Normal Anterior Endoderm Corrects the Heart Defect in Cardiac Mutant Salamanders (*Ambystoma mexicanum*)

Abstract. *Recessive mutant gene* c *in axolotl embryos results in an absence of heart function. Normal (+/+) anterior endoderm cultured with mutant* (c/c) *hearts totally corrects the defect.*

A naturally occurring recessive genetic mutation, designated *c* for "cardiac lethal," was discovered in an imported stock of Mexican salamanders, *Ambystoma mexicanum* (*1*). Homozygous (*c*/*c*) embryos show a lack of heart function, even though initial development of the organ appears normal. Mutant (*c*/*c*) embryos are obtained by mating heterozygous (+/*c* × +/*c*) adults and can be first distinguished from their normal (+/+ or +/*c*) siblings at stage 34 when the normal embryos develop contracting hearts (*2*). The mutant embryos at this stage are normal in appearance, except that their hearts do not beat properly since only the conus region shows slight contractions; no circulation is established. In later stages, the mutant embryos develop ascites, and their hearts become distended and thin-walled. In spite of the absence of blood circulation, mutant embryos live approximately 3 weeks beyond the time when the heart normally should have begun to beat, presumably by simple diffusion of oxygen directly into the tissues. Skeletal muscle is not affected by gene *c* and the mutant embryos are able to swim normally.

Morphological and biochemical studies comparing normal and mutant heart development from stage 34 (heartbeat stage) through stage 41 (when mutant embryos die) have been reported (*3, 4*). Electron microscopy shows that normal ventricular cardiomyocytes contain well-organized sarcomeric myofibrils at stage 34. By stage 41, the normal ventricular myocardium shows extensive trabeculation and is composed of highly differentiated muscle cells. The mutant myocardium remains a single cell layer in thickness throughout development and no trabeculae form. Mutant ventricular heart cells at stage 34 contain sparsely scattered thin (6 nm) and thick (15 nm) filaments and an occasional Z body. Some mutant myocardial cells at stages 34 through 41 show a partial organization of myofilaments; however, distinct sarcomeric myofibrils are absent (*3, 4*). Mutant cells contain instead amorphous proteinaceous collections in their peripheral sarcoplasm where myofibrils initially organize in normal cells (*4, 5*). Studies with sodium dodecyl sulfate polyacrylamide gel electrophoresis (*4, 6*), heavy meromyosin binding (*4, 6*), tropomyosin incubation (*7*), radioimmunoassay (*6, 8*), and immunofluorescence microscopy (*9*) extend these morphological findings and demonstrate that myosin and tropomyosin are reduced in mutant hearts. These studies also show that actin, while abundant in mutant heart cells, is stored in an unpolymerized, nonfilamentous form. It is obvious that gene *c*, when homozygous, alters normal heart cell differentiation. The most striking deficiency in mutant hearts is a lack of organized sarcomeric myofibrils.

Humphrey (*10*) transplanted mutant (*c*/*c*) heart primordia into the heart regions of normal (+/+ or +/*c*) recipients at stages 29 and 30 and found that the mutant hearts beat. In reverse transplants of normal (+/+ or +/*c*) hearts into mutant (*c*/*c*) hosts, no heartbeat was observed. In further experiments, stage 25 normal and mutant siblings were linked parabiotically; the cardiac deficiency was not corrected in mutants, and the normals were not adversely affected. Such conjoined animals lived indefinitely, and the mutant twins, except for their nonbeating hearts, appeared normal. These studies suggest that gene *c* affects the heart specifically and indicates that the failure of normal heart development in cardiac-lethal embryos results from abnormal inductive effects from tissues in the pericardial area. It is evident that such effects are restricted to the heart region and are not circulating in the blood.

The heart, as with most vertebrate organs, requires inductive interactions for normal development; these interactions control and direct heart formation. The anterior dorsolateral endoderm of early vertebrate embryos is the most potent (and important) heart inductive tissue. This has been established experimentally in both amphibians (*11, 12*) and chicks (*13*), and is probable for mammals (*14*). These inductive interactions have been demonstrated to occur in hanging drop organ cultures from the salamander *Taricha torosa* (*11*). For example, stage 16 heart mesoderm cultured by itself resulted in 13 percent of the explants beating. When anterior dorsolateral endoderm was placed in culture with the mesoderm, 100 percent of the hearts were observed to beat. On the basis of this and a number of other culture experiments, it was demonstrated that the anterior endoderm in salamanders is a potent heart inducer that increases the frequency and rate of heart differentiation (*11*).

