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EARLY CARDIOGENESIS IN THE NEWT EMBRYO

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Abstract

The migration of cardiogenic cells and the formation of a tubular heart in newt embryos were examined mainly by scanning electron microscopy (SEM). Cardiogenic cells are known to localize at the border region of lateral mesoderm migrating in the space between the ectoderm and the endoderm. They initially (before stage 20 or mid-neurula) appeared to attach to the basal surface of the ectoderm, whereas later (after stage 22 or late neurula) they changed their scaffold to the endoderm. On the scaffold cell surface, very fine fibrils of extracellular matrix (ECM) were found. These fibrils were proved to be composed partly of fibronectin by the immunofluorescence method as well as by immunoSEM using latex bead-labeled antibody, suggesting their seemingly important role in migration of cardiogenic cells. At stage 26 or the early tail bud stage, when the tips of bilateral cardiogenic areas begin to fuse under the foregut, several free vasoformative cells are seen there and the mesodermal sheet itself splits into two layers to produce a coelomic cavity. The splanchnic wall of the coelomic or pericardial cavity was recognized to form a trough consisting of cobblestone-like myocardial cells not yet covered with the epicardium.

KEY WORDS: Newt (Cynops pyrrhogaster), heart development, cardiogenic cells, migration, extracellular matrix, immunocytochemistry, fibronectin.

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Introduction

Before dealing with the specialized aspects of early cardiogenesis in the amphibian embryos, some brief comments on the more general aspects of the development of the vertebrate heart will be made. In this regard, we cite Table 1 from our recent papers (Hirakow, 1985, 1986), which is a summarized form of the previous descriptions of cardiac cell differentiation with regard to ontogenesis (Hirakow and Gotoh, 1980). As shown in Table 1, we have proposed that cardiogenic events in the vertebrate can reasonably be considered in four major periods.

Concerning the first, or precardiogenesis, period, comparative embryological descriptions were made most comprehensively by Mollier (1906), although he unfortunately created a misleading term, "myoepicardialer Mantel", to designate the outer wall of the primitive tubular heart. This term implying in situ differentiation of the myocardium and the epicardium does not represent a real situation and should be eliminated from textbooks of embryology, since it is already definitely established that the outer wall of early tubular heart is composed of myocardial cells only and the epicardium is formed later by migrating mesothelial cells (Hiruma and Hirakow, 1985b; Ho and Shimada, 1978; Manasek, 1969). Experimental embryological investigations on the differentiation of primordium of the heart during the precardiogenesis period have been performed by several eminent embryologists (see reviews of Copenhaver, 1950; DeHaan, 1965).

Hitherto, the majority of cardiac embryologists have concentrated their attention on the second (cardiogenesis I) and the third (cardiogenesis II) periods, especially on septum formation in higher vertebrates, since it is practically important from the standpoint of cardiac malformations. Finally, the fourth, or cardiogenesis III, period has been added to these two major periods by us to distinguish mammalian myocardial cell differentiation from that of other groups of vertebrates by formation of the T-tubule system at this period.

Reflecting on these circumstances, valuable contributions by means of SEM technique have been concentrated mainly on heart development at the periods of cardiogenesis I and II (Hay et al.,

Table 1. DEVELOPMENTAL EVENTS OF THE VERTEBRATE HEART

DEVELOPMENTAL PERIOD	ORGANOGENESIS	HISTOGENESIS	CYTODIFFERENTIATION
1. Precardiogenesis		Lateral Mesoderm	Cardiogenic and Angiogenic Cells
2. Cardiogenesis I	Primitive Tubular Heart Looping Segmentation	Myocardium Endocardium Epicardium Cardiac Jelly Cushion Tissue Cardiac Vessels	Myocardial Cells Endocardial Cells Mesothelial Cells Mesenchymal Cells
3. Cardiogenesis II	Septation	Cardiac Valves Cardiac Skeleton	
4. Cardiogenesis III			T-tubules in Mammalian Myocardial Cells

1984; Ho and Shimada, 1978; Layton and Manasek, 1980; Shimada and Ho, 1980; Pexieder, 1981). In contrast, the precardiogenesis period has rarely been a subject of SEM study. To our knowledge, except for our investigation on amphibian embryos (Hirakow et al, 1984a, b; Hiruma and Hirakow, (1983), only one work on chick embryo precardiogenesis has been published recently (Linask and Lash, 1986).

On the basis of our SEM studies dealing with chick embryos (Hiruma and Hirakow, 1985a, b) and amphibian embryos (Hirakow et al., 1984a, b; Hiruma and Hirakow, 1983; Komazaki, 1984; 1986), as to SEM observations on precardiogenesis period, it can be stated that the amphibian embryos are easier to handle than chick embryos. In this connection, we attempted to bring together our previous studies, which have been presented fragmentarily on various occasions. We believe that our findings give a typical example of vertebrate cardiogenesis. Moreover, it is our hope to present our work in English, which has mainly been published in Japanese. Thus, this paper will serve as a mini-review rather than an original paper.

