HEART DEVELOPMENT IN CARDIAC MUTANT AXOLOTLS

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Since the early days in the field of embryology, the question of how different cells and tissues arise during development has intrigued researchers. Although regional variations in oocyte cytoplasm were recognized, it was not clear how pre-patterning could account for all the complexities of organogenesis and cytodifferentiation. A major breakthrough in beginning to understand the process of organ differentiation came with the experiments of Spemann and Mangold (1924) on the "primary organizer" in urodele amphibians. They found that if they transplanted the dorsal lip of the blastopore from one early amphibian embryo to another, a secondary embryo developed along the flank of the host under the organizing influence of the transplanted dorsal lip. As a consequence, the concept of induction became widely accepted as a basic mechanism by which specialized vertebrate cells and tissues form from relatively undifferentiated tissue in the early embryo. Induction could be defined then as a phenomenon whereby one group of cells and tissues influence another group to differentiate in a specific manner and in a specified direction. These issues are discussed at length in Slack (1991).

Studies on the mechanisms of induction now have moved to the cellular and molecular levels. With modern research tools, it will be possible to answer the questions of what molecules induce specific tissues or organs and how these molecules work.

The cardiac nonfunction gene in axolotls, designated as gene *c*, is a simple Mendelian recessive mutation, which would appear to provide an excellent model for examining inductive processes affecting heart differentiation. Embryos homozygous for gene *c* fail to develop hearts which beat, and the mutant embryos die from a lack of circulation.

Morphological and biochemical studies comparing normal and mutant heart development from stage 34, when the normals develop beating hearts, through stage 41, when mutant embryos die, have been reported (Lemanski, 1973; Lemanski et al., 1980; Starr et al., 1989). Normal heart cells contain a few organized sarcomeric myofibrils beginning at stage 34 and by stage 41 have formed into highly differentiated muscle cells. Mutant cells show a few poorly-defined filamentous structures, however, organized sarcomeric myofibrils fail to form even as late as stage 41. The ultrastructural data make it very clear why the mutant hearts do not contract normally; they lack organized sarcomeric myofibrils (Lemanski, 1973).

With regard to an examination of heart induction in mutant axolotls, several earlier studies have suggested that gene *c* might exert its effect by way of abnormal inductive processes. The first experiments suggesting this possibility were done by Humphrey (1972), who transplanted mutant heart primordia into normal hosts at the early tailbud stages 29-30 and found that the mutant organs beat in their new environment. In the reverse transplants, normal presumptive heart tissue into mutant hosts, no heartbeat was observed. In further studies, Humphrey (1972) parabiotically linked normal and mutant sibling embryos at stages 24-25. This did not correct the defect in mutant embryos, nor were the normals adversely affected by this procedure. Such conjoined animals lived indefinitely, and the mutant parabionts appeared completely normal except that their hearts failed to beat; eventually, the mutant organs formed into a vessel draining the head in younger specimens, while in older pairs the heart of the c/c member largely disappeared (Humphrey, 1972). These experiments indicated that in mutants the inductive factor(s) in the heart region were lacking, or, alternatively, that some inhibitory influence affected mutant heart development. When Humphrey's findings were considered in combination with earlier studies by Jacobson and Duncan (1968) and Fullilove (1970), that anterior endoderm in salamanders is a potent inducer of heart differentiation, the possibility emerged that gene *c* might involve abnormalities in heart induction processes from the anterior

endoderm. A series of experiments were done in our laboratory to address this hypothesis.

To test the possibility that there might be a generalized inhibitor of heart differentiation in mutant embryos which was keeping the heart from beating, Hill and Lemanski (1979) simply removed normal and mutant hearts at stage 35 and organ cultured them individually in hanging drop cultures of Holtfreter's solution. The normal hearts (19 out of 23 or 83%) continued to beat vigorously for 5 to 7 days in culture, and all of those examined by electron microscopy showed numerous organized sarcomeric myofibrils. On the other hand, none of the mutant hearts beat throughout their lengths in the cultures, although slight contractions in the conus regions were noted in 7 out of 27 mutant organs (26%); moreover, myofibrils were not visualized by electron microscopy in any of the mutant hearts examined.

