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An inductive role for the endoderm in *Xenopus* cardiogenesis

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SUMMARY

Heart induction in *Xenopus* has been thought to be dependent primarily on the interaction of the heart primordia with the Spemann organizer. We demonstrate, however, that signals derived from the deep dorsoanterior endoderm during early gastrulation are also essential for heart formation. The presence of deep endoderm dramatically enhances heart formation in explants of heart primordia, both in the presence and absence of organizer. Likewise, extirpation of the entire endoderm can decrease the frequency of heart formation in embryos that retain organizer activity. Finally, we show that the combined presence of both endoderm and organizer is necessary and sufficient to induce heart in ventral mesoderm explants that would not otherwise form heart tissue. *Xenopus* heart induction, therefore, may be a multistep process requiring separate dorsalization and cardiogenic signalling events. This is the first demonstration of a heart-inducing role for the endoderm in *Xenopus*, indicating that the mechanism of heart formation may be similar in most vertebrates.

Key words: endoderm, heart induction, organizer, *Xenopus laevis*

INTRODUCTION

In the vertebrate embryo, the heart arises from paired regions of dorsolateral mesoderm which are thought to be specified by inductive interactions with neighboring tissues during early development (for review, see Jacobson and Sater, 1988). The heart primordia migrate to a common anterioventral destination where cardiomyocyte differentiation occurs. The fused heart rudiments will form a primitive tube which eventually gives rise to the looped, multichambered morphology of the mature beating heart.

In the anuran amphibian, *Xenopus laevis*, the two regions of presumptive heart-forming mesoderm flank the Spemann organizer (Keller, 1976; Sater and Jacobson, 1990). The organizer, defined as the dorsal 60° of marginal zone (Stewart and Gerhart, 1990), is believed to specify dorsoventral axis configuration in the frog. The induction of cardiac mesoderm has been attributed to interaction of the heart primordia with the organizer during gastrulation, a contention supported by observations that correlate heart formation with the development of dorsoanterior characteristics. For example, embryos that have been manipulated to develop excessive dorsoanterior structures by LiCl treatment often have large, radial hearts (Kao and Elinson, 1988). Likewise, UV-treatment results in ventralized embryos with reduced or absent heart formation (Scharf and Gerhart, 1983). Recent experiments also directly implicate the Spemann organizer in heart induction (Sater and Jacobson, 1990). Inclusion of the intervening organizer region in explants of heart primordia from early gastrulae can support beating heart formation in culture (albeit poorly), while extirpation of the organizer region at the onset of gastrulation can prevent heart formation. Furthermore, in some cases, organizer grafts to the ventral side of a host embryo that induce a second axis can also lead to the induction of a second heart associated with the duplicate axis. Thus, *Xenopus* heart induction appears to be dependent to some extent on the activity of the Spemann organizer. However, the poor ability of the organizer to induce hearts in explants isolated from early gastrula (stage 10), suggests that the mechanism of cardiogenesis in *Xenopus* involves additional inductive signals.

In other vertebrates, the endoderm has been shown to be a likely source of heart-inducing signals. In urodele amphibians, the inductive capacity of the endoderm has been most clearly defined due to the accessibility of the embryonic tissues and ease of explant culture. The anterior (or pharyngeal) endoderm in urodeles is in direct apposition to the dorsolateral regions of cardiogenic mesoderm long before their commitment to form heart (Holtfreter, 1938). Several lines of experimentation suggest that this endoderm possesses heart-inducing activity. Jacobson (1960, 1961) and others (Balinsky, 1939; Nieuwkoop, 1947; Chuang and Tseng, 1957) showed that removal of the entire endoderm at early neurula stages prevents heart formation in several urodele species. If the endoderm is removed at successively later stages, however, both the frequency and extent of heart formation observed in the operated embryos increases, indicating that the completion of heart specification occurs during this period. Furthermore, the inclusion of anterior, but not posterior, endoderm increases the rate, frequency and complexity of hearts observed in explant cultures of primordia from newt and salamander neurulae, and compensates for the ablation of endoderm in whole embryos, suggesting that heart-inducing potency is located in the anterior endoderm in these species (Stohr, 1924; Jacobson and Duncan, 1968; Fullilove, 1970). Although the endodermal role in heart induction is most clearly defined in urodeles, endodermal tissue has also been implicated in the specification of heart

mesoderm in other vertebrates, including avian and mammalian species (Orts-Llorca, 1963; Orts-Llorca and Gil, 1965; DeHaan, 1965; Hommes, 1957). In contrast, a heart-inducing role for the organizer in urodeles, or the analogous organizer-like tissues in other vertebrates (i.e Hensen's node, primitive streak) has been neither proposed nor investigated. Thus, heart induction in most vertebrates is thought to be primarily dependent on signals from the endoderm.