An Outline of Early Cardiogenesis in the Newt Embryos

Refering to previous descriptions (Mollier, 1906; Copenhaver, 1950) and results obtained from our own SEM studies as well as from light and transmission electron microscopic observations, we have made diagrams to facilitate the understanding of early developmental sequences in the newt embryo heart (Fig. 1. reprinted from Hirakow, 1985). Developmental stages were defined after Ichikawa (1966). These schematic drawings and legends are self-explanatory.

Cardiogenic Cell Migration

Several synonyms have been used to designate cells which have the potency to give rise to the heart: "heart-forming cells", "primordial heart cells", "precardiac (mesoderm) cells", "cardiogenic cells", and so on. In this paper, we use the term "cardiogenic cells" to signify cells located at the border region of the lateral meso-

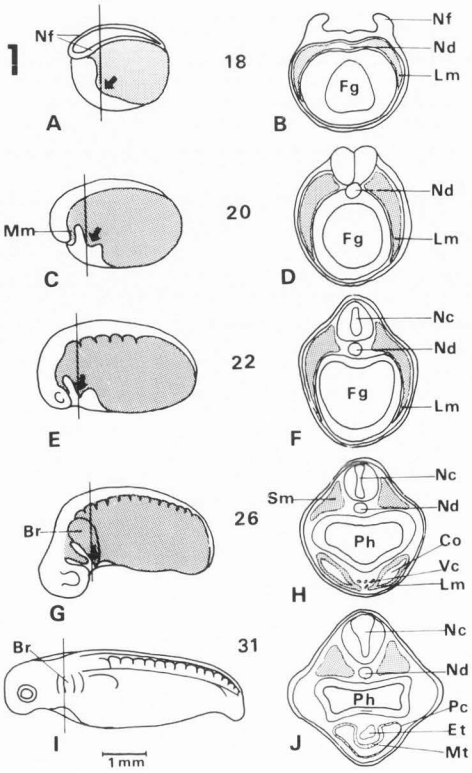


Fig. 1. Diagrams to explain sequential events of early cardiogenesis in neurulae (stages 18, 20, 22) and tail bud embryos (stages 26, 31) of the newt (stage after Ichikawa, 1966). A, C, E, G and I illustrate left-side view of the embryos; transverse sections at the vertical lines in these drawings are shown in B, D, F, H and J. Shaded areas indicate mesodermal materials. Arrows in A, C, E and G signify directional movement of the cardiogenic area in the lateral mesoderm (Lm), with eventual fusing under foregut (Fg) or pharynx (Ph) to form a myocardial tube (Mt). Abbreviations; Br, branchial region; Co, coelom; Et, endocardial tube; Fg, foregut; Lm, lateral mesoderm; Mm, mandibular mesoderm; Mt, myocardial tube; Nd, notochord; Nf, neural fold; Nc, neural canal; Pc, pericardium; Ph, pharynx; Sm, somite; Vc, vasoformative cells.

