A Reevaluation of the Cardiac Mutant

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Introduction

In 1968, Rufus Humphrey reported discovery of a new axolotl mutation, which he named *cardiac (c)*. He followed this up with a more detailed study, published in 1972. On the basis of his work, he concluded that *cardiac* appeared to be a simple autosomal recessive trait affecting only heart development. At stage 35, when the heartbeat is normally established, no contractions were seen, and, as a consequence, the affected embryos developed a marked ascites, assuming a characteristic pear shape. Affected larvae never fed and died within a week of hatching.

About the same time, Jacobson and Duncan (1968) reported that, in the newt, heart induction involves an interaction with the pharyngeal endoderm and begins during neurulation. Recently, we have confirmed these results for the axolotl (Smith and Armstrong, 1990), but we have found that the induction seems to be complete by the end of neurulation (stg. 20), somewhat earlier than in the newt.

In one of his experiments, Humphrey (1972) reported that wild-type heart mesoderm did not form a beating heart when transplanted into a *c/c* embryo at late tailbud stages. He therefore suggested that the failure of *c/c* hearts to begin beating could be caused by either a failure of mutant anterior endoderm to induce the heart mesoderm, or by heart differentiation being inhibited by the surrounding mutant tissues.

In the converse experiment, Humphrey (1972) reported that *cardiac* mesoderm placed in a wild-type host began to beat normally. In subsequent studies, Lemanski and colleagues demonstrated that fully-formed (stage 35) mutant hearts would beat, usually within about 24 hr, when cultured with anterior (pharyngeal) endoderm from stage 28-30 wild-type embryos (Lemanski et al., 1979). As well, RNA-containing extracts of endoderm, also taken from stage 28-30 wild-type embryos, were capable of stimulating the onset of beating (Davis and Lemanski, 1987). Since, at the time, it was not appreciated that induction was over long before this stage, the results seemed consistent with the proposal that the *cardiac* mutation prevented the endoderm from providing a normal inductive signal. However, no direct test of which tissue was the focus of the mutation has been reported. At least in part, this was likely due to problems identifying, before they reached heart-beat stage, which embryos were mutant in a spawning of two heterozygotes. Our solution to this, reported below, is to remove the heart primordium from only one side of a stage 14 embryo [since each heart primordium can form a heart independently (Copenhaver, 1955)], or the mid-ventral endoderm, and then allow the embryo to heal and continue development to the heart-beat stage. This approach allows us to unequivocally conclude that it is the responding tissue, the mesoderm, rather than the inductive endoderm that is defective. A reevaluation of the published results is, therefore, warranted.

Methods

Wild-type and mutant embryos were obtained from spawnings between animals maintained at the University of Ottawa axolotl colony. Embryos were maintained in 25% modified Holtfreter's saline supplemented with 100 mg/L each penicillin and streptomycin sulfate. Surgery was performed, and explanted tissues were maintained as previously described (Smith and Armstrong, 1990). Heart mesoderm was removed from only one side of each potential *c/c* embryo. The embryo was then allowed to heal and develop until the mutant phenotype could be identified with certainty. Embryos from which inductive endoderm was partially removed were also allowed to heal and develop.

Results

We first tested the inductive ability of mutant endoderm by culturing a portion of it with uninduced stage 14 wild-type heart mesoderm. Pieces of mid-ventral pharyngeal endoderm were used in all cases. As shown in Table 1, wild-type heart mesoderm was found to be as capable of differentiating into beating tissue when induced by *c/c* endoderm as

Table 1. Inductive ability of mutant endoderm

Phenotype of endoderm	No. beating/Total (% ± SE)
wild-type	25/29 (86.2 ± 6.4)
cardiac	10/11 (90.9 ± 8.7)

Note: the mean time until the onset of beat was the same for both series.

when wild-type endoderm was used. Clearly, mutant anterior endoderm is fully capable of providing normal inductive signals.

Since the ability of mutant endoderm to induce myocardial differentiation is normal, the ability of *c/c* heart mesoderm to respond to normal induction was also examined. Heart area mesoderm was removed from one side of a series of embryos. Each mesodermal anlage was then placed in culture with a piece of endoderm from another donor. All embryos were from +/*c* X +/*c* spawnings and were maintained post-operatively to positively identify their phenotypes (see Methods). The results confirm that mutant endoderm is capable of inducing wild-type heart mesoderm (Table 2). Furthermore, they clearly demonstrate that *c/c* heart mesoderm is unable to respond to the normal inductive signals produced by wild-type anterior endoderm.

Table 2. Identification of defective mutant tissue

Phenotype		
Mesoderm	Endoderm	No. beating/Total (% ± SE) [a]
wt	wt	40/43 (93.0 ± 3.9)
wt	c	12/12 (100.0 ± 0.0)
c	wt	1/10 (10.0 ± 9.5) [b]
c	c	1/5 (20.0 ± 17.9) [b]

[a]Mean time to begin beating was the same for all series.
[b]A few cells were observed twitching for a short time.