In *Xenopus*, the role of the endoderm in heart induction is unclear. As in other amphibians, endoderm tissues are in intimate association with the heart primordia during the specification of heart mesoderm and are therefore a possible source of inducing signals. Unlike urodeles, the pharyngeal endoderm in *Xenopus* arises from the overlying superficial layer of the marginal zone. It is known that this tissue can be ablated with no effect on heart formation (Sater and Jacobson, 1989). In contrast, the potential role of the deep dorsoanterior endoderm, which lies beneath the heart mesoderm, has not been determined. Presumably, this is because endodermal and mesodermal tissue layers do not become entirely distinct in this species until after the heart mesoderm has become specified (Nieuwkoop and Faber, 1967). However, it is possible to approximate a boundary between the involuting marginal zone and the deep endoderm cells during gastrulation by utilizing the results of fate-mapping studies and differences in cell morphology (see Hausen and Riebesell, 1991). In order to investigate the potential role of the endoderm in *Xenopus* heart induction, we therefore compared combinations of tissues containing few endoderm cells to those in which a substantial mass of the adjacent deep endoderm was deliberately included, and assayed for the formation of a beating heart. We demonstrate that *Xenopus* dorsoanterior endoderm is the source of an essential heart-inducing activity at early gastrula stages, and plays a direct role in heart induction, both in vivo and in explant culture. In addition, we show that the organizer is capable of inducing heart in non-heart-forming (ventral) mesoderm only in the presence of substantial amounts of endoderm tissue. Furthermore, the ability of the endoderm to induce beating heart in *Xenopus* is facilitated by prior or simultaneous organizer influences. Our results suggest a multistep process of heart induction in *Xenopus* in which dorsalized mesoderm responds to direct heart-inducing signal(s) from the deep dorsoanterior endoderm.

MATERIALS AND METHODS

Embryos

Xenopus laevis embryos were obtained by in vitro fertilization as described (Smith and Slack, 1983). Immediately before use, embryos were dejellied in 2% cysteine-HCl (pH 7.8), washed and maintained in 0.1× Marc's modified Ringer's solution (MMR; Peng, 1991). The rate of development was controlled by varying the temperature at which pools of embryos were cultured (14-22°C) in order to most efficiently supply embryos at particular stages. Embryos were staged according to Nieuwkoop and Faber (1967).

Microdissection

All microdissection was performed in sterile 0.75× MMR on a bed of 1% agarose. Watchmaker's forceps and eyebrow/eyelash knives were used for all microsurgical manipulations. Explants were taken from various regions of marginal zone around the circumference of early gastrulae (stage 10 or stage 10.5) as diagrammed in Results (Figs 1, 2, 4, 6). The location of the dorsal midline was estimated from the midpoint of the pigment line which precedes dorsal lip formation (stage 10), or from the midpoint of the blastopore groove itself (stage 10.5). The ventral midline was taken to be 180° opposite the dorsal midline. In explants that included deep endoderm, a small mass of the associated endoderm cells was left attached to the isolated marginal zone tissue. In explants that did not include endoderm, care was taken to gently scrape the larger endoderm cells away from the more tightly associated mesodermal cells, without disturbing the head or axial mesoderm. A flap of ectoderm covering most of the explant was included to promote healing (for example, see Fig. 1A). Explants were allowed to heal for at least 3 hours in sterile 0.75× MMR in small round agarose wells before being transferred to 24-well culture dishes containing sterile 0.75× MMR supplemented with 25 μg/ml gentamycin sulphate.

Lineage labelling and histology

For lineage labelling, donor embryos were injected at the 2-cell stage with 10 ng (in 2 nl) of rhodamine-conjugated lysinated dextran (RLDx, M_r 10×10^3; Molecular Probes) and cultured until use in 0.1× MMR. For routine histology, embryos and explants were fixed in MEMFA (Harland, 1991) for 1-2 hours at 4°C, rinsed in 70% PBS, dehydrated through an ethanol series and embedded in JB-4 resin (Polysciences). 8-10 μm sections were cut and collected on coated glass slides (Superfrost plus, Fisher). Fluorescently labelled sections were mounted with 80% glycerol containing 4% N-propyl gallate (Sigma) and photographed using epifluorescence illumination.

Statistics

Explants from each trial were classified as having beating hearts or failing to undergo heart formation after 7 days. The homogeneity of contingency tables tabulated from different trials was analyzed by a conditional exact test, calculating the probabilities using the Tribo StatXact computer package (Cytel, Cambridge, MA). If the results from different experiments were judged to be not significantly different (exact test: $P\geq0.05$), they were pooled and expressed as a percentage of total cases (n). Where the data was not significantly homogeneous (exact test: $P<0.05$), the results of each trial are shown in table format.

RESULTS

Inclusion of endoderm in dorsal marginal zone explants increases frequency of heart formation

Previous studies (Sater and Jacobson, 1990), which defined the heart-inducing role of the Spemann organizer during gastrulation, employed explant culture of regions of the dorsal marginal zone (DMZ). These explants may have included an indefinite number of deep endoderm cells due to the incomplete distinction of endoderm and mesoderm at this stage of development (A. Sater, personal communication). The potential influence of the endoderm on heart formation is therefore unclear from these studies.