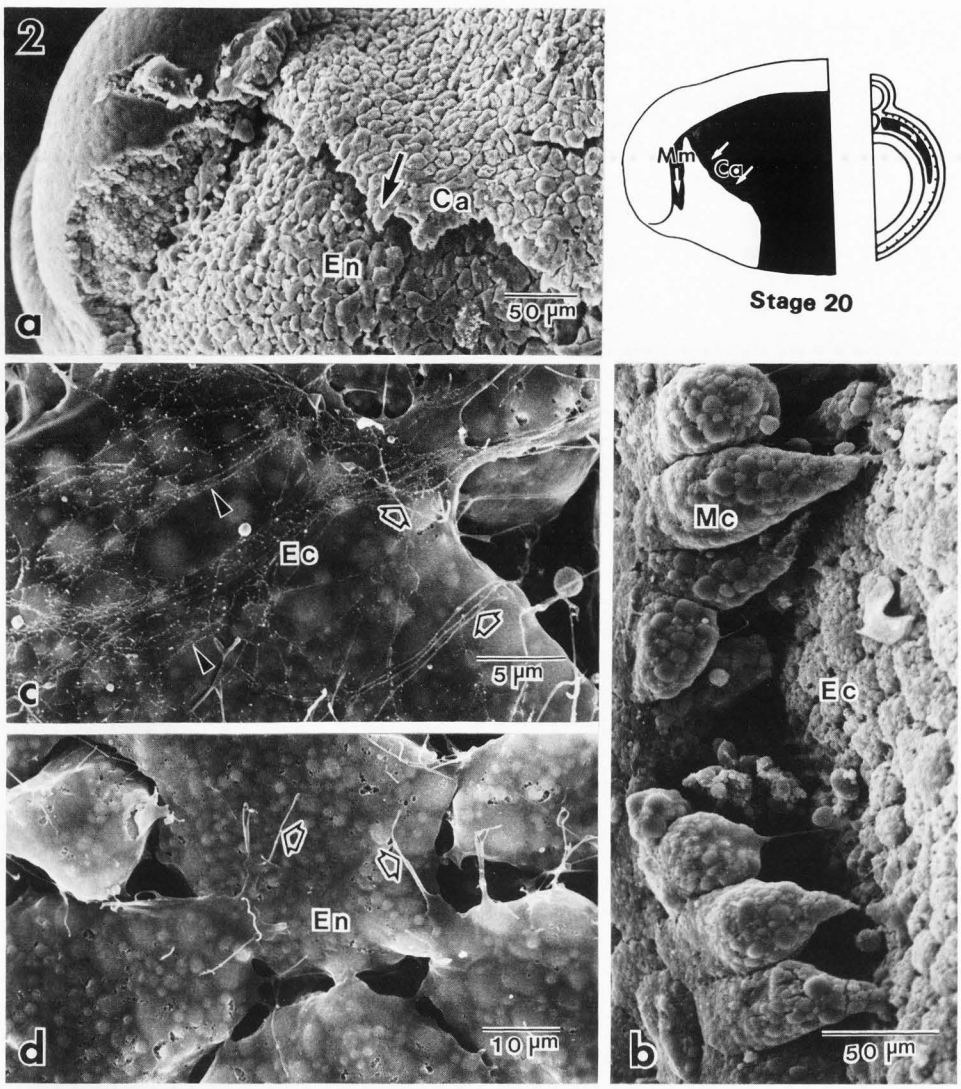
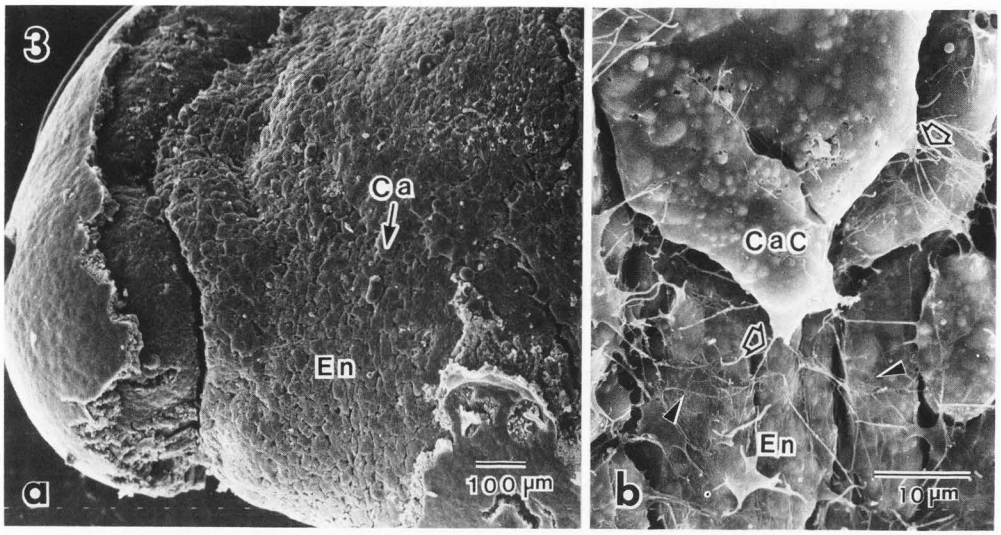
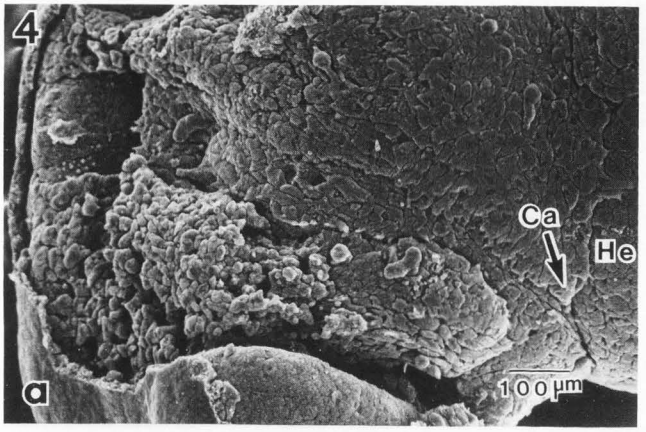
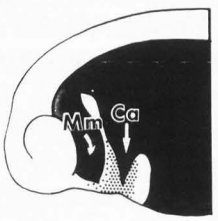


Fig. 2. SEM of a newt neurula at stage 20, at low magnification (2a), and at higher magnification (2b, 2c, 2d). The picture of 2a corresponds approximately to the left figure of the inset. The right figure of inset diagrams illustrates a transverse section of the cardiogenic mesodermal region. Arrow in 2a and the inset drawings shows migration of cardiogenic mesoderm (Ca) as well as that of mandibular mesoderm (Mm). At this stage, the mesodermal cells (Mc) appear to make contact with the undersurface of the ectoderm (Ec), as shown in 2b. On the undersurface of the ectodermal cells (Ec), fine fibrils of ECM (arrowheads) are found (2c), whereas on the endodermal cells (En), no such fibrils are present (2d). Note filopodial processes (open arrows) of these cells.