In a related set of experiments, Lemanski et al. (1979) co-cultured stage 29-30 normal embryonic anterior endoderm with mutant heart tubes at stage 35, when mutants could be distinguished with certainty from their normal siblings. When mutant hearts were placed in hanging drops with normal anterior endoderm, they began to contract throughout their lengths within 12-24 hours and by 48 hours were contracting as vigorously as the normal controls. The corrected mutant hearts showed well-organized myofibrils in the electron microscope and, in fact, were very similar morphologically to normal controls after 48 hours in culture. A total of 17 out of 21 (81%) of the mutant hearts were "rescued" in these anterior endoderm cultures. Mutant hearts cultured in Holtfreter's solution alone or in epidermal vesicles, or with somites or posterior endoderm, did not beat (0 out of 44), nor were organized sarcomeric myofibrils detectable by electron microscopy in any of the 18 mutant hearts examined. Thus, these studies clearly demonstrated that mutant heart cells were corrected by culturing them in the presence of normal anterior endoderm. We suggested previously (Lemanski et al., 1979) that these data supported the hypothesis that gene *c* acts by way of abnormal influences at the differentiation step of development and possibly results from lack of some kind of "final inductive influence" from the anterior endoderm. We further suggested at that time that if there were inhibitory factors operating in the mutant embryo, then the inhibition could be overcome by the presence of normal anterior endoderm.

Davis and Lemanski (1987) did further studies in an attempt to better understand the mechanisms by which normal anterior endoderm was able to influence the differentiation of mutant hearts. In one set of experiments, mutant hearts were organ-cultured in Holtfreter's medium which had been conditioned with normal anterior endoderm. This endoderm-conditioned medium rescued the mutant hearts, demonstrating that induction was mediated by a diffusible substance from the anterior endoderm, which influenced the mutant hearts. To characterize the unknown substance(s), a series of enzymes were immobilized on agarose beads and were used to digest the conditioned medium in an effort to destroy or reduce its rescuing ability. Treatment with neuraminidase and trypsin did not have a statistically significant effect on the rescue activity, and, even after treatment, the medium still promoted beating of the mutant organs. Boiling of the conditioned medium for three minutes also did not reduce the rescuing effect. The addition of ribonuclease, however, totally eliminated the ability of the medium to rescue mutant hearts (0 out of 18); in conditioned medium controls for that particular experiment, 14 out of 26 or 54% of the mutant hearts were corrected. Corollary experiments were then performed in which RNA was extracted from normal anterior endoderm, liver and neural tube tissues of stage 29 normal embryos. The RNA from endoderm corrected 9 out of 10 or 90% of the mutant hearts, while the RNA from nonendoderm tissues showed no effect at all (0%). In very recent dose-response experiments (La France et al., 1992) (summarized below), we have extended these experiments and have confirmed with very rigorous control studies that, indeed, mutant hearts are rescued by RNA from the heart regions (endoderm and heart mesoderm) of normal embryos.

Smith and Armstrong (1990) also have reported recent studies on heart induction in cardiac mutant axolotls. They found that the pharyngeal endoderm from normal embryos when cultured with mutant hearts corrected the mutant defect, thus confirming the results of Lemanski et al. (1979). In addition, Smith and Armstrong (1990) reported experiments using RNA extracted from stage 14 and stage 30 normal embryonic endoderm and found that both were capable of inducing stage 35 *c/c* hearts to begin beating; this corroborates the earlier studies of Davis and Lemanski (1987), which showed that normal endodermal

RNA rescued the mutant hearts in organ culture. Smith and Armstrong (1990) concluded that both stage 14 and stage 30 endoderm were effective in inducing the mutant hearts to beat with the stage 14 preparations being somewhat less active. Unfortunately, the numbers of hearts used in each of their individual experiments (only 5 cultures at each stage with 2 out of 5 at stage 14 and 4 out of 5 at stage 30 being corrected), were too low to demonstrate a statistical difference between stage 14 and 30 with a high degree of reliability. Nevertheless, taken together, these experiments are in full agreement with the earlier published findings from our laboratory (Davis and Lemanski, 1987) and are in full agreement with our just-completed dose-response experiments (La France et al., 1992).

In very recent studies, La France, et al., (1992) performed detailed dose-response experiments to analyze the correction of stage 35 mutant hearts in organ cultures by using highly purified RNA at different concentrations purified from medium conditioned by tissues of the heart regions (containing both anterior endoderm and pre-heart mesoderm) of stage 25 normal (+/+ wild-type) embryos. Concentrations of the RNA ranged from 0.025 ng to 4 ng RNA/µl of Steinberg's solution. The data show that there is an increase in the percent of correction in cardiac mutant hearts that correlates with the increase in RNA concentration in the culture medium. Rigorous proteinase K treatment does not significantly reduce the activity of the RNA preparation, suggesting that protein is not the active factor. However, RNAase treatment completely abolishes the activity, confirming our earlier experiments (Davis and Lemanski, 1987). Normal sibling control hearts cultured under the same conditions continued to beat vigorously throughout the 48 hour culture period. These data are summarized in Table 1. Electron microscopic studies of the corrected mutant