Since the inductive role of anterior endoderm on heart development in salamanders is well established and since Humphrey's (*10*) transplantation experiments suggested that gene *c* in axolotls acts by way of abnormal induction, we undertook the present study to determine whether the cardiac defect could be corrected by culturing mutant (*c*/*c*) hearts with normal (+/+) anterior endoderm (*15*).

Cardiac mutant (*c*/*c*) embryos and their normal (+/+ or +/*c*) siblings were obtained by mating adult axolotls heterozygous for gene *c* (+/*c* × +/*c*). Additional normal (+/+) embryos were derived from wild-type (+/+ × +/+) matings. The embryos were reared in 25 percent Holtfreter's solution (*16*) and were staged according to the Schreckenberg and Jacobson (*2*) system. Mutant spawnings were allowed to develop to stage 35, the first point at which mutant (*c*/*c*) embryos can be distinguished with certainty from their normal (+/+ or +/*c*) siblings. The normal wild-type (+/+) embryos were permitted to develop to stage 30. The developmental stages were controlled by rearing the embryos at temperatures of 12° or 18°C so that the mutant (*c*/*c*) embryos and their normal (+/+ or +/*c*) siblings were at stage 35 when wild-type normals (+/+) were at stage 30. Thus, stage 35 mutant and normal siblings and stage 30 normal wild-type embryos provided the tissues for the organ

Table 1. Summary of organ culture experiments after a 48-hour culture period.

Type of culture	Cultures (No.)	Hearts with vigorous propagated beats	Heart ventricles (No.)	
			Examined by electron microscopy	With organized sarcomeric myofibrils
Normal hearts	23	21 (91 percent)	7	7 (100 percent)*
Mutant hearts plus normal anterior endoderm	21	17 (81 percent)	12	12 (100 percent)*
Mutant heart controls	44†	0 (0 percent)	18	0 (0 percent)

*Beating hearts only. †Includes all controls described in the text.

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culture experiments. The culture medium consisted of sterile 100 percent Holtfreter's solution (*17*) containing 1 percent antibiotic-antimycotic (Grand Island Biological Company). The tissues were cultured in a drop of medium on dental wax located inside an airtight, plastic Falcon culture dish (60 mm). Extra drops of sterile medium were placed in the dish to provide a humid environment. The mutant hearts at stage 35 were extirpated with glass needles; a portion of the ventral epidermis overlying the heart was left intact so that epidermal "sandwiches" (*18*) could be made. The hearts were placed in the drop of culture medium with a Spemann transfer pipet (*18*). The stage 30 normal anterior endoderm was handled similarly and placed in the drop with the mutant heart. Special care was taken to ensure that the normal anterior endoderm was free of any adhering heart mesoderm cells by scraping the ventral surface of the endoderm with a hair-loop (*18*) prior to extirpation. An epidermal sandwich, derived from mutant epidermis as described above, was used to enclose the stage 35 mutant heart with the stage 30 normal anterior endoderm. Controls included culturing mutant hearts alone or in epidermal vesicles, or with somites or posterior endoderm in epidermal vesicles. An additional control included culturing of stage 35 normal sibling hearts by themselves or in epidermal vesicles.