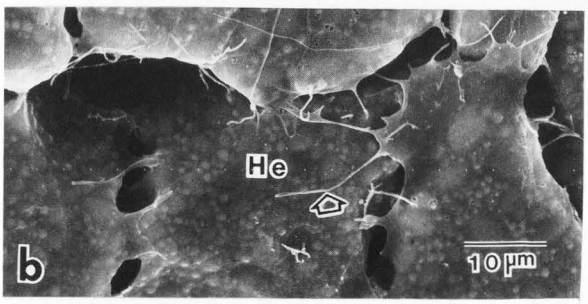
derm (arrows in Fig. 1 A, C, E), which differentiate into two different types of cells in the primitive heart, endocardial cells and myocardial cells. However, since the former is not specific to the heart, but common to the universal vascular system, the characteristics for differentiation into the heart must reside in the latter, which are capable of pulsation. Thus, the term "cardiogenic cells" implies primarily cells which are fated to differentiate into myocardial cells. In fact, using this criterion, a number of experimental embryological studies on cardiac differentiation of the amphibian embryo have been carried out (e.g., Holtfreter, 1938; Jacobson and Duncun, 1968; Willens, 1955; for others see reviews by Copenhaver, 1950; DeHaan, 1965). According to these authors, the cardiogenic area in the amphibian embryo was definitively established to be at the border zone of the lateral mesoderm, which migrate extensively during the gastrulation and neurula stages. It has also been shown that the lateral mesoderm apart from the cardiogenic area can be "induced" to form myocardium by the foregut endoderm (Jacobson and Duncun, 1968).

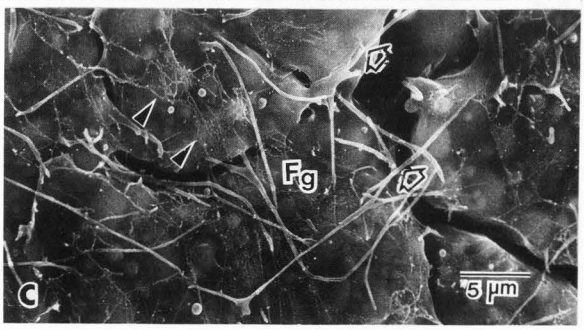

3
Ca
En
100 µm
a
CaC
En
10 µm
b


4
Ca
He
100 µm
a


Mm
Ca

Stage 24


He
10 µm
b


Fg
5 µm
c


1 µm
d

For ordinary SEM observations of the mesodermal mantle, the ectoderm was peeled off manually with tungsten needles from the embryos fixed with 2.5% glutaraldehyde and 3% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.0, at room temperature. They were then critical point dried in carbon dioxide and coated with platinum.

At earlier stages studied, stages 18 and 20, the migration mode of the lateral mesodermal or cardiogenic cells was found to be essentially identical. Therefore, we present here SEM studies of an embryo at stage 20 (Fig. 2). At this stage, it is worthy of note that migrating mesodermal cells appeared to attach to the basal surface of ectoderm (Fig. 2b) rather than to that of endoderm. This condition was recognized during embryo manipulation, since the removal of the mesoderm from the ectoderm was much more difficult than from the endoderm. It was intriguing to have found a fine fibrillar network of the ECM on the ectodermal cell surface (Fig. 2c), which is presumed to be used as the scaffold for movement. On the other hand, the basal surface of the endodermal cells at this stage is smooth and has no fibrillar materials, possessing only a few filopodial processes (Fig. 2d).

At later stages, the cardiogenic area became more prominently localized (Fig. 3a) and remarkable changes were found in the surface structures of the endodermal cells, such as the formation of numerous filopodial processes and precipitation of abundant fine fibrillar materials (Fig. 3b). It was also felt that the mesodermal cells attached more firmly to the endoderm than to the ectoderm.

When embryonic development progresses further, the localization of the cardiogenic area is more and more distinctly defined, assuming a wedge-like shape (Fig. 4a). During stage 24 regional differences in the endodermal cell surface

