Heart Induction in the Cardiac Mutant Axolotl: A Reply

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In the last issue of the *Axolotl Newsletter*, Lemanski and coworkers (1992) presented their interpretation that the cardiac mutation (*c*) in the axolotl leads to an inductive failure. Those who have followed the recent literature on cardiac undoubtedly know that we have presented evidence that, instead, it is the heart-forming mesoderm that fails to respond to a correct inductive signal (Smith and Armstrong 1991a, b). (We have also proposed a reaction-diffusion mechanism for the control of heart development, but whether or not our model is correct does not affect the basic dispute and will not be discussed here.)

What we will do here is go over some old and new data in the hope of being more convincing. However, before we begin, we wish it to be clearly understood that the ultimate effect of the *c* mutation is not in dispute. Lemanski's previous work has demonstrated quite convincingly that mutant myocardium fails to form organized sarcomeres. The question is: Is the lack of myofibrillogenesis due to inductive failure (as Lemanski and colleagues maintain), or is it caused by a failure of the heart mesoderm to respond to normal inductive influences (as we have proposed)?

***In Vitro* Evidence.** In our last report in the Axolotl Newsletter (1991a), and in a subsequent formal report (Smith and Armstrong 1991b), we showed that, when cultured with wild-type endoderm, normal heart mesoderm forms functional myocardial tissue in the majority of cases. More importantly, we also demonstrated that +/+ heart mesoderm began beating in all cases when cultured with *mutant* inductive endoderm.

Lemanski and colleagues apparently feel that there is some uncertainty in these results, since "... appropriate controls ... were not reported..." (Lemanski et al. 1992). However, the controls in question (mesoderm from stage 14 wild-type embryos cultured alone) were reported in our first study, and we felt it unnecessary to report them again (Smith and Armstrong 1990, Figure 1). In this study, we showed quite clearly that, when cultured alone, stage 14 heart mesoderm began beating in only 19% of cases. Furthermore, those which began beating took an average of 7.7 days to begin (Heart Differentiation Coefficient = 7).

In comparison, stage 14 wild-type mesoderm began beating in 86-100% of cases when cultured with endoderm from either wild-type OR *c/c* embryos (Smith and Armstrong 1991a, b). These cultures also took less time to begin beating (between 4.9 and 6.4 days with +/+ endoderm, HDC = 44-62; and 4.9 days with *c/c* endoderm, HDC = 61). Clearly, the wild-type heart mesoderm in our studies could not have already been induced, as Lemanski and coworkers suggest. Therefore, mutant anterior endoderm MUST be as inductive as its wild-type counterpart.

Even more important is our report that stage 14 *c/c* heart mesoderm almost never began beating when cultured with wild-type inductive endoderm (1/10 cases, 13 days to begin beating; HDC = 2). We believe that this result, more than any other, clearly shows that the *cardiac* mutation impairs the ability of the mesoderm to respond to a normal inductive signal.

The Humphrey Transplants. Lemanski's argument is based largely on Humphrey's (1972) work, in which he transplanted wild-type hearts into mutant hosts, and *vice versa*. When Humphrey transplanted mutant heart primordia into normal recipients at stages 29-30, beating hearts formed. When normal heart mesoderm was transplanted into mutant hosts, heartbeat was not observed. One interpretation of this result is that the mutant endoderm fails to induce the mesoderm, and that mutant heart mesoderm is induced by contact with normal endoderm.

However, these operations were performed at considerably later stages than when heart induction normally occurs in the axolotl (see Smith and Armstrong 1990). Therefore, Humphrey must have transplanted *fully induced wild-type mesoderm* into his *c/c* hosts—mesoderm which should have been capable of forming tissue *in vitro*, but which did not in the mutant environment. Seen in this light, Humphrey's results suggest strongly that

there must be an inhibitor of cardiomyocyte differentiation present in mutant embryos (an alternative proposed by him). But, if it is indeed present, what is the source of this inhibitor?

Transplantation of Heart Mesoderm in Stage 20 Embryos. Our *in vitro* results suggest that heart mesoderm is directly affected by the *c* gene. If true, then (Humphrey notwithstanding) replacing mutant heart tissue with wild-type mesoderm should correct the defect. We tested this prediction by replacing the heart mesoderm in a series of embryos from several *c*/+ X *c*/+ spawnings. Since we have shown that the induction is essentially complete by stage 20, we replaced both heart areas with tissue from known wild-type embryos at this stage. Since mutant heart mesoderm does not begin beating in culture, but wild-type mesoderm usually does (Smith and Armstrong 1990), we were able to determine the phenotypes of the hosts by maintaining their heart mesoderms in culture.