Discussion

Clearly the *c* gene does not affect the inductive endoderm as previously suspected. Instead, the mutation directly affects the precardiac mesoderm, rendering it incapable of responding to normal inductive signals. How, then, does this affect interpretation of earlier results, especially those of Lemanski and coworkers?

Biochemical, ultrastructural, and immunocytochemical analyses of mutant hearts have shown that most of the contractile proteins are present, but are not organized into regular (functional) sarcomeric arrays (see, for example, Lemanski, 1978; Fuldner et al., 1984; Shen and Lemanski, 1989, and additional references cited therein). Some experiments suggest that tropomyosin is deficient (Starr et al., 1989). With the inductive failure model, one would be forced to say that most of the proteins were induced normally, and suppose that there was a separate induction of tropomyosin, and/or perhaps some other factor responsible for organizing the proteins into myofibrils and ultimately sarcomeres. However, if the induction is normal, one might propose that the organizing factor (perhaps tropomyosin) is, itself, altered by the mutation, or perhaps not produced as a conse-

quence of a mutated promoter or other control sequence.

What then of the rescue experiments of both Humphrey (1972) and Lemanski (Lemanski et al., 1979; Davis and Lemanski, 1987)? As the rescue appears to be mediated by an RNA, one might throw caution to the wind and suggest that it is the mRNA for the defective component, but it is not a proposal we like very much, nor one that explains why a fully induced wild-type heart should be inhibited when transplanted into a mutant (Humphrey, 1972; Smith, 1990). What we would like to suggest, instead, is that the organization of the contractile proteins is controlled by a reaction-diffusion system of activator and inhibitor.

We had suggested such a model previously (Armstrong, 1989), but at that time did not have the data showing that the focus of the mutation lay in the mesoderm. In brief, what this model proposes is that the mesoderm responds to induction by setting up a two component reaction-diffusion system that partitions the heart field into heart-forming and non-heart regions. Within the heart-forming region, one of the components (probably the activator)[1] promotes synthesis of an organizing factor (perhaps tropomyosin, based on Lemanski's data) which causes sarcomeric arrays to organize. Our reasons for favoring such a model, other than that we can fit most of the present data to it, have been discussed previously in this Newsletter (Armstrong, 1989).

The defect, then, would lie in the setting up of the reaction-diffusion system, which is still a mesodermal function, rather than in some structural component of the sarcomere. It could be that the activator is altered by the mutation, or is not produced in adequate amounts. Conversely, the inhibitor could be overproduced, or not be degraded fast enough. Since Lemanski finds the other major contractile proteins to be present in *cardiac*, one would have to assume that they are not controlled by the reaction-diffusion system, but by some other mechanism such as the endodermal induction.

How does the model explain the rescue experiments? Let us suppose that the inhibitor is overproduced. Reaction-diffusion inhibitors have short 1/2-lives and diffuse rapidly, so when Humphrey transplanted a *cardiac* heart into a wild-type host, he would have been surrounding this tissue with a very effective sink into which the inhibitor could diffuse, thus creating a more normal activator/inhibitor balance in the heart itself. Lemanski's explant experiment, where the *cardiac* heart was rescued by wild-type endoderm, might be explained the same way. In the converse experiment of a wild-type heart into a mutant, the higher-than-normal inhibitor levels in the surrounding heart field tissue would be expected to suppress activation in the heart.

What about Davis and Lemanski's (1987) RNA experiments? One possibility is that the RNA is the activator, and when enough is added exogenously, it overrides the inhibition. Another is that it is something that, perhaps quite accidentally, binds to and inactivates the inhibitor. Of course the former is the more exciting possibility, and we hope it is the correct one.

Cardiac remains a fascinating puzzle, slow to give up its secrets. What is the prognosis for future research? The RNA story obviously needs to be pursued. In particular, its distribution in the embryo needs to be determined, as that should help to establish which model is correct. Our prediction is that it would be found in highest concentration in the heart itself, but a contrary finding would not make it less interesting, or necessarily rule out a reaction-diffusion model. However, all the correlations in the world will probably not make converts out of the skeptics. We need to isolate and characterize the putative activator (should it be something other than the RNA), and especially the inhibitor. The latter will not be easy, given the prediction (inherent in a reaction-diffusion model) that it will be a small molecule with a short 1/2-life that is likely active at very low concentrations.

For further reading on reaction-diffusion models, the reader is referred to Harrison (1987) for a general review, and Meinhart (1989) for some variations on the basic scheme. In addition, Geier and Meinhardt's (1972) original model for explaining patterning in hydra remains a 'must'.

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[1]The "activator' is called such because it activates production of both itself and the inhibitor, while the 'inhibitor' inhibits production of activator. In principle, either or both could be morphogens (Harrison, 1987).

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