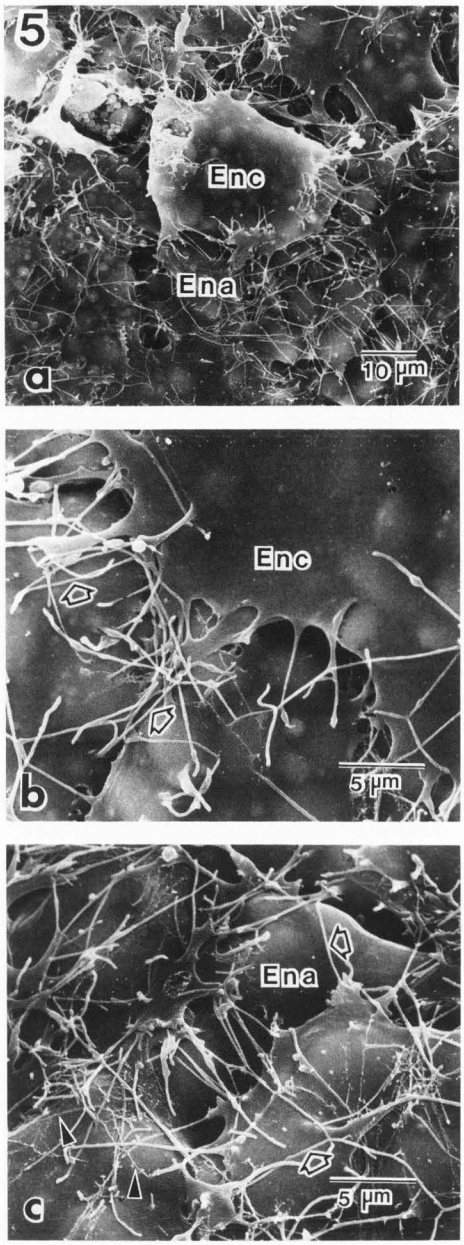


Fig. 5. SEM to show structural differences of the endodermal cellular surface before (Ena) and after (Enc) it is covered by mesodermal cells, at lower (5a) and higher magnification (5b, 5c). It is evident that ECM fibrils disappear and filopodial processes decrease in number from the surface of endodermal cells (5b), in contrast to the preceding state (5c).

Fig. 3. SEM of a newt neurula at stage 22. Note cardiogenic area (Ca) migrating ventrally as indicated by an arrow in 3a, a low magnification SEM of left-side view of the anterior region. At higher magnification (3b), a tip of cardiogenic cells (CaC) provided with filopodia (open arrows) is shown to be in contact with endodermal cells (En), on which fine fibrillar networks (arrowheads) are recognized.

Fig. 4. SEM of a newt tail bud embryo at stage 24. Compare with those of preceding figures (2 and 3). At low magnification SEM (4a), the cardiogenic area (Ca) is seen to assume a wedge shape directing its tip toward the ventral side of the foregut (Fg). On the surface of the hepatic endoderm (He), no fibrils are discernible on the cell surface (4b),whereas abundant fibrils are found on the endodermal cell surface of the foregut (Fg) and are presumed to be utilized as the scaffold of the migrating cells (4c). These fibrils are shown at higher magnification in 4b by arrowheads. Filopodial processes extending from the cell surface are seen (open arrows in 4b, 4c, 4d).

were noticed in terms of the absence (Fig. 4b) or presence (Figs. 4c, 4d) of ECM fibrils. The former, i.e., the hepatic endodermal area, remains uncovered, whereas the latter, or foregut area, is used by cardiogenic mesodermal cells as migration route. However, once the endoderm is covered by mesoderm, ECM fibrils disappear from the endodermal surface (Figs. 5a, 5b). In contrast, there are still abundant ECM fibrils on the surface of the endoderm ahead of the migrating mesoderm (Figs. 5a, 5c). From these facts, it is evident that ECM fibrils on the cell surface play an important role in mesodermal cell migration as will be mentioned below.

Myocardial Tube Formation

Around stage 25 or 26, the early tail bud stage, the bilateral cardiogenic mesoderm or primordia of the heart meet beneath the ventral side of the foregut (Fig. 6). Simultaneously, free cells presumed to be endocardium-forming cells (Vc in Fig. 1H and Fig. 6) are liberated from the mesoderm. Within the mesoderm, a body cavity or coelom has been produced by the separation of the mesodermal layers into visceral and somatic layers (Fig. 1H).

After complete fusion of the border of the lateral mesoderm, both visceral layers unite and bulge toward the coelomic, now pericardial, cavity, with the ventral mesocardium remaining as a fusion site of the lateral mesoderm (Fig. 7). This is an initial state of the myocardial tube, which is really not a tube, but trough-shaped. It then elongates cranio-caudally and bends normally to the right, forming a c-shaped loop (Fig. 8). At this stage, the heart begins to beat. Although there have been no comprehensive investigations on epicardium formation in the amphibian embryo, it is reasonably assumed to take place in essentially the same manner as described in the chick embryo (Hiruma and Hirakow, 1985b; Ho and Shimada, 1978; Manasek, 1969).

Extracellular Matrix (ECM) in the Newt Cardiogenesis

It is now widely accepted that the various components of ECM materials play a significant role in the developmental processes of organisms (see review of Hay, 1984). Recently, we have also presented a brief review (Hirakow, 1986) on the ECM in vertebrate cardiogenesis, referring to our previous investigations and those of other workers.

In the above, we already paid special attention to ECM fibrils in describing the results of ordinary SEM observations, as have been shown in Figs. 2b, 3b, 4c, 4d. Below, we present two different immunocytochemical investigations carried out to detect fibronectin in ECM materials during the cardiogenesis in the newt embryo.

