Histological Study of the Proximal and Distal Segments of the Embryonic Outflow Tract and Great Arteries

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ABSTRACT

The normal development of the ventricular outlets and proximal region of the great arteries is a controversial subject. It is known that the conus, truncus arteriosus (truncus), and aortic sac participate; however, there are some doubts as to the actual prospective fate of the truncus. Some authors propose that it gives origin to the proximal region of the great arteries and that the myocardial cells of its wall become smooth muscle. Nevertheless, others think that the truncus only forms the arterial valve apparatus and that therefore the myocardial cells transform into fibroblasts. As a first approach to beginning to elucidate which process occurs, the aim of this article was to study the histological changes in the wall of these components of the developing heart in chick embryos whose hearts had been labeled at the truncoconal boundary at stage 22HH, tracing the changes up to stage 36HH. Also, the histological constitution of the wall of the pulmonary arterial trunk and its valve apparatus were studied in the posthatching and adult hearts of chickens and rats. The conus and truncus walls were always encircled by a myocardial sleeve from the outset of their development. Between stages 26HH to 28HH, the truncal myocardial cells adjacent to the mesenchymal tissue of the ridges began to lose cell-to-cell contacts and invaded the extracellular matrix. At stage 24HH, the aortic sac began to project into the pericardial cavity and became divided into two channels by the aortic-pulmonary septum at stage 26HH. The wall of the aortic sac is mostly constituted by a compact mesenchymal tissue. Initially, it does not have smooth muscle but this starts to appear at stage 30HH. The insertion ring of the valves, a broad structure, was formed by mesenchymal tissue. Both structures were always covered by a myocardial sleeve. The leaflets developed from the truncal ridges, the segment immediately proximal to the aortic sac. Our results indicate that the proximal region of the pulmonary and aortic arteries do not originate from the truncus arteriosus; rather, we found that they take origin from the aortic sac. Thus, our findings agree with the proposal that the myocardial cells of the external sleeve of the truncus become fibroblastic and suggest that the insertion ring of the arterial valves has a dual origin: fibroblasts produced by truncal myocardial transdiferentiation and the mesenchymal tissue of the proximal region of the truncal ridges, while the leaflets have their origin from the truncal ridges. We discuss the fact that, because the truncus arteriosus does not give origin to the trunks of the aortic and pulmonary arteries, it may be necessary to modify terminology. Based on our results, together with the new findings obtained by in vivo labeling, immunostaining, a chimeric approach, and ultrastructural studies, we propose a developmental model that correlates the fate of the conus, truncus, and aortic sac with the normal morphogenesis of the ventricular outlet tracts and the trunks of the great arteries.

Key words: heart development; embryonic cardiac outflow; truncus arteriosus development

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At present, developmental biology combines embryology, genetics, molecular biology, and the study of human birth defects. For this reason, cardiac developmental biology seeks to understand the cellular interactions and genetic pathways that determine the normal development of each component of the mature heart, studying the selective expression of the genes over time in each segment of the embryonic heart, which is formed by the progressive integration of different segments, each of which is an undifferentiated morphogenetic unit (de la Cruz and Markwald, 1998). Thus, in order to understand the molecular and genetic basis of the heart development, it is necessary to know the precise stage at which each primitive cardiac segment appears, its limits, and its actual prospective fate.

A great deal of interest has been paid to the normal development of ventricular outlets and great arteries because they are abnormal in truncoconal congenital heart diseases. However, despite their importance in pathology, their normal embryogenesis remains one of the most controversial subjects of developmental cardiovascular biology. Although it is known that the conus, truncus arteriosus (truncus), and aortic sac participate in the normal morphogenesis of the ventricular outlets and the proximal region of the great arteries, there are some doubts as to their actual prospective fate. It is essential to distinguish between these regions, to know their limits and the stage of development at which they appear and then, in the future, to investigate the normal and pathologic processes at the ultrastructural, molecular, and genetic levels.

By means of in vivo labeling, it was demonstrated in the chick heart that the conus gives rise to the pulmonary infundibulum and participates in the development of the aortic vestibulum of the mature heart (de la Cruz et al., 1977; de la Cruz, 1998). However, there is no consensus as to the embryonic components of the trunks of the aortic and pulmonary arteries and their valves. Some authors think that both the arterial trunks and valves have their origin in the embryonic truncus of the outflow track (Kramer, 1942; Streeter, 1942; De Vries and Saunders, 1962; Grant, 1962; Van Mierop, 1969; Anderson et al., 1974; Thompson and Firtzharris, 1979; Steding and Seidl, 1980). One implication of this idea is that some myocardial cells of the truncus wall become smooth muscle cells of the arterial wall or they somehow retract into the conus region. Still, other researchers have concluded that the truncus does not participate in the formation of the proximal region (trunks) of the great arteries. They speculate that it only gives origin to the pulmonary and aortic valves (Tongue, 1868; Pexieder, 1978; Orts Llorca et al., 1982; Qayyum et al., 2001). This conclusion supports the idea that the truncus myocardial wall will transform (transdifferentiate) into fibrous connective tissue (Argüello et al., 1978).

Owing to the importance of knowing the precise limits between the three components of the arterial pole of the embryonic heart (conus, truncus, and aortic sac), as a first approach we determined whether the myocardial wall of the truncus actually becomes connective tissue, participating only in the development of the arterial valve apparatus (but not in the proximal region of the great arteries). The aim of this article, then, was to study the histological changes in the wall of these components of the developing heart and that of the aortic sac in chick embryos whose hearts had been labeled at the truncoconal boundary at stage 22HH, tracing the changes up to stage 36HH (fully defined mature heart). Also, the histological constitution of the pulmonary arterial trunk wall and its valve apparatus were studied in posthatching and adult hearts of both chickens and rats. Based on our results, we propose a developmental model that correlates the fate of the conus, truncus, and aortic sac with the normal morphogenesis of the ventricular outlet tracts and that of the trunks of the great arteries, pointing out the stage at which these primitive cardiac segments and aortic sac appear, their boundaries, and their prospective fates.