As predicted, beating hearts developed in most of the recipient embryos, including those identified as mutant by explants of their heart mesoderm (Table 1). As well, circulation commenced in most of these embryos, and the secondary characteristics associated with the mutant phenotype were not observed in these embryos. There was no edema, and all of the mutant embryos containing wild-type heart with mesoderm had large, well-developed gills, normal pigmentation patterns, and apparently normal heads. In fact, only one mutant characteristic remained in such "rescued" larvae (see accompanying paper).

In another series of transplants performed at stage 20, only one of the two primordia was replaced. Both donor and host embryos were from *c*/+ X *c*/+ spawnings. Mutant recipients were identified by explanting one heart primordium, and mutant donors were identified by allowing them to heal and continue developing (see Smith and Armstrong 1991). Most of these embryos also formed beating hearts and developed circulation, including those which contained both mutant and wild-type heart primordia (Table 2).

Interestingly, the presence of only one wild-type heart primordium is sufficient to stimulate the formation of functional myocardial tissue. This corroborates our previous report that wild-type heart mesoderm can stimulate the formation of beating tissue by mutant mesoderm *in vitro* (Smith and Armstrong 1991a, b). Furthermore, the stimulation is *post-inductive*, occurring after the induction is essentially complete (stage 20; Smith and Armstrong 1990).

Finally, our results do not support the contention that further induction is necessary after stage 20, since replacing both (or even one) of the two heart primordia at stage 20 was sufficient to stimulate the formation of beating hearts in mutant embryos. Alternatively, if further inductive influences are required, *c*/*c* tissues must be able to supply them.

Table 1. Bilateral Transplantations of Heart Mesoderm

Host Phenotype[a]	Number of Cases	Number with Beating Heart	Number with Circulation
wt	33	31	28
c	7	6	6

[a] wt = wild-type (+/*c* and +/+); c = mutant (*c*/*c*).

Admittedly, these results alone cannot tell us whether the endoderm or the mesoderm is defective in *cardiac* (even if it does agree with the prediction from our explant cultures). If the induction is essentially complete by stage 20, and we replace *c*/*c* heart mesoderm with wild type tissue at that stage or later, it could be argued that we are simply bypassing the induction. However, if this were true, why didn't Humphrey's transplants (done at even later stages) work?

Humphrey Revisited. The results shown above appear to contradict those reported by Humphrey (1972). Therefore, in addition to

Table 2. Unilateral Transplantations of Heart Mesoderm

Phenotype[a] Donor	Host	Number of Cases	Number with Beating Heart	Number with Circulation
wt	wt	35	35	32
wt	c	7	6	2
c	wt	13	13	8
c	c	1	0	0

[a] wt = wild-type (+/*c* and +/+); c = mutant (*c*/*c*).

transplanting stage 20 heart mesoderm, we also transplanted heart tissue at the later stages examined by Humphrey (stage 29). At this point, it must be noted that Humphrey reports that he transplanted the area of heart mesoderm defined by Copenhaver (1955). However, Copenhaver delineates two areas of heart mesoderm: the heart-forming mesoderm (which actually forms the heart under normal circumstances), and the wider area of heart-field mesoderm (which does not normally contribute to heart tissue, but has the capability to do so when the heart-forming region is extirpated). Humphrey did not specify which of these areas he transplanted.

In an attempt to verify Humphrey's results, we first replaced the entire area of heart field mesoderm in stage 29 embryos (from *c*/+ X *c*/+ spawnings) with tissue from known wild-type donors. The phenotypes of the recipients were unequivocally identified by maintaining their heart-field mesoderm in culture. In contrast to Humphrey's result, beating hearts formed in all mutant hosts (N=8), as well as in control (wild-type into wild-type) embryos (N=23).

However, when only the actual heart-forming mesoderm (the tissue between, and posterior to, the tips of the mandibular arches, as defined by Copenhaver, 1955) was transplanted in stage 29 embryos, beating hearts did not form in 8 of 31 cases (25.8%). Unfortunately, we could not confirm from explants that all 8 were definitely *c*/*c*, as this smaller piece of tissue frequently did not maintain its integrity in culture. However, the proportion corresponds well to that expected for a spawning of two heterozygotes, and, more importantly, is consistent with Humphrey's (1972) results.