An indirect immunofluorescence study was performed to find fibronectin on the basal surface of the ectoderm in embryos at stages 20 to 22. Embryos were fixed briefly with 3% paraformaldehyde, to which rabbit anti-human fibronectin (Wako Pure Chemicals Ind.) was reacted and labeled indirectly with FITC (fluorescein isocyanate) -conjugated goat anti-rabbit IgG (Litton Bionetics Inc.). The labeled network was observed under fluorescence microscopy (Fig. 9a; also refer to Figs. 2b, 2c). When embryos at these stages were freeze-dried at -40°C after similar fixation and observed under SEM, the ECM network was revealed abundantly in the space between the ectoderm and the endoderm (Fig. 9b), establishing satisfactory preservation of ECM materials. On the basis of these experiments, immuno-SEM was attempted to locate fibronectin in this ECM material. Rabbit anti-human fibronectin (IgG) labeled with latex beads (0.3 μm in diameter) was used. As a result, clusters of beads were found in the space between the ectoderm and the endoderm (Fig. 9c).

Considering these immunocytochemical findings and ordinary SEM images, we assume that the ECM materials observed so far are composed of fibronectin (the only defined component) and other macromolecules such as glycosaminoglycans. At present, however, it is difficult to determine whether the fibrillar structure of ECM materials is native or barely artifact. At any rate, the role of ECM materials as represented by fibronectin in amphibian cardiogenesis may be the same as has been shown in amphibian gastrulation in terms of cellular migration (Boucaut et al., 1985).

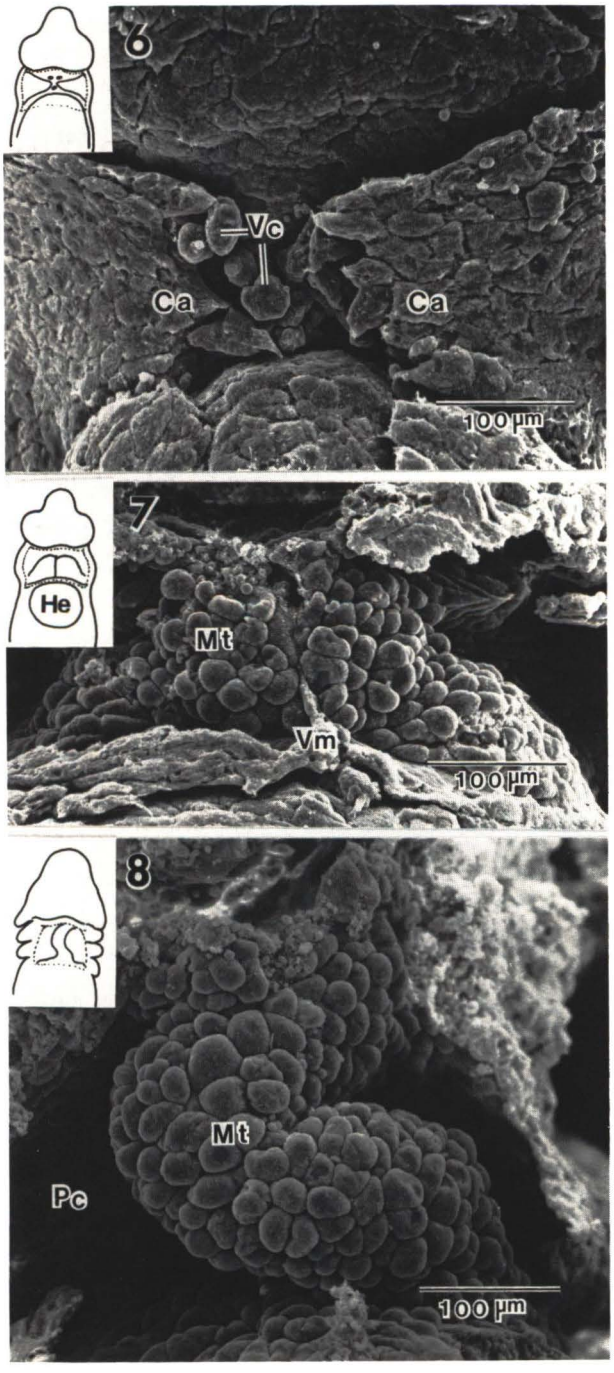
Conclusions

The migration pattern of cardiogenic cells located at the border region of the lateral mesoderm was revealed with SEM at a higher magnification. During the mesodermal cell migration period, cellular interactions between the mesoderm and other germ layers were found to change markedly. ECM materials, which are assumed to play an important role in the movement of the cardiogenic cells, were also observed. The morphology of the primitive heart after fusion of the cardiogenic mesoderm is considered to be essentially identical to that of the chick embryo.

Fig. 6. SEM of ventral view of a newt tail bud embryo at stage 26, as shown by inset drawing. Bilateral cardiogenic areas (Ca) are going to fuse with each other, liberating seemingly vasoformative cells (Vc).