To examine the role of the deep dorsoanterior endoderm in the specification of heart mesoderm, we utilized similar explant culture experiments, with slight modifications (see Fig. 1A). We explanted the dorsal 150° of the marginal zone from early gastrula stage embryos, 75° on either side of the dorsal midline. These DMZ explants included both the Spemann organizer, a 60° arc centered at the dorsal midline, (30° to each side), and the two flanking regions of dorsolateral mesoderm, (30°-75°). Although the heart primordia are minimally defined as the dor-

solateral 30°-45° (Sater and Jacobson, 1990), larger explants were used in our experiments. We have found these tissues to have heart-forming potential similar to the smaller regions extirpated in previous experiments but, in our hands, the larger explants survive longer in culture, facilitating a more thorough analysis of complete heart-forming capacity.

Incisions were made from the vegetal aspect of the embryo to retain a substantial mass of deep endodermal cells (Fig. 1A). This extra deep endoderm tissue was either left attached to the explant, or gently scraped away to leave as few deep endoderm cells as possible associated with the marginal zone. Isolated explants were cultured and monitored daily for the formation of beating hearts over a period of 7 days. During this time, the explants gradually became transparent and rhythmically beating heart tissue was easily distinguished with a dissecting microscope.

The results are summarized in Fig. 1B. Explants removed at stage 10 with only minimal deep endoderm cells developed hearts at a frequency of 24% of total cases (n=92). In contrast, 98% (n=55) of the stage 10 DMZ explants that included a substantial amount of deep endoderm formed beating hearts. At stage 10.5, the frequency of explants that formed beating hearts remained high (>95%) whether or not endoderm was included in the explant, indicating that the effect of the endoderm is irrelevant by stage 10.5.

Endoderm alone induces heart formation in explants of heart primordia

The above experiments reveal the ability of the deep dorsoanterior endoderm to induce heart in the presence of organizer. We then investigated the ability of the endoderm to induce heart in the absence of simultaneous organizer influences. Previous work by Gerhart and co-workers delimited the organizer to the dorsal-most 60° of the marginal zone (Stewart and Gerhart, 1990). To remove organizer signalling, DMZ explants were excised as above, except that the dorsal 60° was then removed from the explanted marginal zone, as diagrammed in Fig. 2A. The two heart primordia were left connected by a small bridge of ectoderm, preserving their relative spatial orientation. These bridged primordia explants were then allowed to heal with or without associated deep endoderm.

The results of these experiments are shown in Fig. 2B and Table 1. In the absence of interactions with organizer and substantial amounts of anterior endoderm, the presumptive heart primordia never formed beating hearts when explanted at stage 10 (n=34). These explants rarely formed dorsoanterior structures and were predominantly composed of skeletal muscle and pronephric tissue. Inclusion of endoderm, however, resulted in heart formation at a frequency of 46% (n=68) of cases. The hearts observed were morphologically complex, often with three chambers. Although it is possible that these explants included some organizer cells, we note that, with or without endoderm, they rarely developed dorsoanterior characteristics such as pigmented retinal epithelium or cement glands, suggesting that most organizer activity is absent (Fig. 3). The inclusion of deep endoderm appeared sufficient to induce heart in stage 10 dorsolateral mesoderm, with little or no organizer influence.

Stage 10.5 bridged primordia explants, lacking both organizer and endoderm tissue, formed hearts in an average