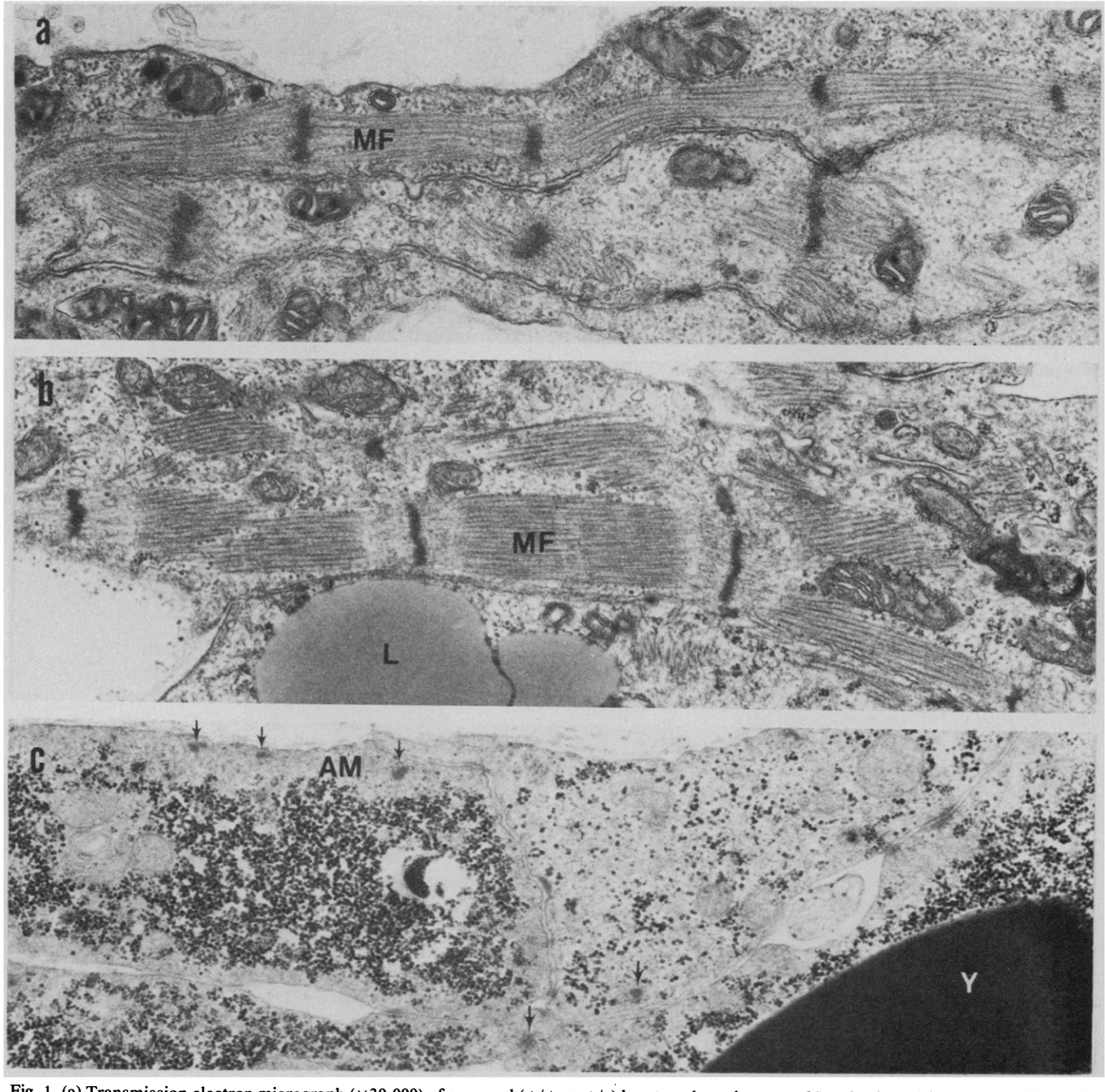

Fig. 1. (a) Transmission electron micrograph (×30,000) of a normal (+/+ or +/*c*) heart explanted at stage 35 and cultured for 48 hours. The cells contain numerous well-organized myofibrils (*MF*). (b) Transmission electron micrograph (×30,000) of a mutant (*c*/*c*) heart explanted at stage 35 with stage 30 normal (+/+) anterior endoderm and cultured for 48 hours. The cells contain numerous well-organized myofibrils and are virtually identical in appearance to normal myocardial cells; *L*, lipid. (c) Transmission electron micrograph (×30,000) of a control mutant (*c*/*c*) heart explanted at stage 35 in an epidermal vesicle and cultured for 48 hours. Myofibrils could not be found in the cells; however, amorphous proteinaceous collections (*AM*) with associated Z bodies (arrows) are prominent. The mutant heart controls cultured alone or in epidermal vesicles with or without posterior endoderm or myotomes were similar in appearance; *Y*, yolk.