Fig. 7. SEM of ventral region of a newt tail bud embryo at stage 28, as shown by inset drawing. primitive myocardial tube (Mt), just formed by fusion of the paired cardiogenic areas, is exposed by removing the parietal wall of the pericardial cavity or coelom. Ventral mesocardium (Vm) remains. The hepatic region (He in inset) is not covered by the mesoderm.

Fig. 8. SEM of a C-looping myocardial tube (Mt) at pulsation onset period (stage 34). Myocardial cells are exposed to the pericardial cavity (Pc) without the epicardium. The ventral somatopleure (body wall and pericardium) has been removed.



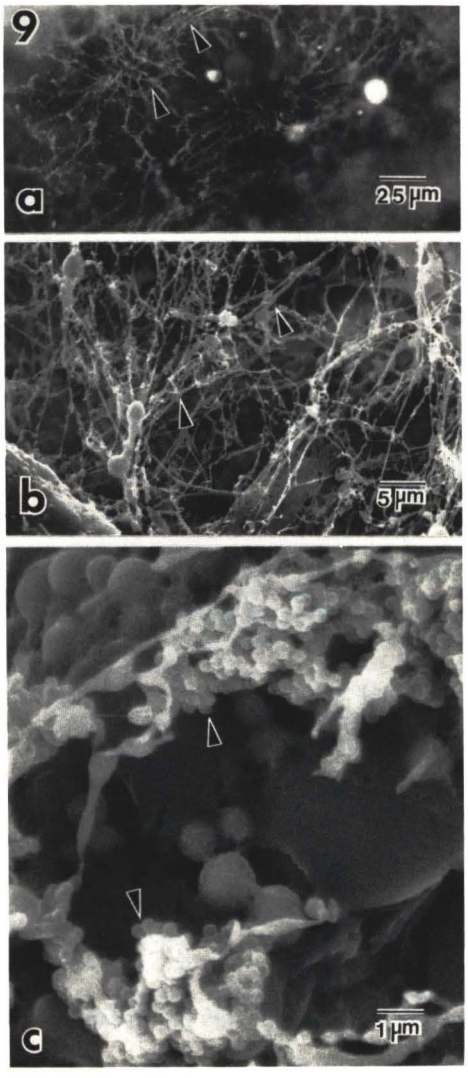


Fig. 9. ECM materials in newt embryos at stage 22 as observed by means of various methods; indirect immunofluorescence for fibronectin using FITC-conjugated antibody on the undersurface of ectoderm (arrowheads in 9a), SEM of ECM fibrils found in the space between the ectoderm and the endoderm in an embryo after freeze-drying (arrowheads in 9b) and immunoSEM for fibronectin using latex bead-labeled antibody (arrowheads in 9c).

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We are grateful to Dr. M. Hayashi, Ochanomizu University, for his cooperation to supply rabbit anti-human fibronectin (IgG) labeled with latex beads.

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Discussion with Reviewers

T. Pexieder: Are the granulations seen under the cell membrane in Fig. 2b yolk particles, or do the authors have another interpretation of this phenomenon, not observed in chick and mammalian embryos?
Authors: They are certainly yolk granules. The early amphibian embryonic cells contain a remarkable abundance of yolk granules of a relatively large size, although there are some differences in their ultrastructural appearance with regard to the germ layers as well as to the developmental stages.

T. Pexieder: What kind of evidence can the authors provide for more firm attachment of mesodermal cells to endoderm at stages older than 20?
Authors: We have two types of circumstantial evidence; the first is based on the observation of cellular processes which attach to the ectoderm before stage 20 (Fig. 2b), and subsequently to the endoderm (Fig. 3b); the second is based on our experience in removing the germ layers during the manipulation of the embryos.

T. Pexieder: What kind of proof do you have for the endocardial nature of Vc cells in Figs. 14 and 6?
Authors: The primitive heart wall is composed of only two layers, outer myocardium and inner endocardium. Careful examination of serial sections of embryos at appropriate stages shows that cells labeled as Vc give rise to endocardial cells (See Copenhaver, 1950).

T. Pexieder: The cardiogenic cells in the newt have much more cobblestone-like appearace than those of chick, mouse, rat, dog or human embryo. Do you have any explanation for this difference or is it an artefact?
Authors: No, this feature is not an artifact, but it is due to the large size of the amphibian cells. For example, the newt's embryonic cells of the

primitive heart (Figs. 7, 8) are about three times larger (ca. 30 μm in diameter) than those of the chick (ca. 10 μm) as seen in SEM.

Y. Shimada: Fig. 3b and Fig. 9b are pictures of ECM fibrils of stage 22 embryos seen at the same magnification. Why are ECM fibrils much more abundant in Fig. 9b than in Fig. 3b? Is it because of the different specimen preparation method?
Authors: Yes, it is. Fig. 3b represents a SEM micrograph of a specimen processed by the conventional method which tends to preserve the ECM more poorly than in a freeze-dried specimen as shown in Fig. 9b.