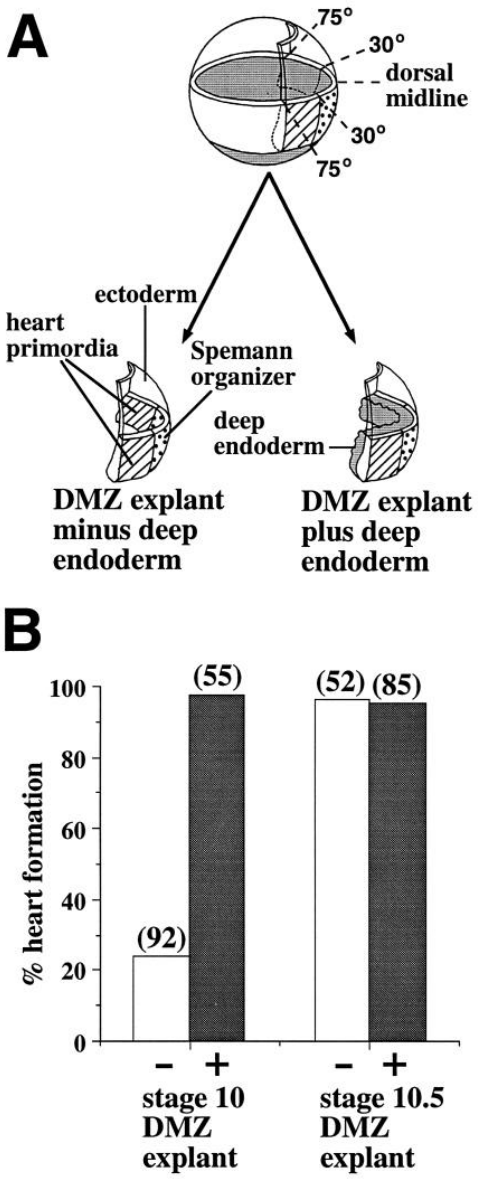

Fig. 1. The deep dorsoanterior endoderm enhances heart formation in explants of dorsal marginal zone (DMZ). (A) Regions of the DMZ were explanted which encompassed the dorsal 150° of marginal zone, and included the Spemann organizer (30° to each side of the dorsal midline; stippled), lateral mesoderm including the two heart primordia regions (30° to 75° lateral to the dorsal midline; hatched), and substantial amounts of the underlying deep endoderm (shaded). The endoderm was either left associated with the explant or removed after explantation. A flap of dorsal ectoderm (depicted as transparent for clarity) was also included to promote healing. Explants were cultured for 7 days and scored for the formation of a beating heart. (B) The effect of deep endoderm on heart formation in DMZ tissues explanted at stage 10 and stage 10.5 is presented as a percentage of total cases undergoing heart formation. The results from all trials were pooled to construct the histogram, since the data from individual experiments were judged to be not significantly different by a conditional exact test calculating the probabilities using the Turbo StatXact computer package (exact test: $P \geq 0.05$; see Materials and Methods). The number in parentheses, (n), represents the total number of explants. + and – refer to presence or absence of deep endoderm, respectively.

of 26% (*n*=49) of cases, indicating that stage 10.5 primordia are, to some extent, already specified to form heart. Although the range of experimental variability in these particular explants was considerably broad (see Table 1), inclusion of anterior endoderm consistently enhanced heart formation (78%; *n*=49). Thus endoderm alone, in the absence of organizer, is sufficient to increase the frequency of heart formation in these tissues to the majority of cases, suggesting that the dependence on organizer activity is diminished by stage 10.5.

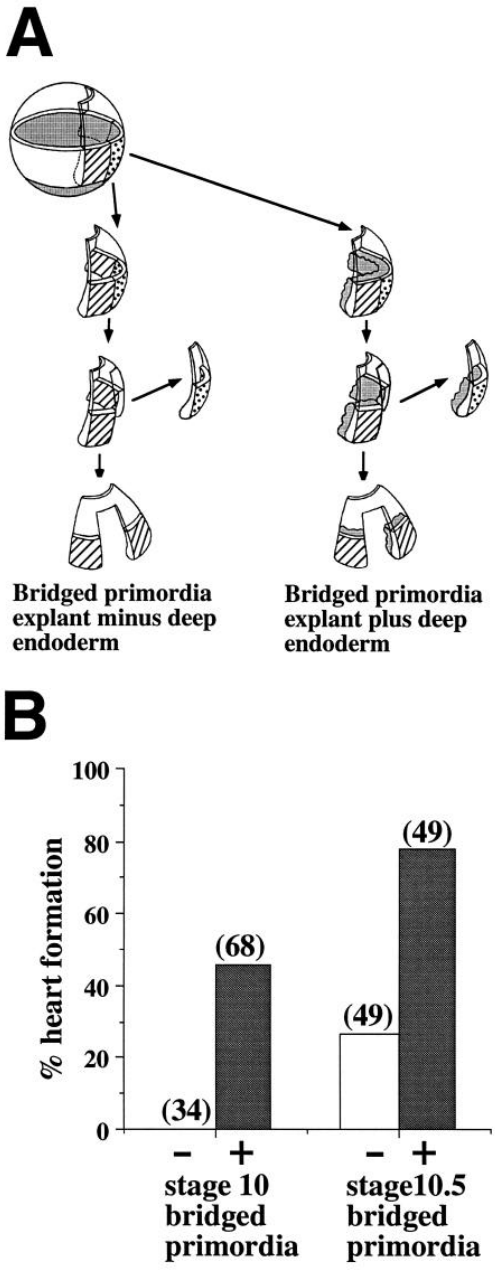

Fig. 2. The deep endoderm can induce beating heart in the absence of organizer. (A) Bridged primordia explants were made by explanting regions of the DMZ as in Fig. 1A, and subsequently removing the organizer region (dorsal 60° of marginal zone) from the excised tissue, while leaving the two primordia connected by a narrow bridge of ectoderm. Explants were cultured with their associated endoderm or deprived of deep endoderm tissue. (B) The effect of deep endoderm on the frequency of heart formation in bridged primordia was assessed in explant culture. The results of different trials were analyzed statistically, and some were found to be not significantly homogeneous (exact test: $P<0.05$; see Table 1). For consistency, the data from individual experiments have been pooled and represented here as a percentage of total explants with beating hearts as in Fig. 1B. Number in parentheses (*n*) represents the total number of explants. + and – indicate the presence or absence of deep endoderm, respectively.