Table 1

Dose-Response Experiments on Cardiac Mutant Hearts

RNA Concentration	Ratio	Correction %	n*
40ng/10µl	19/30	63	5
20ng/10µl	14/33	42	3
10ng/10µl	13/30	43	4
2.5ng/10µl	8/36	22	5
0.25ng/10µl	3/30	10	3
0.0ng/10µl	4/82	5	15
Proteinase K-treated			
40ng/10µl	15/30	50	3
RNase A-treated			
40ng/10µl	1/30	3	3

*n values represent number of independent experiments.

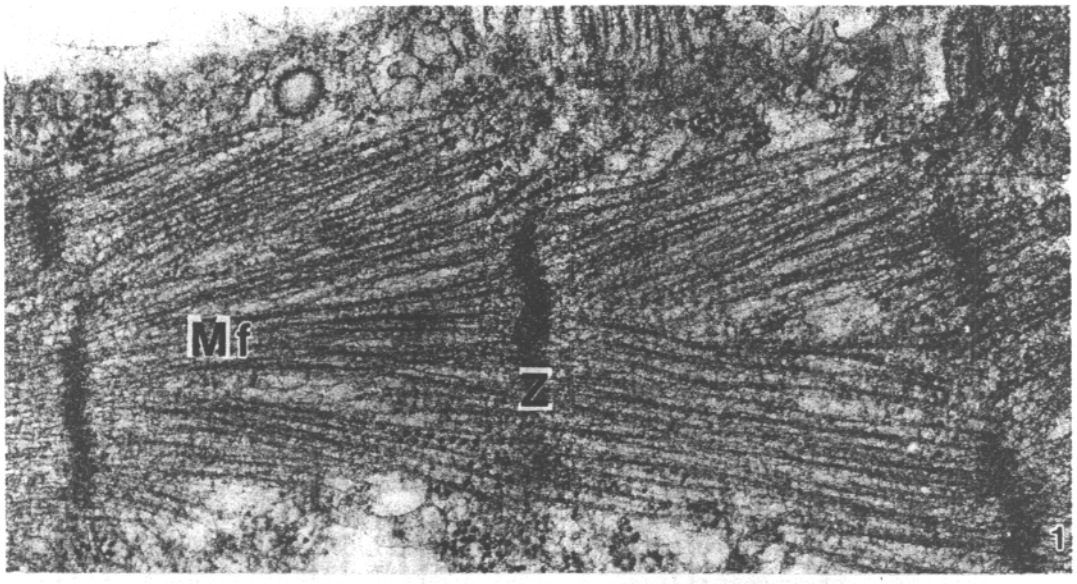
These data show that there is an increase in the percent of correction in cardiac mutant hearts that correlates with the increase in RNA concentration in the culture medium. Rigorous proteinase K treatment does not significantly reduce the activity suggesting that protein is not the active factor. However, RNase A treatment totally abolishes the rescuing activity, confirming our earlier experiments.

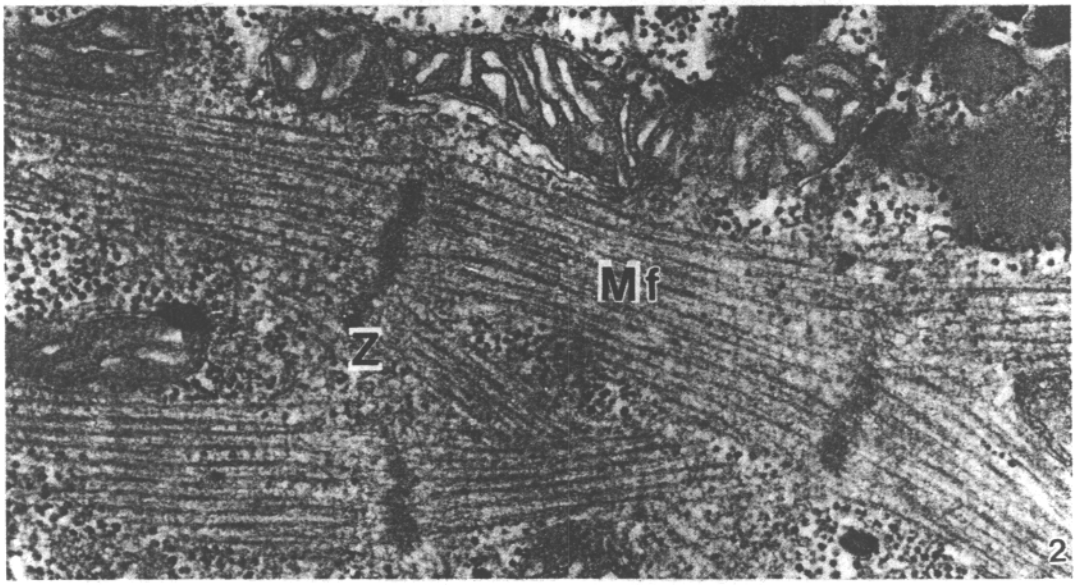
Normal control hearts cultured under all of the conditions specified above continued to beat throughout the culture period.