Y. Shimada: In Fig. 9c, aggregates of beads were seen. Did the beads adhere around complexly running and branching ECM fibrils (grape-like appearance of decoration is not an artifact), or did the beads form clusters before decoration or in the course of their adhesion to fibrils (beads did not uniformly cover fibrils)?
Authors: No, we do not think so, since control experiments did not show figures as presented in Fig. 9c.

R.R. Markwald: What evidence is there that the cardiogenic splanchnic mesoderm (an epithelium) actually "migrates"? Does this epithelial cell sheet actually translocate by migratory mechanisms or is it passively "moved" by the morphogenetic events associated with the formation of the body folds or closure of the foregut? Do the presumptive cardiogenic cells display any cell surface characteristics of migrating cells, e.g. filopodia, pseudopodia or "protrusal" activity on the leading edge of the migratory cell population?
Authors: Please refer to the paper of Willens (1955). This paper demonstrated the movement of the cardiac mesoderm by means of vital staining as was carried out earlier by Vogt (1929) to reveal amphibian morphogenetic movement. Consequently, we assume that the migration of cardiogenic mesodermal cells is a final phase of the gastrulation or morphogenetic movement. Migration of these cells may also be inferred from slender filopodia extending from the peripheral edge of the cells as seen in the SEM micrograph (Fig. 2b).

R.R. Markwald: Was fibronectin antibody localized on both surfaces of the cardiogenic mesoderm, i.e. between the splanchnic mesoderm and the ectoderm on one side and the mesoderm and endoderm on the other? If so, did fibronectin staining diminish at stage 22 when the cardiogenic mesoderm was "felt" to have become more definitely "attached" to the endoderm? Do the authors have any hypotheses as to why the precardiac mesoderm might differentially attach to ectoderm initially but endoderm at a later stage? Have the authors considered experiments to determine if the precardiac mesoderm has cell surface receptors for fibronectin or if the "movement" of the cardiogenic mesoderm can be modified by treatment with peptides having sequences (e.g. Arg-lys-Asp) specific for the putative fibronectin receptor?
Authors: At the present stage of our immunocytochemical investigation, we cannot adequately answer this question. In order to resolve these problems essential for understanding the interaction between cells and ECM, a specifically designed experiment must be undertaken.

R.R. Markwald: Would the authors care to speculate as to the origin of cardiac endothelium? For example, why do the authors consider this endothelium to be identical to that of vascular (arterial or venous) endothelium in view of the observation that cardiac endothelial cells have a biphasic pattern of differentiation (i.e. some but not all "transform" into prevalvular mesenchyme).
Authors: This question is related to the principal subject of developmental biology. The origin of the vascular endothelial cells has been considered to be so called "angioblasts". However, we have no critical idea whether there is a difference in the origin between the endothelium of the heart and that of the blood vessels or not. It seems to us that no one can answer this question until an effective cellular marking method has been devised.

R.R. Markwald: Did the authors observe any contribution of neural crest (ectodermal cells) to the precardiac mesoderm during early development? Is there a reason to think that some of the ECM associated with the endoderm becomes "encased" within the forming myocardial tube?
Authors: No, we did not observe the neural crest incorporated into the cardiac mesoderm. It is conceivable that the ECM on the endoderm may be "encased" within the primitive heart tube. However, definitive evidence cannot be obtained from simple observation. A different technique such as autoradiography may provide an answer to this question.

R.R. Markwald: Have the authors verified their observation of reduced ECM following migration of the cardiac mesoderm by other means, e.g., anti-fibronectin staining or TEM?
Authors: We did not examine this systematically. This is a subject to be studied further.

D.A. Hay: Regarding your use of anti-fibronectin-FITC markers: Have you compared the fibronectin labeling patterns of premigratory endodermal surfaces to those of postmigratory endodermal surfaces? It is difficult to discern with what structures the fibronectin-latex beads are associated within the ectodermal/endodermal interface. Are they associated with the same fibrillar materials demonstrated in figures 2c-d?

Although the presence of fibronectin at the ectodermal/endodermal interface appears to be verified by immunocytochemistry, there is no real evidence that the presence of fibronectin is responsible for, or necessary for, the migration of cardiogenic mesoderm.
Authors: The main purpose of this paper is to describe early events of the newt cardiogenesis, not to deal extensively with the immunocytochemistry of fibronectin or its role. Here we only describe preliminary work to detect fibronectin by means of two kinds of immunocytochemistry, using fluorescence and immuno-SEM methods, simply because this material of ECM has drawn the attention of biologists in terms of its role in e.g., cellular migration (see e.g., Boucaut et al., 1985; Linask and Lash, 1986). However, the fibrillar material shown in Fig. 2c (not 2d where no fibrils

are present) and that demonstrated in Fig. 9 are quite different in appearance and structure due to the different methods used for demonstration. In this respect, in our present study, it was impossible to discern which type of fibril among the ECM materials was composed of fibronectin. What we can state in any case is that fibronectin exists in the space between ectoderm and endoderm in the newt embryos during the period of cardiogenic cell migration.