsal aortic sinus are the valvular regions which show greater predisposition to develop cartilaginous tissue during the first six weeks of life.

The results of the  $\chi^2$  contingency test substantiate that in the aortic valves of the hamsters aged 41 days and older, the incidence of cartilaginous deposits is not at random. It is significantly higher in the two valvular regions in which cartilage had been recorded in previous studies (Kelsall and Visci 1970; Sans-Coma et al. 1994), namely, (1) in the territory that comprises the collagenous condensation of the ventral commissure and the ventro-lateral and proximal fibrous attachments of the right leaflet to its sinus, and (2) in the proximal fibrous attachment of dorsal leaflet to its sinus (Fig. 3 A). The high incidence of cartilaginous foci in these two regions might be the product of intense mechanical tensions generated during the cardiac cycle following the dorso-ventral plane of the heart (Fig. 3 B).

In summary, the present findings suggest that mechanical action might play an inductive role in the formation of the cartilaginous tissue in the aortic valve of mammals. In addition, they suggest that locally intense mechanical stimulation is responsible for the differentiation of the anticipated cartilaginous tissue into hyaline cartilage. Nonetheless, it is well known that the incidence of valvular cartilage varies widely

between species and between individuals of the same species (Matumoto 1938; Hueper 1939; Hollander 1968; Wexler 1964; Sans-Coma et al. 1994; López et al. 2001). This denotes that, as in reptiles (Matumoto 1938; Kashyap 1959; Young 1994) and birds (Matumoto 1938; Tsusaki et al. 1956; López et al. 2000), the presence of cartilage is not a prerequisite for normal performance of the valve. Thus, the true functional significance of the valvular cartilage is still an open question. Moreover, it remains uncertain why some neural crest cells occurring in the sinus boundaries of the cardiac semilunar valves differentiate into chondrocytes, while others do not.

The possible role of the genotype in the development of the aortic valve cartilages is unknown, and the present findings allow no speculation on this topic. All the specimens reported herein belonged to a substrain in which the incidence of cartilaginous tissue in aortic valves of adult specimens amounts to 100%. It is known, however, that not all Syrian hamsters form cartilage in the aortic valve. Sans-Coma et al. (1994) detected cartilaginous foci in only 51% of 169 hamsters aged 40 to 771 days. This suggests an area for further study, namely, the elucidation of whether the presence or absence of valvular cartilage is a characteristic influenced by genes. At this point in time, however, a significant limitation in this study is that no breeding experiments based on pre-known phenotypes can be performed, because the occurrence of valvular cartilage can only be assessed after the death of the animals.

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### **REFERENCES**

- Angelini A, Ho SY, Anderson RH, Devine WA, Zuberbuhler JR, Becker AE (1989) The morphology of the normal aortic valve as compared with the aortic valve having two leaflets. *J Thorac Cardiovasc Surg* 98:362-367
- Bachnou N, Saule S, Dieterlen-Litvre F (1996) An identical effect mediated by thyroid deficiency or oncogene v-erbA in the chick embryo. *Int J Dev Biol* 40:491-497
- Bergwerff M, DeRuiter MC, Poelmann RE, Gittenberger-de Groot AC (1996) Onset of elastogenesis and downregulation of smooth muscle actin as distinguishing phenomena in artery differentiation in the chick embryo. *Anat Embryol* 194:545-557
- Bergwerff M, Verberne ME, DeRuiter MC, Poelmann RC, Gittenberger-de Groot AC (1998) Neural crest cell contribution to the developing circulatory system. Implications for vascular morphology? *Circ Res* 82:221-231
- Broom ND (1998) Connective tissue function and malfunction: a biomechanical perspective. *Pathology* 20:93-104