Endoderm deficiency reduces heart formation in early gastrulae

The above experiments indicate that the adjacent deep endoderm, like the organizer, can have a direct inducing influence on presumptive heart primordia. To define when the endoderm signal is conveyed in vivo, we extirpated the entire endoderm from whole embryos at various stages and monitored them for heart development. In order to assure that as much endoderm as possible was removed, stage 10 or stage 10.5 gastrulae were partially bisected along their ventral midline (Fig. 4A). The deep endoderm was gently scraped away from the two halves of the marginal zone accessible through the large incision. The resultant endoderm-deficient embryos healed as the ectoderm halves fused, and the marginal zone tissue invaginated into the cavity left by the operation.

The results of these manipulations are shown in Fig. 4B. When the endoderm was removed at stage 10, only 25% (*n*=20) of these embryos had beating heart tissue. In contrast, when the endoderm was removed at stage 10.5, 83% (*n*=18) of the resultant embryos possessed beating hearts. Regardless of the developmental stage at which the operation was performed, the embryos developed fairly normal axes with well-defined dorsoanterior structures (Fig. 5). Control bisected embryos, which retained endoderm, developed normally and all possessed beating hearts (Fig. 5A). However, the endoderm-deficient embryos lacked much of the gut and associated structures (Fig. 5B,C). These results suggest that the endoderm is crucial for heart induction in vivo before stage 10.5, and is unnecessary thereafter.

The Spemann organizer requires endoderm to induce hearts in non-heart-forming ventral tissues

The results above suggest that endoderm provides an essential signal for heart induction before stage 10.5. However, grafts of organizer tissue, transplanted to the ventral side of gastrula stage embryos, frequently induce a second axis that contains a second heart or shares an enlarged heart with the primary axis (Sater and Jacobson, 1990). One explanation for the organizer's apparent capacity to induce heart in this experiment, without the influence of the dorsoanterior endoderm, is that the transplanted organizer acts in conjunction with adjacent ventral endoderm to induce heart tissue. To evaluate potential interactions between the organizer, endoderm and ventral marginal zone (VMZ) under these circumstances, we tested the ability of explants of organizer to induce heart in isolated ventral tissues.

Small explants of donor organizer tissue (15° to either side of the dorsal midline), from which the deep endoderm was removed, were implanted into unlabelled VMZ tissue comprising 75° on either side of the ventral midline (see Fig. 6A). To distinguish endodermal effects, they were made in the presence or absence of the ventrovegetal endoderm. These

Table 1. Effect of endoderm on heart formation in bridged primordia explants

	Stage 10 bridged primordia						Stage 10.5 bridged primordia					
	Minus endoderm			Plus endoderm			Minus endoderm			Plus endoderm		
Expt.	Hearts	No hearts	Total	Hearts	No hearts	Total	Hearts	No hearts	Total	Hearts	No hearts	Total
1	0	12	12	6	6	12	7	4	11	13	0	13
2	0	11	11	3	10	13	0	8	8	8	3	11
3	0	11	11	13	7	20	0	12	12	7	4	11
4				7	4	11	0	2	2	10	4	14
5				2	10	12	0	8	8			
6							6	2	8			
Totals	0	34	34	31	37	68	13	36	49	38	11	49

recombinants were constructed to recreate the interactions that occur in the ventral side of a host embryo during an organizer transplant graft.

Hearts were never observed in the resultant embryoids when endoderm was not included in the explant recombinants (*n*=29, Fig. 6B), although extensive dorsoanterior axial organization was evident. Even in the presence of the associated ventral endoderm, beating heart formation was rarely observed (2%, *n*=85). The low percentage of heart formation in these experiments may indicate that the in vitro transplants lack an essential heart-inducing factor.

We also investigated whether the addition of dorsoanterior endoderm could contribute to heart formation in VMZ recombinants. Organizer explants were combined with VMZ tissues as described above, except that the organizer components retained substantial dorsoanterior deep endoderm (see Fig. 6A). These recombinants displayed a dramatically increased ability to form hearts. Over 30% of the recombinants containing both dorsoanterior and ventral endoderm had beating heart tissue (*n*=21, Fig. 6B). Lineage labelling of the organizer donor embryo with RLDx (see Materials and Methods) revealed that ventral tissues were induced to contribute to the heart tissue in these recombinants (Fig. 7). These results