Figure 1. Electron micrograph of a portion of a cardiac mutant axolotl cardiomyocyte from a mutant heart organ-cultured for 48 hours in Steinberg's medium containing 1 ng RNA/µl. The RNA had been extracted from conditioned medium prepared from the heart regions (anterior endoderm and precardiac mesoderm) of normal (+/+) embryos. Organized sarcomeric myofibrils (Mf) complete with Z-lines (Z) are obvious in the cell. X66,500

Figure 2. Electron micrograph of a portion of a control cardiomyocyte from normal heart organ-cultured 48 hours. The myocytes show sarcomeric organization very similar to the rescued mutants in Figure 1. Mf, myofibril; Z, Z-line. X66,500

Figure 3. Electron micrograph of a mutant heart organ-cultured in Steinberg's solution without RNA. The mutant hearts do not beat and organized sarcomeric myofibrils are not discernable. However, there are indications of myofibrillar-type structures in the form of dense areas which resemble "Z-bodies" (Zb) as well as filamentous structures (F). X66,500


Mf
Z
1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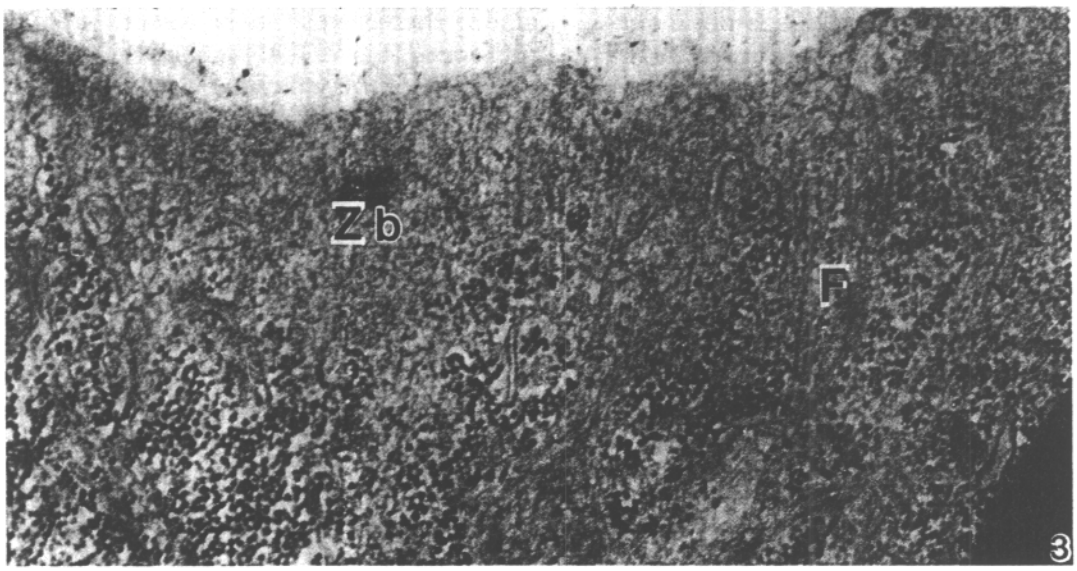
Z
2


Zb
F
3

hearts confirmed that they formed organized myofibrils of normal morphology (Fig. 1) and, in fact, were indistinguishable from the normal controls (Fig. 2). Control mutant hearts, which were cultured without the endoderm RNA, did not contract nor did they form organized myofibrils (Fig. 3).

These experiments were designed to unequivocally test the hypothesis that RNA from conditioned medium produced by the anterior endoderm/heart regions of normal embryos was capable of rescuing the mutant hearts in a dose-dependent fashion. Our results show, without question, that RNA from the normal heart regions has a positive inductive influence on the mutant hearts and, in fact, is able to turn noncontracting mutant organs into vigorously contracting hearts which are phenotypically very similar to normal.