- Fernández MC, Durán AC, Real R, López D, Fernández B, De Andrés AV, Arqué JM, Gallego A, Sans-Coma V (2000) Coronary artery anomalies and aortic valve morphology in the Syrian hamster. *Lab Anim* 34:145-154
- Hall BK, Miyake T (1992) The membranous skeleton: the role of cell condensations in vertebrate skeletogenesis. *Anat Embryol* 186:107-124
- Hall BK, Miyake T (1995) Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int J Dev Biol* 39:881-893
- Hiruma T, Hirakow R (1992) Histogenesis of tunica media of the chick aorta. *Acta Anat Nippon* 67:749-761
- Hokken RB, Bartelings MM, Bogers AJJC, Gittenberger-de Groot AC (1997) Morphology of the pulmonary and aortic roots with regard to the pulmonary autograft procedure. *J Thorac Cardiovasc Surg* 113:453-461
- Hollander CF (1968) Cartilaginous focus at the base of the noncoronary semilunar valve of the aorta in rats of different ages. *Exp Gerontol* 3:303-307
- Hueper WC (1939) Cartilaginous loci in hearts of white rats and mice. *Arch Pathol* 27:466-468
- Jiang X, Rowtich DH, Soriano R, McMahon AR, Sucov HM (2000) Fate of the mammalian neural crest. *Development* 127: 1607- 1616
- Junqueira LCV, Bignolas G, Brentani RR (1979) Picrosirius staining plus polarization microscopy, a specific method for collagen detection in tissue sections. *Histochem J* 11:447-455
- Kashyap HV (1959) The reptilian heart. *Proc Natl Inst Sci India* 26 B: 234-252
- Kelsall MA, Visci M (1970) Aortic cartilage in the heart of Syrian hamster. *Anat Rec* 166:627-634
- Kosher R (1983) The chondroblast and the chondrocyte. In: Hall BK (ed) *Cartilage. Volume 1. Structure, function and biochemistry*. Academic Press, New York, pp 5-85
- López D, Durán AC, Sans-Coma V (2000) Formation of cartilage in cardiac semilunar valves of chick and quail. *Ann Anat* 182: 349-359
- López D, Fernández MC, Durfin AC, Sans-Coma V (2001) Cartilage in pulmonary valves of Syrian hamsters. *Ann Anat* 183: 383-388
- López D, Durán AC, De Andrés AV, Guerrero A, Blasco M, Sans-Coma V (2003) Formation of cartilage in the heart of the Spanish terrapin *Mauremys leprosa* (Reptilia, Chelonia). *J Morphol* 258:9-105
- Matumoto K (1938) Beiträge zur Kenntnis der vergleichenden Anatomie des Herzknotens. *Keijo J Med* 2:114-169
- McKay RA, Smith A, Leung MP, Arnold R, Anderson RH (1992) Morphology of the ventriculoaortic junction in critical aortic stenosis: implications for hemodynamic function and clinical management. *J Thorac Cardiovasc Surg* 104:434-442
- Miller EJ (1976) Biochemical characteristics and biological significance of the genetically distinct collagens. *Mol Cell Biochem* 13:165-192

- Miller EJ, Matukas VJ (1969) Chick cartilage collagen: a new type of  $\alpha 1$  chain not present in bone or skin of species. *Proc Natl Acad Sci USA* 64:1264-1268
- Sans-Coma V, Franco D, Durán AC, Arqué JM, Cardo M, Fernández B (1994) Cartilage in the aortic valve and its relationship with the aortic valve morphology in Syrian hamsters. *Acta Anat* 149:255-263
- Sans-Coma V, Fernández B, Durán AC, Thiene G, Arqué JM, Muñoz-Chápuli R, Cardo M (1996) Fusion of valve cushions as a key factor in the formation of congenital bicuspid aortic valves in Syrian hamsters. *Anat Rec* 244:490-498
- Scott JE, Dorling J (1965) Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solution. *Histochemie* 5:221-233
- Stiefel K (1926) Das Herz des melanotischen Seidenhuhns. *Anat Anz* 61:177-201
- Sumida H, Akimoto N, Nakamura H (1989) Distribution of the neural crest cells in the heart of birds: A three dimensional analysis. *Anat Embryol* 180:29-35
- Swiderski RE, Daniels KJ, Jensen KL, Solursh M (1994) Type II collagen is transiently expressed during avian cardiac valve morphogenesis. *Dev Dyn* 200:294-304
- Thubrikar M (1990) The aortic valve. CRC Press Inc., Boca Raton
- Tsusaki T, Sugiyama H, Ishimitsu K, Eida T, Kojima R, Shirai M, Tanabe A (1956) Ober die Entwicklung der Herzknorpel bei Gallus gallus domesticus Brisson. *Yokohama Med Bull* 7: 86-98
- Waldo K, Lo CW, Kirby M (1999) Connexin 43 expression reflects neural crest patterns during cardiovascular development. *Dev Biol* 208:307-323
- Waldo K, Miyagawa-Tomita S, Kumiski D, Kirby M (1998) Cardiac neural crest cells provide insight into septation of the cardiac outflow tract: aortic sac to ventricular septal closure. *Dev Biol* 196:129-144
- Webb GJW (1979) Comparative cardiac anatomy of the Reptilia: III. The heart of the crocodilians and an hypothesis on the completion of the interventricular septum of crocodilians and birds. *J Morphol* 161:221-240
- Wexler BC (1964) Spontaneous coronary arteriosclerosis in repeatedly bred male and female rats. *Circ Res* 14:32-43
- White FN (1956) Circulation in the reptilian heart (Caiman sclerops). *Anat Rec* 125:417-431
- White FN (1959) Circulation in the reptilian heart (Squamata). *Anat Rec* 135:129-134
- Yablonka-Reuveni Z, Schwartz SM, Christ B (1995) Development of chicken aortic smooth muscle: expression of cytoskeletal and basement membrane proteins defines two distinct cell phenotypes emerging from a common lineage. *Cell Mol Biol Res* 41:241-249
- Yablonka-Reuveni Z, Christ B, Benson JM (1998) Transitions in cell organization and in expression of contractile and extracellular matrix proteins during development of chicken aortic smooth muscle: evidence for a complex spatial and temporal differentiation program. *Anat Embryol* 197:421-437
- Young BA (1994) Cartilago cordis in serpents. *Anat Rec* 240: 243-247