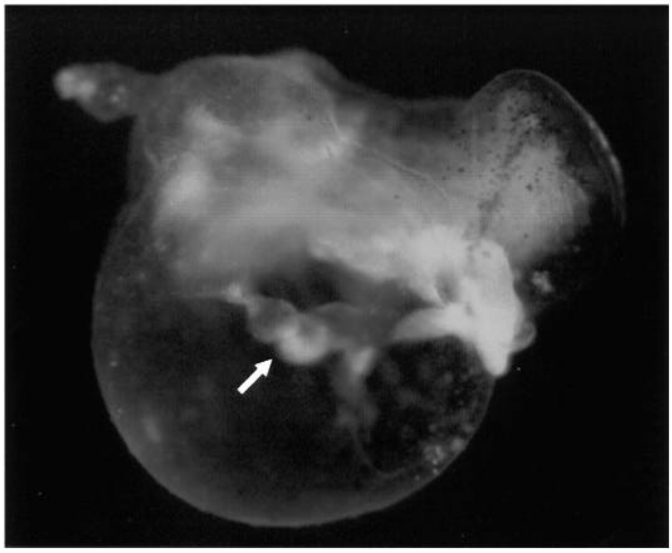

Fig. 3. Beating hearts form in the absence of dorsoanterior structures. Stage 10 bridged primordia explant with endoderm, after 7 days in culture. Arrow points to location of beating heart.

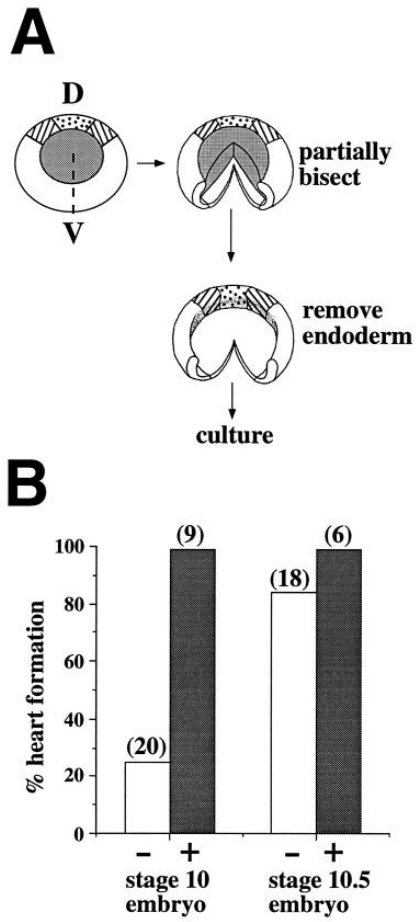

Fig. 4. Endoderm deficiency reduces the frequency of heart formation in whole embryos. (A) Stage 10 or stage 10.5 gastrulae were partially bisected along the ventral midline to access the endoderm-mesoderm boundary. The endoderm tissue was gently scraped away from the two halves of marginal zone and discarded. Embryos then healed upside-down in round-bottom wells for 30 minutes in 0.75× MMR, during which time the ectoderm halves fused and the marginal zone invaginated into the cavity left by the operation. The embryos were then transferred to 0.1× MMR + gentamycin for culture, and assessed for the presence of beating heart tissue. (B) The effect of endoderm removal on heart formation in intact embryos is compared to partially bisected controls whose endoderm was not removed. Data from different trials is statistically homogeneous (exact test: $P \geq 0.05$), and presented as a percentage of total (*n*) explants with beating hearts.

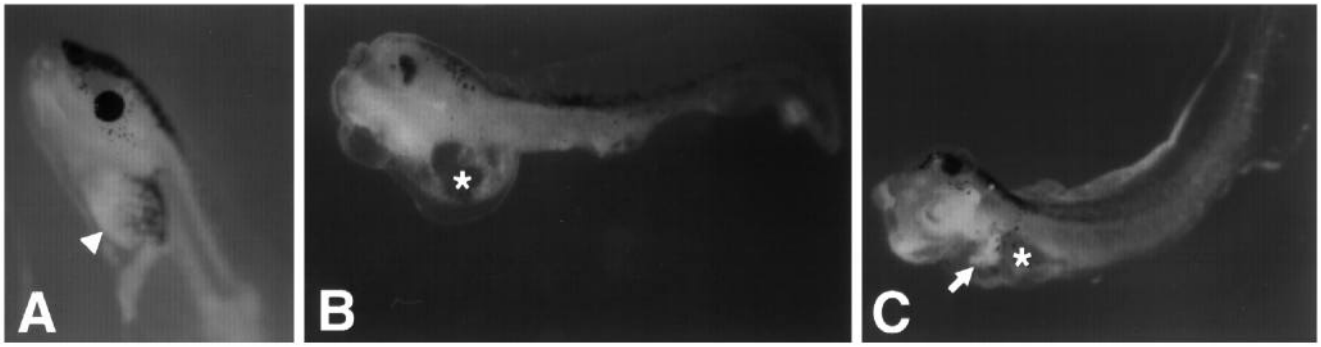

Fig. 5. Removal of endoderm at stage 10 can prevent heart formation in whole embryos. Embryos were cultured for 7 days after the operation. (A) Control bisected embryo (stage 10.5) whose endoderm was left intact. All controls had beating heart tissue and normal gut (arrowhead). (B) Embryo deprived of entire endoderm at stage 10. No beating heart tissue was evident in this explant. Asterisk indicates lack of gut development. (C) Embryo deprived of endoderm at stage 10.5. Location of beating heart is indicated by arrow. Gut development is absent (asterisk).

indicate that ventral mesoderm is competent to form heart in the combined presence of organizer and dorsoanterior endoderm.