These results have important implications for interpreting Humphrey's (and later) studies. First, our transplants suggest that Humphrey transplanted only the heart-forming region, and not the entire heart field. Since beating tissue does not form when only the narrower area of mesoderm is replaced with (fully induced) heart tissue, we must again conclude that the wild-type tissue is inhibited in the mutant milieu. But now, we have some indication as to the source of the inhibition. Beating tissue did not form when heart-forming tissue was replaced, but did when the entire field was replaced. This strongly suggests that the inhibition is present *within the heart-field mesoderm itself.*

Our work also requires that Hill and Lemanski's (1979) findings be reinterpreted. They reported that mutant hearts did not begin beating when placed in organ culture. While originally interpreted as indicating that no inhibitor was present, it is now clear that the inhibitor is in the heart tissue itself. Thus, merely removing the hearts from the mutant environment could not, and did not, reverse the inhibition.

The RNA Story. What is the role of the RNA discovered by Davis and Lemanski (1978)? We agree that something in these preparations is capable of stimulating myofibrillogenesis in fully-formed *c*/*c* hearts. However, we doubt that the RNA preparations contain the inducer for a number of reasons.

First, we have shown that mutant endoderm is inductive. This is corroborated by the fact that hearts (although nonfunctional) form in mutant embryos. If the heart mesoderm remained uninduced in *c*/*c* embryos, as Lemanski maintains, we think it more likely that hearts would not form at all, or that they would be composed of naive, multipotent (*i.e.*

uninduced) mesodermal cells. However, Lemanski's group reports that mutant heart cells contain most or all of the contractile proteins characteristic of differentiated (*i.e.* induced) cardiomyocytes (Fuldner et al. 1984, Lemanski 1976, 1978, Lemanski et al. 1976; 1980, Shen and Lemanski 1989, Starr et al. 1989). Only the final phase of cardiomyocyte differentiation (myofibrillogenesis) is abnormal.

As Lemanski et al. (1992) mention, we confirmed their reports that RNA extracts stimulated the formation of beating tissue in mutant hearts. However, in that same study we also reported that the same RNA preparations did not induce stage 14 wild-type heart mesoderm to form beating tissue (Smith and Armstrong 1990, Table 3). This clearly indicates that the RNA preparation cannot contain the inducer.

We also doubt that anterior endoderm is the source of the active substance in the RNA preparations. As we have shown, normal stage 14 endoderm does not stimulate the formation of beating tissue by stage 14 *c/c* mesoderm *in vitro*, even when the tissues remain in contact for up to two weeks. Furthermore, RNA extracts from neurula-stage endoderm are less effective than those from stage 29 endoderm. And *c/c* endoderm clearly cannot stimulate the onset of beating in mutant hearts, even though it is fully inductive.

On the other hand, we have shown both *in vitro* and *in vivo* that induced wild-type mesoderm can stimulate the formation of beating tissue by *c/c* tissue, as has Lemanski's group (Fransen et al. 1990, Lemanski et al. 1992). Therefore, we feel that the active principle in Davis and Lemanski's (1987) extracts is normally produced by the heart mesoderm itself, after it has been induced. We also believe that the factor is diffusible (hence the ability to condition medium). Thus, tissue which has had prolonged, intimate contact with normal induced heart mesoderm (stage 29 endoderm) would be expected to contain some of the active factor, whereas tissue without such a history (stage 14 wild-type endoderm; *c/c* endoderm from any stage) would not. This is the only explanation which we can envisage which will explain all of the apparently contradictory results.

However, while Lemanski's group may not have isolated the inducer, they may well have another factor (produced by induced heart mesoderm) which is important for controlling the final phase of cardiomyocyte differentiation—myofibrillogenesis. As well, the report that this factor is found in a species as far removed as sheep (La France et al. 1989, Lemanski et al. 1992) opens the exciting possibility that this is a generalized factor responsible for controlling cardiomyocyte differentiation in most or all vertebrates.

At present, we cannot say with certainty what the function of this factor may be, but we suggest that it may either be necessary for actively stimulating myofibrillogenesis (thus we have termed it the "activator"), or for binding or otherwise removing or degrading the excess inhibitor present in mutant heart tissue.

In conclusion, we suggest that we often speak too casually about heart induction, as though it were a single event. Even if we restrict our discussion to the events necessary to get a spontaneous beat (the end-point most of us use in our assays), we are talking about a complex series of events which may require more than one step. Thus, we feel that the Lemanski group's continued elucidation of the identity and role of a factor which is involved in the control post-inductive cardiomyocyte differentiation is very important, even if it is not the long-sought heart inducer.

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