Table 1. Cartilage in aortic valves of Syrian hamster

Age in days	n	nC <sup>+</sup>	%	Topographic regions of the aortic valve											
				VC	RDC	LDC	R1	R2	R3	L1	L2	L3	D1	D2	D3
0	26	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	28	6	1.4	5	0	0	3	0	0	0	0	0	0	0	0
2	26	13	50.0	2	0	0	7	0	2	0	2	1	0	0	1
3	25	14	56.0	1	0	0	7	1	4	0	3	0	0	2	3
4-40	25	25	100.0	11	1	3	14	4	10	0	9	1	5	9	18
>40	151	151	100.0	128	43	66	95	62	90	12	78	38	53	85	128

n = number of specimens examined; nC<sup>+</sup> = number of specimens with cartilaginous deposits in the aortic valve. See the text and figure 1 for the definition of the topographic regions of the aortic valve.

Table 2. Contingency table of location of the cartilaginous foci in the aortic valve and results of the  $\chi^2$  contingency test E = expected values; O = observed values: tn = total number of cartilaginous foci; 1 = p < 0.001; 2 = p < 0.05. See the text and figure 1 for the definition of the topographic regions of the aortic valve.

	VC	RDC	LDC	R1	R2	R3	L1	L2	L3	D1	D2	D3	tn	$\chi^2$
O	128	43	66	95	62	90	12	78	38	53	85	128	878	
E	73.2	73.2	73.2	73.2	73.2	73.2	73.2	73.2	73.2	73.2	73.2	73.2	878	
$\chi^2$	41.02 <sup>1</sup>	12.46 <sup>1</sup>	0.71	6.49 <sup>2</sup>	1.71	3.86 <sup>2</sup>	51.17 <sup>1</sup>	0.31	16.93 <sup>1</sup>	5.57 <sup>2</sup>	1.90	41.02 <sup>1</sup>		183.15

E = expected values; O = observed values: tn = total number of cartilaginous foci; 1 = p < 0.001; 2 = p < 0.05. See the text and figure 1 for the definition of the topographic regions of the aortic valve.

Fig. 1. Location of the topographic regions of the aortic valve. See the text for the definition of each region. DS = dorsal aortic sinus; LS = left aortic sinus; RS = right aortic sinus.

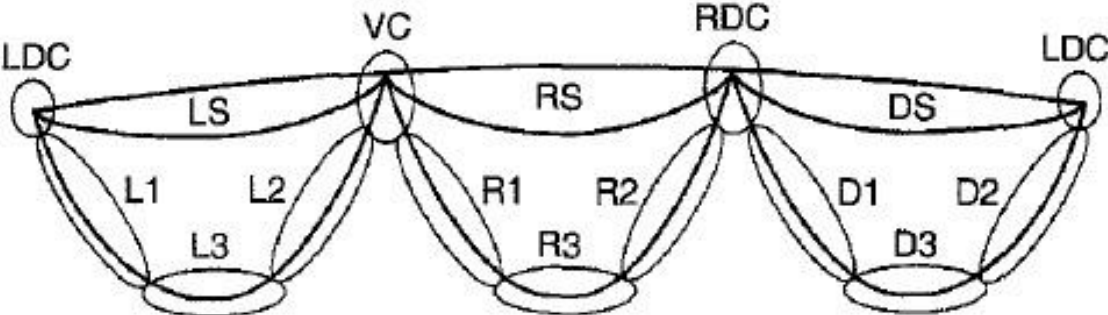


Fig. 2. Transverse (A-F, H) and longitudinal (G) sections of aortic valves from Syrian hamsters. A-D, F, H: Type II collagen immunostaining, counterstained with haematoxylin-eosin; E: Alcian blue stain; G: Weigert-van Gieson stain.