MATERIALS AND METHODS

Histological changes of the truncus and the conus walls were studied in embryonic chick hearts at stages 22HH, 24HH, 26HH, 28HH, 30HH, and 36HH. To distinguish between the conus and the truncus, we first delimited the ventral wall of the conus and therefore the anatomic structure in which it participated. The hearts of embryos at stage 22HH were labeled in vivo at the level of the conoventricular fold, and in the cephalic boundary of the conal ridges (i.e., the conotruncal limit). The latter corresponded to the place in which the outlet track region of the primary heart tube bends or changes orientation from a caudocephalic direction to a ventrodorsal one. The embryos were reincubated to the desired stage; their ages were calculated based on the schedule of Hamburger and Hamilton (1952). Six embryos of each stage were selected, fixed in alcoholic Bouin solution for 24 hr, and embedded in paraplast using cedar oil. Frontal or transversal serial sections at 5 μm were made and stained with hematoxylin and eosin (H&E). The histological constitution of the wall of the pulmonary arterial trunk and its valve, in posthatching and adult chicken and rat hearts, were also studied. Adult chickens were obtained from the Universidad Nacional Autónoma de México (UNAM) farm and rats from the animal house at Hospital Infantil de México Federico Gómez. Fertilized White leghorn chicken eggs were incubated at 37°C and 85% relative humidity for 21 days to produce posthatching specimens. Adult chicken hearts were dissected to separate the right ventricular outlet and the trunk of the pulmonary artery. All the specimens were fixed in 3.5% formol in phosphate-buffered saline for 24 hr at room temperature and embedded in paraplast. Frontal serial sections were made at 5 μm and stained with H&E or by the pentachromic Movat's technique (Russell, 1972).

RESULTS

We defined the truncus as the segment of the embryonic heart located between the aortic sac and the cephalic boundary of the conal ridges. On the other hand, we consider the aortic sac to be the segment that is just upstream from the arterial arches and downstream from the truncus. The aortic sac is initially embedded in the branchial mesenchyme located ventral to the foregut, at the site where the pharyngeal arteries progressively appear. After the torsion and looping processes are completed, it becomes an intrapericardial structure that distally connects to successive pairs of arterial arches. The endocardial ridges and myocardial cuff of the truncus do not continue into the aortic sac. Instead, the wall of the aortic sac is mostly composed of mesenchymal tissue. We made use of these histological features to distinguish the truncus from the aortic sac and to position the in vivo markers.

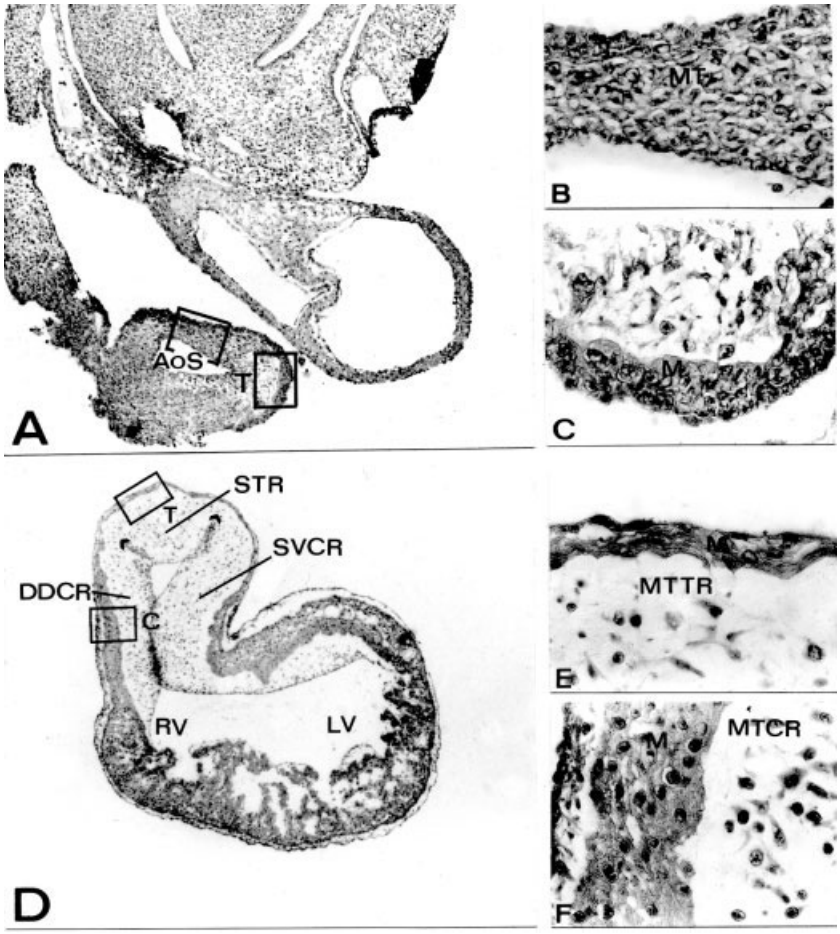

Fig. 1. Photographs of histological sections of the chick embryo heart at stage 24HH, stained with H&E, showing that the wall of the aortic sac is mesenchyme but that the wall of the truncus and the conus is formed by myocardial cells. **A:** Transversal section at the level of the aortic sac (AoS). Note that its wall is a continuation of that of the truncus. **B:** Higher magnification of the wall of the aortic sac formed by mesenchymal tissue (MT). **C:** Higher magnification of the wall of the distal region of the truncus, which contains myocardial cells (M). **D:** Frontal section showing a small part of the truncus (T), the conus (C), and the trabeculated region of the ventricles. Observe that the dextrodorsal (DDCR) and the sinistroventral (SVCR) conal ridges are not yet fused. Also notice the indentation (arrowheads) between the conal ridges and the superior truncal ridge (STR). E and F: Higher magnification of the walls. **E:** Proximal region of the truncus. **F:** Conus. RV, apical trabeculated region of the right ventricle; LV, apical trabeculated region of the left ventricle; MTR, mesenchymal tissue of the ridges of the truncus; MTCR, mesenchymal tissue of the ridges of the conus.