We then studied the ability of dorsoanterior endoderm alone to induce heart in VMZ tissue in the absence of any organizer activity. A mass of endoderm from the dorsal half of a stage 10 or stage 10.5 embryo was transplanted into the ventral midline of a host embryo, immediately subjacent to the VMZ cells. None of these embryos ever developed a second heart, and always appeared normal except for slight malformations in the tail (data not shown). This experiment was also performed with isolated pieces of VMZ, which likewise failed to form hearts (data not shown). We conclude that interaction with dorsoanterior endoderm alone is insufficient to induce heart in ventral mesoderm that has not been in contact with the organizer.

These results imply that endoderm signals cannot induce heart in ventral tissues in the absence of organizer dorsalization, and that Spemann organizer grafts are unable to induce secondary hearts without additional dorsal endoderm influence. However, our findings demonstrate that the presence of both tissues is necessary and sufficient to induce hearts in naive explants of VMZ that otherwise would not form a heart.

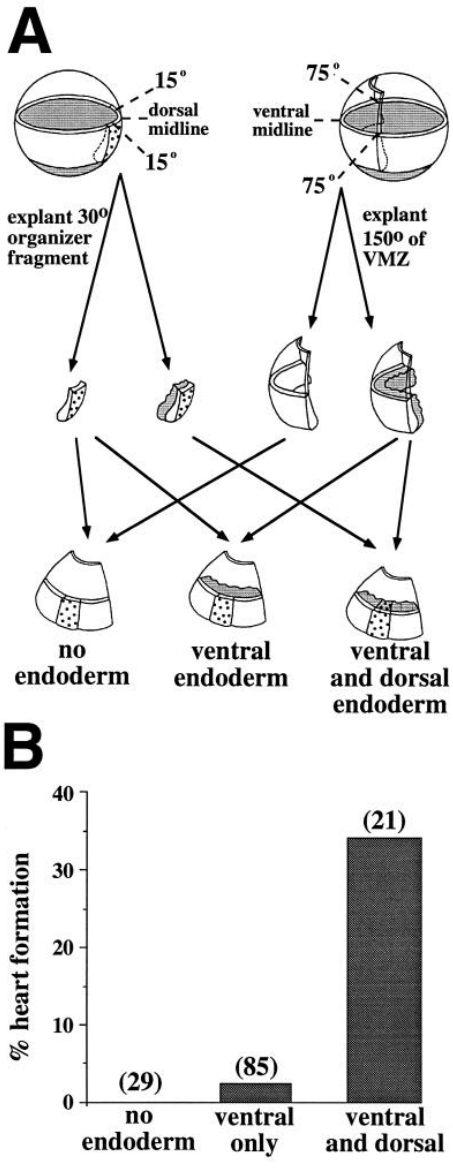

Fig. 6. The presence of both organizer and endoderm is necessary for heart induction in ventral marginal zone (VMZ). (A) Small pieces of organizer (dorsal 30° of marginal zone) were explanted and used with or without their associated dorsoanterior deep endoderm. Regions of the VMZ encompassing the ventral 150° (75° lateral to dorsal midline) of the embryo were simultaneously explanted with or without ventral endoderm. Three different types of recombinants were constructed by implanting the organizer fragment into the ventral midline of the VMZ explant, as in an in vivo organizer transplant graft. (B) The effect of no endoderm, ventral endoderm alone, or ventral and dorsal endoderm, on the frequency of heart formation in VMZ recombinants is expressed as a percentage of explants with beating hearts, since statistical tests indicated significant homogeneity between individual trials (exact test: $P>0.05$). The number in parentheses (n) refers to the total number of recombinants made.