A: Age = 1 day. The arrowheads indicate a small cellular group surrounded by a type II collagen-positive extracellular matrix, located in the ventral commissure (region VC). B: Age = 2 days. The arrowheads point to a group of cells embedded in a type II collagen-positive extracellular matrix, located in the ventral commissure (region VC). C: Age = 10 days. Two groups of cells surrounded by a type II collagen-positive extracellular matrix are present, one (arrow) in the right lateral attachment of the dorsal leaflet to its sinus (region D1), near the right dorsal commissure, and the other (arrowhead) in the left lateral attachment (region D2), near the left dorsal commissure. D: Age = 10 days. An aggregation of cells, embedded in a type II collagen-positive matrix, is located in the ventral commissure (region VC). E: Age = 25 days. A cartilaginous deposit is located in the ventral commissure (region VC), extending to the adjacent regions (regions R1 and L2). F: Age = 35 days. A small group of chondroblasts surrounded by a type II collagen-positive matrix is located in the front of the left-dorsal commissure (star; region LDC). G: Age = 136 days. A deposit of hyaline cartilage (arrow) is located in the proximal attachment of the right leaflet to its sinus (region R3). H: Age = 179 days. A fibrocartilage (arrowheads) is located in the dorso-lateral attachment of the right leaflet (region R2). DS = dorsal aortic sinus; LS = left aortic sinus; RS = right aortic sinus. Bars: F-H = 25  $\mu\text{m}$ ; A-E = 50  $\mu\text{m}$ ; G = 100  $\mu\text{m}$ .