Embryonic and Posthatching Hearts

Stages 22–24HH. At the end of this period, the aortic sac had not yet projected into the pericardial cavity; therefore, its wall is a direct continuation of the wall of the truncus (Fig. 1A). The aortic sac had walls formed by a compact mesenchymal tissue containing abundant small round cells with a light-colored nucleus and scarce cytoplasm (Fig. 1B). Although the truncus is continuous with the aortic sac (Fig. 1A), it is easy to distinguish the boundary between them because both structures have different histological features (compare Fig. 1B with C). The walls of the truncus and the conus were always formed by myocardial cells (Fig. 1D) with well-developed myofibers and a clear large nucleus (Fig. 1C, E, and F). Inside, they were covered by the endocardium. Two endocardial ridges are present within both the truncus and the conus. The boundary between these two sets of ridges is marked by a smooth indentation (Fig. 1D). At the site of this indentation is where we found the in vivo labels at older stages when initially placed at stage 22 at the cephalic end of the conal ridges (conotruncal border). The ridges are formed by mesenchymal tissue in which the fibroblasts are surrounded by a large amount of extracellular matrix (ECM). The myocardial sleeve of the truncal wall is thinner than that of the conus; it is 3–4 cells thick (Fig. 1C and E) and, at stage 22HH, is not yet covered in epicardium. The wall of myocardial sleeve of the conus was 6–8 cell layers thick with some intercellular spaces between them, forming a loosely compacted tissue (Fig. 1F). A thin epicardium is also present.

Stage 26HH. The aortic sac had become positioned within the pericardial cavity. It did not show evidence of endocardial ridges (Fig. 2A) and had begun to be divided by the aortic-pulmonary septum into two channels. Its histological features were similar to what they were at

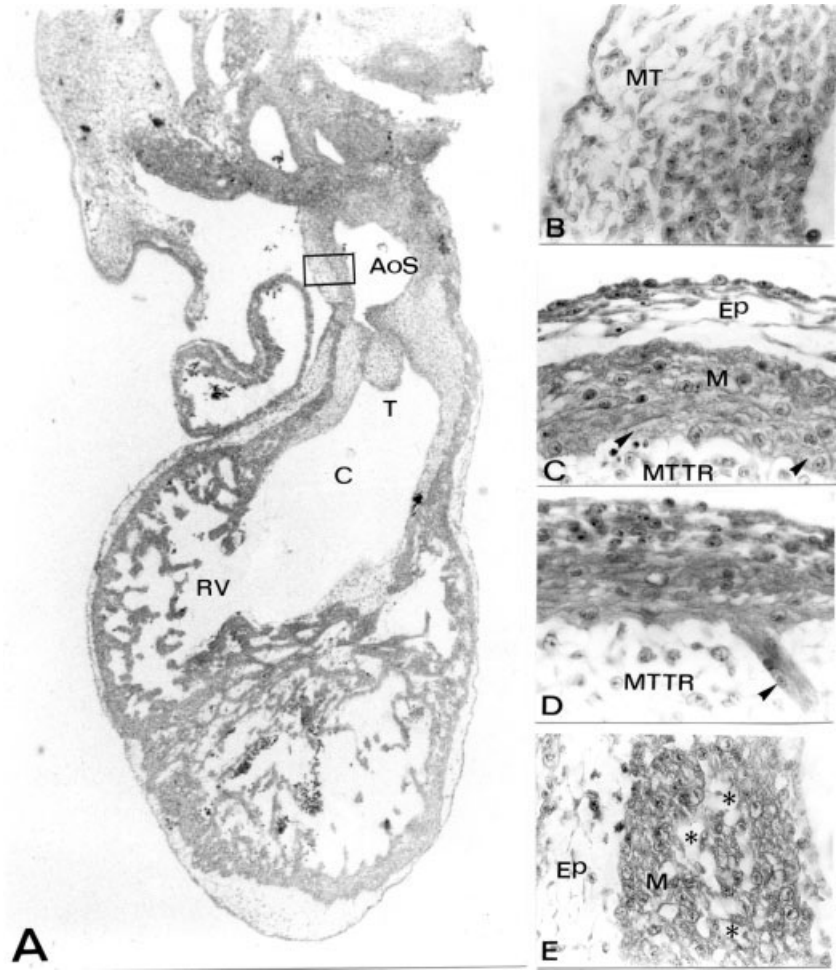

Fig. 2. Photographs of histological sections of the chick embryo heart at stage 26HH, stained with H&E, showing that the aortic sac (AoS) formed by mesenchymal tissue (MT) is projecting into the pericardial cavity and that the external sleeve of the wall of the truncus (T) and the conus (C) remains myocardial (M). **A:** Sagittal section of the heart showing that the aortic sac does not contain any endocardial ridges and that within the distal region of the truncus there are three ridges while in its proximal region there are only two ridges. B–E: Higher magnification of the walls. **B:** Aortic sac. **C:** Transversal section of the distal region of the truncus already covered by epicardium (Ep). **D:** Transversal section of the proximal region of the truncus. Notice that the myocardial cells adjacent to the truncal ridge (arrowheads) are invading the mesenchymal tissue (MTTR). **E:** Sagittal section of the conus. Observe the spaces between the myocardial cells (asterisks), which are not yet covered by endocardium. RV, apical trabeculated region of the right ventricle.