Of 23 normal sibling (+/+ or +/*c*) stage 35 hearts explanted into culture, 21 (or 91 percent) beat vigorously within 15 minutes and continued beating throughout the culture period. Of the 21 mutant (*c*/*c*) stage 35 hearts cultured with stage 30 normal anterior endoderm, 17 (or 81 percent) began beating throughout their lengths within 12 hours and by 24 hours in culture were contracting as vigorously as the normal controls. Control mutant hearts in culture did not contract vigorously although within 15 minutes of explanting there were a few slight contractions visible in the conus regions of some hearts. These slight conus contractions were observed in in vivo hearts as well and are presumed not to be the result of the culture conditions. Even after 48 hours in culture the 44 control mutant hearts failed to contract in their ventricular, atrial, or sinal portions and therefore closely resembled in vivo mutant hearts (*3, 4, 10*).

After 48 hours, the cultures were prepared for study in the transmission electron microscope. The tissues were fixed initially in a mixture of glutaraldehyde, formaldehyde, and picric acid buffered with 0.10*M* phosphate to *p*H 7.3, postfixed in 2 percent osmium tetroxide buffered to *p*H 7.2 with 0.10*M* phosphate, dehydrated in ethanol, and embedded in Epon. Thick sections were made for orientation and to locate the heart ventricles. Thin sections of the ventricles, cut with a diamond knife, were mounted on bare copper grids, stained with uranyl acetate and lead citrate, and viewed at 80 kV in an AEI 801 electron microscope.

The ventricles of seven of the cultured normal control hearts were examined in the electron microscope and, as was expected (*3, 5, 7, 9, 19*), were composed of myocytes containing numerous well-organized sarcomeric myofibrils (Fig. 1a). The stage 35 mutant hearts cultured with stage 30 normal anterior endoderm also contained numerous myofibrils of normal morphology (Fig. 1b). In fact, of the 12 corrected mutant hearts examined by electron microscopy, all were identical in appearance to the normal control hearts. In contrast, when electron microscopy was used to analyze 18 of the control mutant heart ventricles (that is, those cultured alone or in epidermal vesicles with or without posterior endoderm or myotomes), no organized sarcomeric myofibrils could be found (Fig. 1c). These cells contained instead amorphous proteinaceous collections, along with a few thick (15 nm) and thin (6 nm) filaments and thus their ultrastructure closely approximated mutant hearts in vivo at corresponding developmental stages (*3, 4*).

Our results (Table 1) demonstrate that normal anterior endoderm (prior to the onset of heart beat) corrects the heart defect in cardiac lethal mutant axolotl embryos and essentially transforms mutant hearts into normal ones. We believe that this is accomplished because normal anterior endoderm provides an essential "final inductive influence" to cause the mutant hearts to form organized sarcomeric myofibrils and begin beating. Since mutant hearts do not beat normally or form myofibrils when cultured alone, it seems improbable that a general inhibitory effect causes the defect, as was once considered a possibility (*3, 10, 20*). In the unlikely event that there are inhibitory influences operating in cardiac mutant embryos, such effects can be overcome by the addition of normal anterior endoderm. The mechanism of the inductive abnormality in mutants and its relation to gene *c* and to the heart mesoderm remains moot at this time and requires further study. Our study suggests that gene *c* results in an absence of heart function because there is an alteration of the normal heart inductive processes from the anterior endoderm and this prevents final differentiation of the heart.

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Intercellular Communication in Pancreatic Islet Monolayer Cultures: A Microfluorometric Study

Abstract. *Single islet cells in monolayer cultures of neonatal rat pancreas were microinjected with fluorescein and scanned topographically by microfluorometry. Fluorescein spread from an injected islet cell directly into neighboring islet cells, and, in the presence of 16.7 millimolar glucose, significantly more islet cells communicated with the injected cell than in glucose-free medium. Islet cells were also microinjected with glycolytic substrates and activators that produced transient changes in cellular levels of reduced pyridine nucleotides—nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate [NAD(P)H]. Changes in NAD(P)H fluorescence were observed in islet cells incubated first for 18 hours in very low glucose concentrations and then in a glucose-free medium and injected with glycolytic substrates and activators; however, little change of fluorescence occurred in adjacent islet cells. In contrast, after adding 16.7 millimolar glucose to the medium, injection of glycolytic substrates and activators produced transient changes in NAD(P)H fluorescence in the injected cell and in neighboring cells.*

The presence of gap junctions (*1*) between insulin-containing B cells (and other endocrine cells) in the pancreatic islets of Langerhans (*2–4*), as well as electrophysiological studies (*5*), suggests that cell-to-cell communication (*6–11*) may be important for secretory functions of islet cells. The objectives of this study were to determine whether intercellular communication can be directly demon-

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