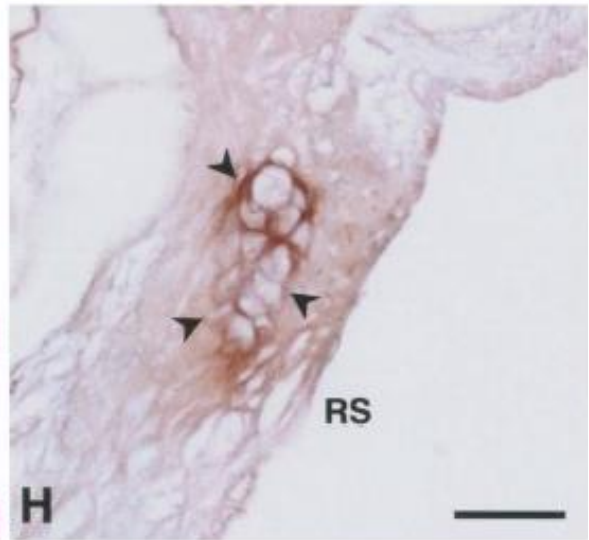
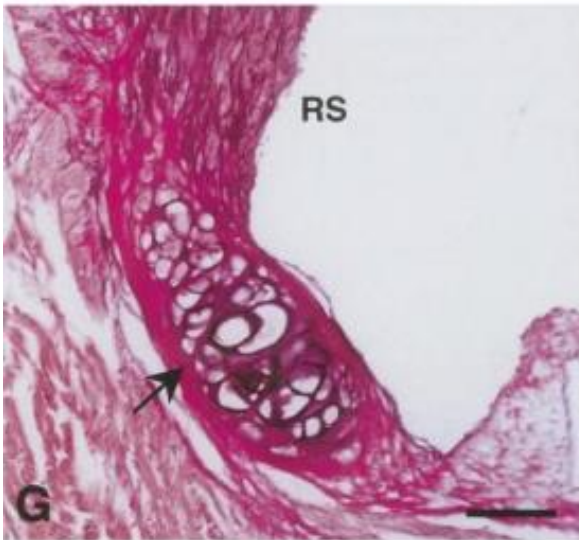
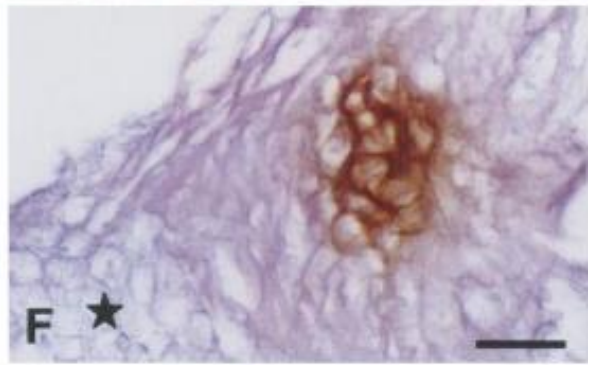
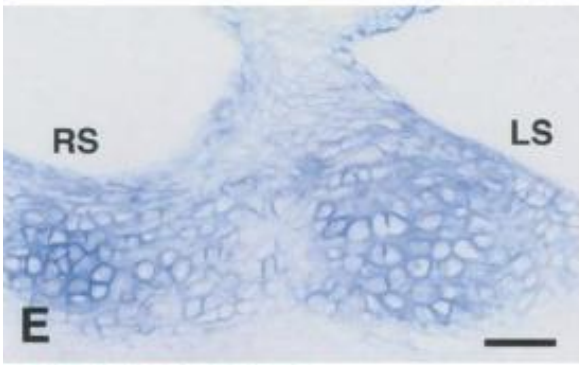
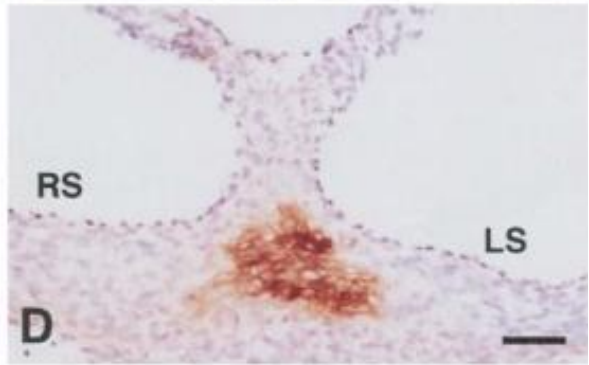
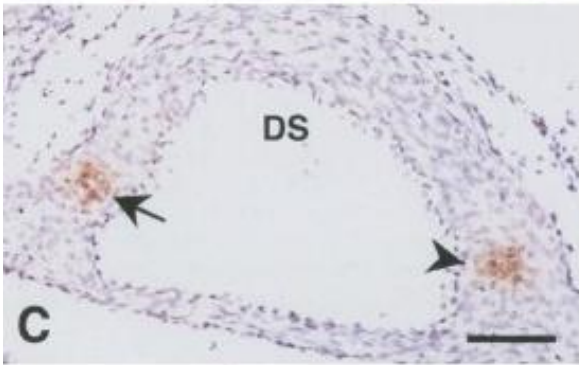
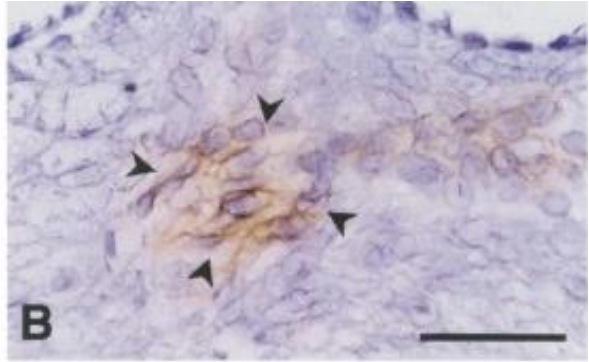
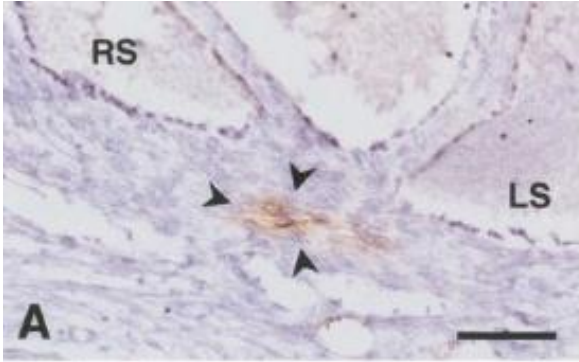


Fig. 3. **A:** Open cut view of the aortic valve, showing the topographic regions with significantly higher incidence of cartilaginous foci (black areas). **B:** View of the cardiac valves after dissecting the atria and great vessels. The topographic regions of the aortic valve with significantly higher incidence of cartilaginous foci are indicated in black. AV -- aortic valve; DS = dorsal aortic sinus; LS = left aortic sinus; MV = mitral valve; PV = pulmonary valve; RS = right aortic sinus; TR = tricuspid valve.