stage 24HH (compare Fig. 1B with 2B). The external wall in both the truncus and the conus remained myocardial while internally the endocardial ridges had expanded into the lumen, showed more mesenchymal cells than in the earlier stages, but had not yet fused (Fig. 2A). The truncal myocardial sleeve remained thinner than that of the conus and more compact. Importantly, in some regions of the truncus, the myocardial cells adjacent to cushion mesenchymal tissue had begun to lose cell-to-cell contacts and had invaded the cushion mesenchyme (Fig. 2C and D). At this stage, we also found three endocardial cushion ridges within the distal region of the truncus while in its more proximal region we only observed two ridges (Fig. 2A). Conversely, the conus myocardial wall increased in thickness and showed an almost trabeculated organization (Fig. 2A and E). This feature was more apparent at the ventricular boundary of the conus. It was at this site that the in vivo markers (labels) were found that were placed originally at stage 22HH at the level of the conoventricular grooves, indicating little movement of the markers. Importantly, the labels initially placed at the cephalic end of the conal ridges, i.e., the conotruncal boundary, also remained at this boundary, which at stage 26HH was located beneath the site where the heart tube changed from a caudocephalic to a ventrodorsal direction.

Stages 28–30HH. Development of the proximal region of the aortic and pulmonary arteries and the ventricular outlet tracts had progressed considerably. The wall of the proximal region of the great arteries had begun to acquire its histological features (Fig. 3A). Fibroblasts had begun to form radial lamellae, although the tunica media and the tunica adventitia were not well defined (Fig. 3B). At stage 30HH, smooth muscle cells were present. The H&E technique did not permit us to visualize any elastic fibers. These vessels were covered on the inside by endothelial

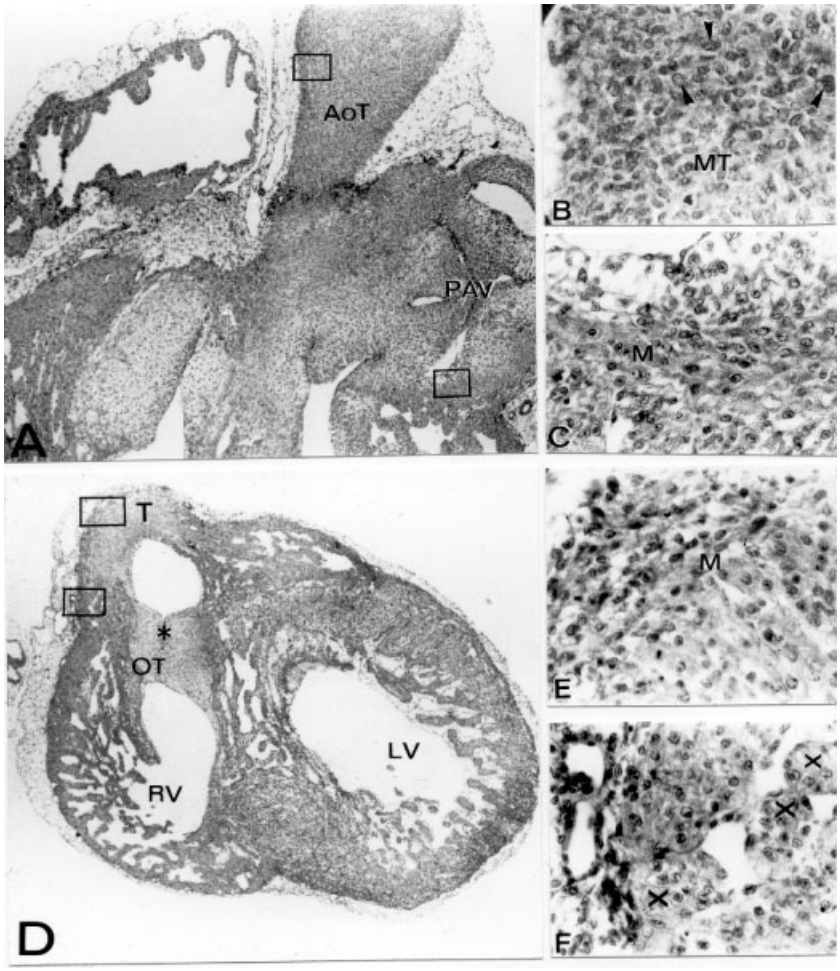

Fig. 3. Photographs of histological sections of the chick embryo heart at stage 30HH, stained with H&E, showing that the wall of the proximal regions of the great arteries has begun to acquire its histological features and that the developing valves of the great arteries are encircled by a myocardial sleeve. **A:** Frontal dorsal section of the heart exhibiting the pulmonary valve (PAV) in the distal region of the truncus and the trunk of the aorta (AoT). B and C: Higher magnification of the walls. **B:** Frontal view of the aortic trunk in which the smooth muscle cells (arrowheads) are mixed with the fibroblast of the mesenchymal tissue (MT). **C:** Transversal section of the external sleeve of the ring of the pulmonary valve formed by myocardium (M). **D:** Frontal ventral section of the heart showing the developing outlet tract (OT) of the right ventricle (RV) and the proximal region of the truncus (T). Notice that the conal ridges have begun to fuse (asterisk). E and F: Higher magnification of the external sleeves. **E:** Proximal region of the truncus formed by myocardial cells (M). **F:** Outlet tract of the right ventricle showing some trabecules (×). LV, apical trabeculate region of the left ventricle.

cells. The valves of the developing great arteries remained encircled by a myocardial sleeve (Fig. 3A and C). Serial sections of the truncus showed that the mesenchyme of the endocardial ridges had been invaded by myocardial cells whose myofibrils were not well organized. These changes in the truncal ridges occurred at the level of the leaflets and the insertion ring (Fig. 3A, C, and E). The ventral wall of the right ventricular infundibulum was now fully trabeculated (Fig. 3D and F) and the conal ridges had begun to fuse (Fig. 3D). As a result, the subvalvar aortic and pulmonary outflows were partially separated. Interestingly, labels initially placed at stage 22HH at the cephalic end of the conal ridges were found at stage 30HH on the ventricular boundary of the insertion ring of the pulmonary valve.