In additional experiments currently in progress, we are assessing "pure endoderm-conditioned medium" and "pure mesoderm-conditioned medium" individually to evaluate each one's capability of rescuing mutant hearts. Our preliminary results using conditioned medium (i.e., the RNA had not been purified) suggest that both the normal anterior endoderm and the normal precontractile mesoderm has the ability to correct mutant hearts. In fact, both showed correction of mutant hearts when cultured for 48 hours; 5 out of 10 (50%) mutants in the endoderm-conditioned medium and 8 out of 13 (62%) mutant hearts in the precardiac mesoderm-conditioned medium were rescued. Given earlier reports from our laboratory of mutant heart rescue by RNA from juvenile axolotl heart (Fransen et al., 1990) as well as sheep heart RNA (La France et al., 1991), this is not a surprising finding. Smith and Armstrong (1991) also found that mutant hearts beat when cultured in the presence of normal heart mesoderm. In that same study, they reported that mutant endoderm was capable of inducing stage 14 wild-type precardiac mesoderm to beat at stage 35 in roughly the same manner as normal endoderms. This is a very interesting observation; unfortunately, appropriate controls to validate these results were not reported. In particular, there were no stage 14 normal precardiac mesoderm cultured alone or with non-endodermal tissues. The solitary explant controls are especially important to confirm that the explanted normal precardiac mesoderm (presumably stage 14) had not already been induced. The uncertainty arises because these authors mention that they often store embryos in the cold to retard development. We have noticed that some morphogenetic movements are retarded by cold storage, so that the gross criteria used for identifying embryonic stages may not match the developmental state of particular tissues. Thus, while the data are potentially interesting, it is impossible at present to draw definitive conclusions.

Once it had been shown that RNA was capable of correcting the mutant heart defect in our laboratory (Davis and Lemanski, 1987; Fransen et al., 1990; La France et al., 1992) and in Dr. Armstrong's (Smith and Armstrong, 1990), we undertook studies to clone and sequence the RNA present in normal anterior endoderm-conditioned medium.

To accomplish this, a cDNA library was constructed using random priming of the total RNA from conditioned medium which showed positive inductive activity. The library was screened with 32P labelled conditioned medium RNA. Hybridization positive clones were amplified and sequenced using Sanger's dideoxy chain termination method. The sequences then were compared to other known sequences in all available databases utilizing the Genetic Computer Group (GCG) DNA analysis package. We found so far that four of ten clones have homology with 28S rRNA from a variety of eukaryotic species and two are homologous to 18S rRNA. No significant homology was detected with the remaining four clones, suggesting the possibility that they may be products of distinct coding genes. The four unique clones contain open reading frames of 273, 186, 186 and 132 bases respectively and show no overlapping regions among themselves. These results indicate the presence of novel RNA species in the conditioned medium, which we are beginning to characterize further as described in the following example.

One of the four above mentioned unique clones was found to have an open reading frame with at least 90 amino acids. By RTPCR (reverse transcription of RNA and PCR amplication) using specific primer-pairs and probes, we have determined that this gene is expressed in different tissues/organs of juvenile axolotls. Interestingly, our results suggest that the level of expression may be tissue-specific (e.g., the expression is maximal in skeletal muscle and brain, followed by heart and lung, but barely detectable in liver). We are in the process of synthesizing a peptide (15 mer) in order to raise anti-peptide anti-

body in rabbits. The antibody, in turn, will be used to study subcellular distributions of the protein in different organs at embryonic stages by immunoelectron microscopy and confocal microscopy. Furthermore, we are in the process of examining whether the antisense oligonucleotide against this RNA has the ability to block heart induction activity of total conditioned medium RNA.

In summary, the cardiac mutant axolotl is proving to be a very interesting model for studying heart development. The mutant gene results in a failure of heart cells to form organized myofibrils and as a consequence, the heart fails to beat. Experiments have shown that mutant hearts can be "rescued" (i.e., turned into normally-contracting organs) by the addition of RNA purified from conditioned media produced by normal embryonic anterior endoderm-mesoderm cultures. These corrected hearts form myofibrils of normal morphology. New advances in recombinant DNA technology applied to this system should provide significant insights into the regulatory mechanisms of myofibrillogenesis as well as the inductive processes related to the control of gene expression during embryonic heart development. In a broader biological sense, the use of gene *c* in axolotls is potentially capable of helping to solve major unanswered questions in modern biology related to the genetic regulation of differentiation in vertebrates.

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