Stage 36HH. The heart as a tetracameral organ was now defined; the ventricular outflow tracts were fully separated, as were the proximal trunks of the pulmonary and aortic arteries. In the proximal walls of pulmonary and aortic trunks, a tunica media and a tunica adventitia were evident (Fig. 4A). Concentric lamellae of fibroblasts and smooth muscle cells had formed in the walls of the proximal regions of the great arteries (Fig. 4A and B). The insertion ring of the arterial valves was broad and comprised of compact fibrous connective tissue. At the most distal boundary, smooth muscle cells were mixed with myocardial cells. At this level, there is no precise limit between the tissues formed by these two types of muscular cells. At the proximal (ventricular) boundary, a collagen-rich ECM was present in which groups of myocardial cells had associated (Fig. 4A). At this limit and time, we found the labels that were originally placed at stage 22HH at the cephalic end of the conal ridges. Importantly, the insertion ring was still covered by a myocardial sleeve (Fig. 4C),

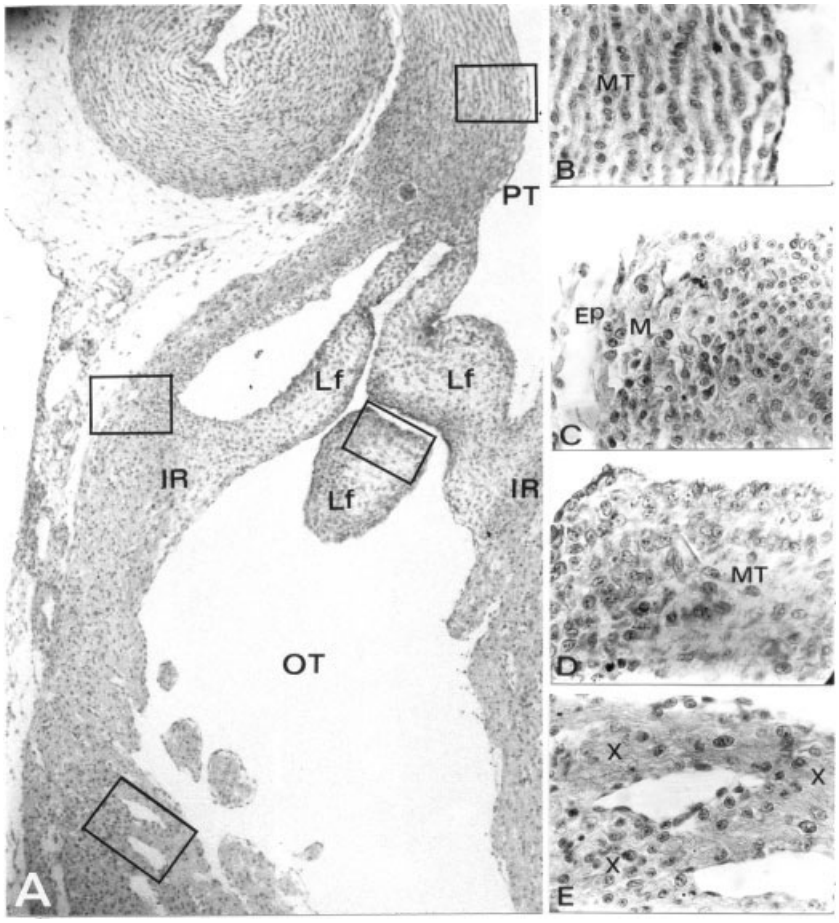

Fig. 4. Photographs of histological sections of the chick embryo heart at stage 36HH, stained with H&E, showing that the wall of the main branches and trunk of the great arteries are well developed and that the pulmonary artery valve is encircled by a myocardial sleeve. **A:** Frontal ventral section of the heart exhibiting the pulmonary trunk (PT), the insertion ring of the pulmonary valve (IR), the leaflets (Lf), and the outlet tract of the right ventricle (OT) whose lateral wall is trabeculated. B–E: Higher magnification of the walls. **B:** Frontal view of the pulmonary trunk in which fibroblast and smooth muscle cells are forming lamella. **C:** External myocardial sleeve covering the insertion ring of the pulmonary valve formed by myocardium (M). **D:** Pulmonary leaflet consists of mesenchymal tissue. **E:** Lateral wall of the outlet tract of the right ventricle, which presents trabecules (×).

which extended caudocephalically, covering the proximal region of the mesenchymal valve leaflets (Fig. 4D). The ventral wall of the infundibulum of the right ventricle remained trabeculated (Fig. 4E).

Posthatching chick. The heart was similar to what it was at stage 36HH (compare Fig 4A with 5A). The walls in the pulmonary and aortic arterial trunks were well developed. The pulmonary valve apparatus was formed by three leaflets in which the connective tissue was loose and had a homogeneous fibrous ECM. The broad insertion ring was formed by denser connective tissue and covered by a myocardial sleeve (Fig. 5A′). Movat's technique showed that its main constituents were the mucopolysaccharides (glycosaminoglycans) with a minor collagen fibrillar constituent. At the proximal boundary of the insertion ring, the limit between the connective tissue and the myocardium was not well defined. Myocardial cells were still present within the ECM of the insertion ring. The wall of the pulmonary infundibulum was muscular.

Adult Hearts

Frontal serial sections of the adults hearts stained using the pentachromic Movat's technique showed the trunk of the pulmonary artery, its valve apparatus, and the infundibulum (Fig. 5B and C).

The walls of the pulmonary arterial trunks in the chicken and rat were both made up of fibroblasts and smooth muscle surrounded by an ECM of collagen and elastic fibers. The internal, media, and adventitia tunicas are present. The limit between the arterial wall and the myocardium in both animals was well defined at the level of the arterial boundary of the insertion ring of the leaflets (Fig. 5B and C). The insertion ring had a complex histological organization. It was constituted by connective tissue with fibroblasts with a large oval-shaped nucleus that were lightly stained in the chick but in the rat the fibroblasts had a smaller and darker nucleus (Fig. 5B′ and C′). In both, the ECM was dense and fibrous, composed of mostly collagen and acid mucopolysaccharides. Abundant

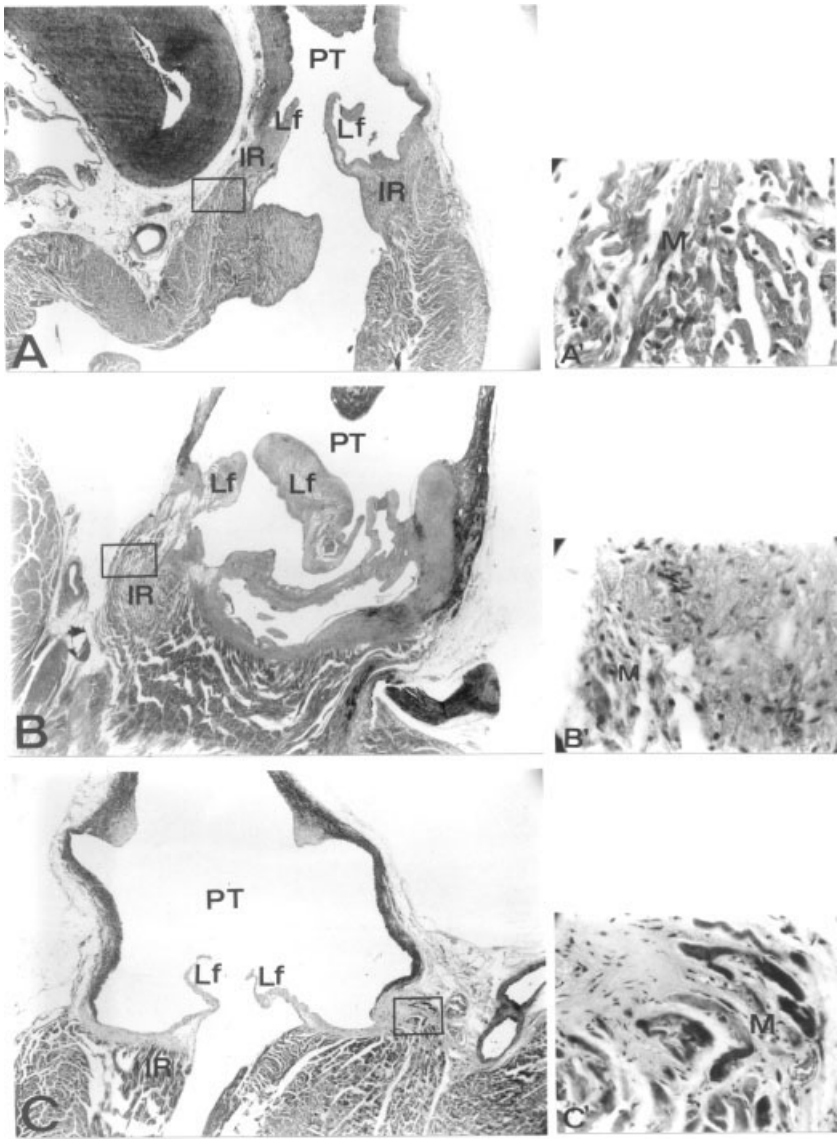

Fig. 5. Photographs of histological sections of posthatching and adult chicken and adult rat hearts stained using Movat's technique. They show the trunk of the pulmonary artery and its valve apparatus. The insertion ring (IR) covered by a myocardial sleeve (M) and the leaflets (Lf) are comprised of mesenchymal tissue. In all cases, the right ventricle infundibulum is trabeculated. **A:** Posthatching chick. **B:** Adult chicken. **C:** Adult rat. **A′–C′**: Higher magnifications of the external sleeve of the insertion ring of the pulmonary valve.

myocardial cells, either individually or in small groups, were present in the ECM of the valvar insertion ring. No precise boundary existed between the myocardial and the connective tissues at the proximal edge of the insertion ring in the chick (Fig. 5B), whereas in the rat this limit was quite precise (Fig. 5C). The leaflets were formed by connective tissue covered by spindle-shaped endocardial cells with single dark nuclei at the center. The fibroblasts and the ECM were similar to those of the insertion ring. The ECM associated with the endocardium was dense at the boundary, but in the central part it formed a looser meshwork. Its main components were mucopolysaccharides. In the rat, the leaflets were thinner than in the chick. In both animals, the wall of the pulmonary infundibulum was myocardial (Fig. 5B and C).

DISCUSSION

Our histological studies of the embryonic hearts and labeling experiments showed that the truncus does not participate in the development of the proximal region of the great arteries. We found that external walls of the truncus and the conus were always encircled by myocardial cells from the outset of their development. These findings were similar to those described by Qayyum et al. (2001). An important finding was that, in the external sleeve of the truncus between stages 26HH and 28HH, the myocardial cells adjacent to the mesenchymal tissue began to lose their cell-to-cell unions and became surrounded by the extracellular matrix (Figs. 2 and 3). On the basis of these findings, we concluded, in accordance with

Argüello et al. (1978), that some of the truncal myocardial cells adjacent to the endocardial ridges become connective tissue fibroblasts. We also observed that at stage 24HH the aortic sac, the wall of which is formed by a compact connective tissue, begins to project into the pericardial cavity (Fig. 1) and that at stage 26HH it is divided into two channels by the aortic-pulmonary septum. Initially, the aortic sac does not have smooth muscle cells but these start to appear at stage 30HH. These data challenge the classically upheld notion that the proximal region of the pulmonary and aortic arteries has its origin in the truncus. Rather, they agree with the statement of Waldo and Kirby (1993) that this region of the great arteries has its origin from the aortic sac.

We also found that the insertion ring of the valves is a broad structure formed by a dense connective tissue. Its ventricular (lower) limit, which corresponds to the place where we found the labels that were originally put at the truncoconal boundary, is not well defined, while its upper limit, i.e., the boundary with the vascular wall, is more clearly defined (Figs. 4 and 5). We thus agree with Qayyum et al. (2001) that the valve leaflets develop from the truncal endocardial ridges adjacent to the aortic sac (Fig. 3). Taken together with our findings regarding the histological changes of the external wall of the truncus, as well as the proposal of Argüello et al. (1978), these facts argue that the insertion ring of the arterial valves has a dual origin: fibroblasts produced by the myocardial transdiferentiation and the mesenchymal tissue of the proximal region of the truncal ridges, while the leaflets have their origin in the distal region of those ridges.

There are various reports concerning the normal development of the ventricular outlet tracts and the trunks of the great arteries using immunofluorescence and retroviral staining, quail-chick chimeras, and ablation studies. All of them provide evidence of cell transdifferentiation processes, leading to the conclusion that the myocardium of the truncal external sleeve becomes the smooth muscle of the arterial wall. However, these conclusions were drawn by analyzing the results based on two unsubstantiated concepts, one describing all the primitive cardiac cavities as being present in the straight tube heart (Davis, 1927), and the other presuming that the truncus gave rise to the proximal region of the great arteries. For example, in some studies, it was thought that the Argüello data provided support for this concept, but in actuality he concluded that the myocardial cells of the truncus were able to (trans)differentiate into cells of a fibroblastic type. He neither mentioned nor provided evidence to support the claim that the truncal myocardium becomes vascular smooth muscle. Another interesting example is the work carried out by Ya et al. (1998), who, in studying the normal development of the outflow tract in rats between E.D.10 and day 2 postnatally, found cells that coexpressed desmin and smooth muscle α-actin. These results were taken as proof that the myocardium had transdifferentiated into smooth muscle. However, a special type of cell (the myofibroblast) is known to exist, which coexpresses the same proteins and also has the ability to invade adjacent tissues and retract them (Gabbiani, 1992; Schürch et al., 1998). Because we found cells between stages 26HH and 30HH in the truncus whose myofibrils were not well organized and had invaded the endocardial ridges (Figs. 2C and D and 3A, C, D, and E), we suggest that myofibroblastic cells described by Ya et al. (1998) may be transitorily arising during the myocardial transdifferentiation process. Their data in fact lend important weight to our suggestion that the prospective fate of the truncal myocardial sleeve is to participate in the development of the connective tissue of the insertion ring of the arterial valves. Additionally, Bergwerff et al. (1998) demonstrated in the chick embryonic heart that the smooth muscle cells of the proximal region of the great arteries had their origin in the neural crest cells (NCCs). They found smooth muscle at stages when the aortic sac was present. Thus, we interpret our findings and those of Ya et al. (1998) and Bergwerff et al. (1998) to indicate that the smooth muscle cells of the wall of the proximal region of the aortic and pulmonary arteries do not arise from the transformation of cells originating from the truncal myocardial wall. Although Burke et al. (1994) also reported that the truncal myocardium transforms into smooth muscle, their conclusion was based on the expression of various ECM proteins (fibronectin, laminin, and collagen I) in the chick embryo heart at stage 18HH, a stage at which the truncus was not yet present (García Peláez and Arteaga, 1993). Interestingly, Burke et al. (1994) did describe that the smooth muscle myosin and collagen VI began to be expressed in the truncus at stage 30HH. This is consistent with our own histological observations, which indicate in the chick embryo that the histodifferentiation process of the arterial wall initiates approximately at stage 30HH once each ventricle has acquired its outlet tract, the arterial valve leaflets have began to develop, and the intrapericardial aortic sac has been divided into two channels.

Thus, we concur with Qayyum et al. (2001) that in the posthatching heart the boundary between the myocardium of the truncus and the fibroelastic tissue of the aortic sac was no longer at the level of the arterial valve leaflets, as it initially started in the stage 22 embryo. Specifically, at hatching, this boundary was located two-thirds of the way down the length of the leaflets (Fig. 5A). This can be explained by the transdifferentiation process described above and by the concurrent caudal/posterior growth/expansion of the right ventricle and the cephalically directed differentiation of vascular tissue at the arterial pole. All these events combine to create an apparent caudal retraction of the myocardial truncal wall and the caudal shift of the vascular wall to a level below the distal limit of the outlet valve leaflets.

In order to explain our previous in vivo labeling results, which led some researchers to conclude that the truncus arteriosus gave rise to the trunks of the great arteries (de la Cruz et al., 1977; Arteaga et al., 1988; de la Cruz, 1998), it is necessary to keep in mind our postulation regarding the dual origin of the insertion ring of the arterial valves and that it is a broad structure with three different zones: one adjacent to the ventricular outlets myocardium (in which we found the labels placed initially at the cephalic end of the conal ridges); another on the boundary with the arterial wall (initially in close continuity with the aortic sac); and between them, a third, small zone. In a key experiment, de la Cruz et al. (1977, 1998) labeled the chick embryo heart at stage 22HH with an in vivo marker positioned either at the cephalic limit of the conal ridges (truncoconal boundary) or at the most distal limit of the embryonic heart (M. Salazar, personal communication). Arteaga et al. (1988) performed similar experiments labeling the same regions as de la Cruz but at stage 24HH. At the end of the experiments (stage 30HH or 36HH), each

research group similarly found that both labels over time tracked to the insertion ring region of the pulmonic valves (de la Cruz et al., 1977; Arteaga et al., 1988; de la Cruz, 1998). Arteaga et al. (1988) also pointed out that the label they put at the truncoconal boundary was found at the distal third of the supraventricular crest. However, they did not carry out a study of the histological features of the labeled structure. Based on our current findings and those originally obtained by de la Cruz et al. (1977), we would predict that a label placed at the truncoconal boundary at stage 22HH or 24HH would end up in the medial region of the insertion ring of the pulmonary artery valve. Our proposal that the arterial trunks are derived from the aortic sac is also supported by the fact that the labels placed at the distal limit of the truncal ridges (truncal-aortic sac boundary) at stage 24HH and incubated until stage 36HH (a fully defined, mature heart pattern) were occasionally found in the wall of the pulmonary artery trunk, adjacent to the insertion ring of the arterial valve (Arteaga et al., 1988). These results indicate that at stage 24HH, the aortic sac is beginning to project into the pericardial cavity.

Recently, it has been speculated that the conus and truncus do not derive from the lateral heart-forming fields. Mjaatvedt et al. (2001) pointed out that the outflow tract of the embryonic heart originates from cephalic cardiogenic mesoderm called the anterior heart field by an inductive interaction with the growing distal end of the primary heart tube (initially the right ventricle). Conversely, Waldo et al. (2001) reported that conotruncal myocardium is originated from a secondary heart field, present in the splachnic mesoderm that underlines the caudal pharynx, which is added to the primary heart tube during the looping process. These results have been interpreted to suggest that the conus and truncus have a potentially different origin than the rest of the primary heart tube. Although our data seem consistent with this idea, we think that in fact they provide further evidence of the importance of the interaction of the myocardium of the primitive right ventricle on the conus and that of the connective tissue of the aortic sac on the truncus. These interactions could play a very important role in determining the different fate of these primitive cardiac segments.

It is still unclear how the conus, truncus, and aortic sac are septated. Hypotheses for the mechanisms of this event are controversial and generally fall into one of two classical models: the division of the outflow lumen occurs by fusion of the endocardial conotruncal ridges/cushions (Kramer, 1942; Van Mierop, 1969; Goor et al., 1972) or rotation and retraction of an outflow septation complex (Thompson et al., 1983, 1985; Thompson and Fitzharris, 1985) that includes the myocardial cuff, the aortic-pulmonary septum, and the vascular channels toward the ventricles. However, Kirby et al. (1985) has described that the aortic-pulmonary septum initially separates the lumen of the aortic sac into the aorta and pulmonary trunks; its continuation into the distal truncus results in the formation of a truncal septum that separates the two arterial valves (Qayyum et al., 2001). Our findings regarding the fate of the truncus and the origin of trunks of the two great arteries support the hypotheses of Kirby et al. (1985) and Qayyum et al. (2001).

To understand development of the arterial pole, we offer a summary based on our current and past findings in the embryonic chick heart concerning the stage at which each segment of the pole appears, pointing out how to distinguish between them and their prospective fate.

The conus begins to appear at stage 12HH (de la Cruz et al., 1977), while the truncus starts to emerge at stages 16–17HH (García Peláez and Arteaga 1993; de la Cruz and Markwald, 1998). Since these primitive cardiac segments appear progressively and are both externally covered by a myocardial sleeve, it is hard to distinguish an external boundary between them. We used the cephalic limit of the conal endocardial ridges at stage 22HH and stage 24HH to mark the internal boundary between the conus and the truncus, while the boundary (limit) between the truncus (myocardium present) and aortic sac (no myocardium) is easily distinguished by their histological features. Since the truncal ridges are shorter than the external truncal myocardial sleeve at stage 24HH, they are not the optimal parameter to establish the distal limit of the truncus. After stage 24HH, the progressive loss of visible truncal myocardium begins and, later, between stages 29HH and 30HH, the overt histodifferentiation of the septated aortic sac into vascular arterial walls begins. When we placed labels at stage 22HH at the cephalic end of the conal ridges, these markers remained at this boundary through stage 24HH, which at more advanced stages of the development were found beneath the place in which the heart tube bends, i.e., changes from a caudocephalic to a ventrodorsal direction. Concerning prospective fate of the conus and truncus, it is clear from in vivo labeling that the anterolateral conus gives rise to the right ventricle infundibulum (de la Cruz et al., 1977), while the fate of the posteromedial conus remains unknown.

In this article, we presented in vivo labeling and histological evidence that the fate of the truncal myocardium is to give origin to the insertion ring/annulus of the arterial valves while the truncal ridges form the leaflets. Together with Waldo and Kirby (1993) and Qayyum et al. (2001), we interpret our histological data to indicate that the aortic sac, which really is a vascular structure, forms the proximal region or trunks of the aortic and pulmonary arteries, following its septation by the aortic-pulmonary septum.

Thus, based on our present findings, the proximal regions or trunks of the pulmonary and aortic arteries do not originate from the truncus. If correct, it may be necessary to reach a new agreement regarding the term "truncus." It might be more accurate to use the terms "proximal" and "distal segments" of the outflow tract instead of "conus" and "truncus arteriosus," respectively. To avoid confusion, the embryonic structure that gives origin to the proximal region of the great arteries should be renamed the "aortic-pulmonary sac."

Our model will be useful for distinguishing between the conus, the truncus arteriosus, and the aortic sac and for resolving the controversy over the commonly used term "outflow tract" to describe the embryonic cardiac segment that connects the developing right ventricle with the aortic arch (Thompson and Fitzharris, 1985). It is obvious that, depending on its stage of development, this region can be formed by one, two, or three components of potentially different origin. The model will also facilitate an evaluation of the knowledge regarding the normal morphogenesis of the ventricular outlet tracts and of the trunks of the great arteries obtained by in vivo labeling and by the descriptive techniques in birds and mammals since we found that the anatomical and histological fea-

tures of these cardiac structures in chicks and rats are similar (Fig. 5B and C).

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