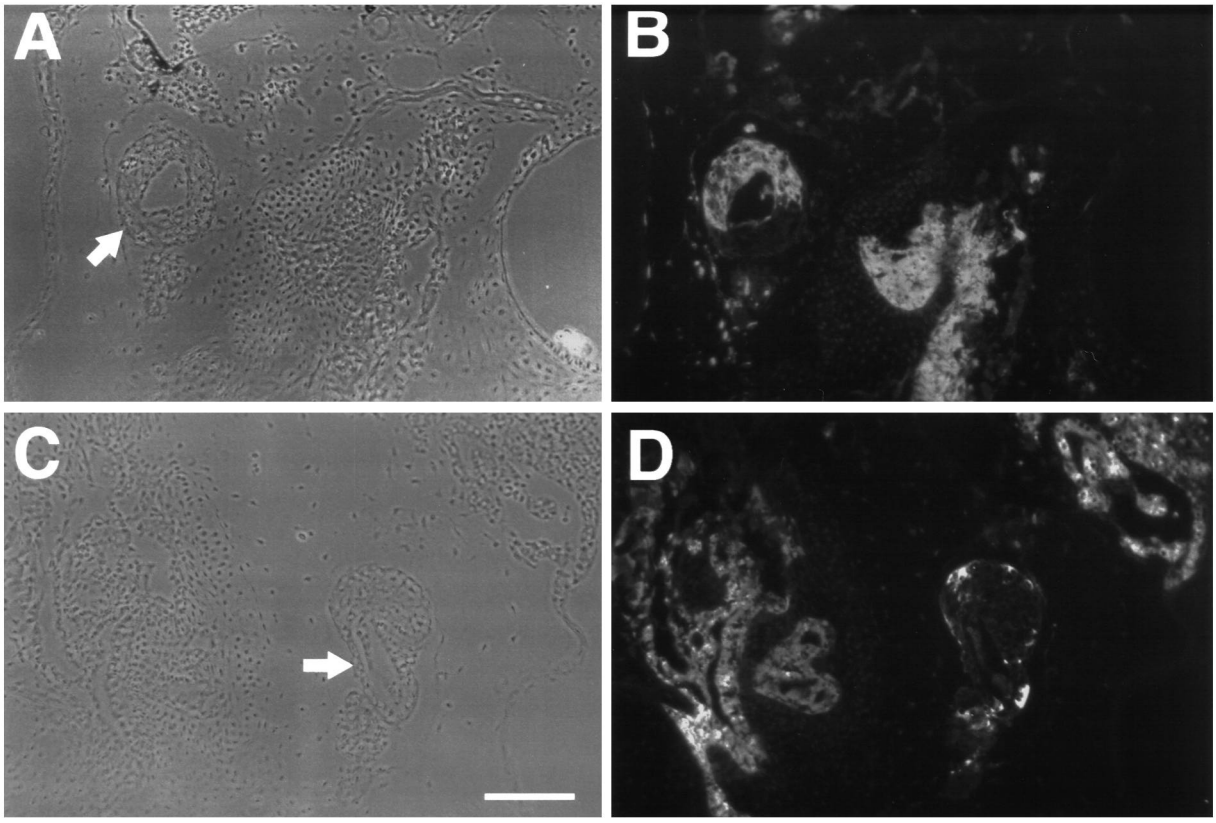

Fig. 7. Ventral mesoderm is induced to form heart tissue in combination with both organizer and dorsoanterior endoderm. A VMZ recombinant was constructed as in Fig. 6A with an RLDx-labelled organizer segment which retained its associated dorsoanterior endoderm. Those with beating hearts after 7 days were sectioned, and the heart tissue was examined by epifluorescence illumination. Two examples are shown. (A,C) Phase micrograph of VMZ recombinant containing dorsal deep endoderm, sectioned at level of heart tissue (arrow). Both myo- and endocardial layers are evident. (B,D) Epifluorescent micrograph of same sections as in A and C. Unlabelled VMZ cells contribute to approximately half of the heart tissue evident in B, whereas they give rise to most of the heart tissue in the explant shown in D. The scale bar in C represents 100 μm.

DISCUSSION

The establishment of heart mesoderm in the frog, *Xenopus laevis*, was previously thought to be contingent on the activity of the Spemann organizer (Sater and Jacobson, 1990). Our results demonstrate, however, that signals derived from the deep dorsoanterior endoderm, in addition to the organizer, are responsible for *Xenopus* heart induction. These results indicate that heart induction in *Xenopus* is similar to that in other vertebrates and may occur via the stepwise actions of organizer and endoderm signals.

A role for the endoderm

Several lines of experimentation reveal the capacity of the deep dorsoanterior endoderm to induce heart in *Xenopus*. We have demonstrated that inclusion of substantial amounts of deep endoderm greatly enhanced the frequency of heart formation in explants of dorsal marginal zone (DMZ; stage 10) which include both the organizer and the flanking heart primordia. In the absence of endoderm, identical explants formed hearts in only 24% of cases (Fig. 1). Moreover, we have found that the addition of deep endoderm allowed explants of heart primordia to form beating hearts in the absence of organizer influence (i.e. bridged primordia), while the absence of both the organizer and the endoderm prevented heart formation in primordia isolated at this same stage (Fig. 2). This suggests that, by the onset of gastrulation, the heart primordia are already competent to form a beating heart in response to endoderm influences and are, to some extent, independent of organizer signalling. Likewise, we have shown that bridged primordia isolated at stage 10.5 also formed hearts when deep endoderm was included in the explant. Unlike stage 10 primordia, however, these older explants varied broadly in their capacity to form hearts in the absence of both organizer and endoderm influences (see Table 1). Such disparate frequencies most likely indicate that events critical to heart induction occur at or just before stage 10.5. Heart formation at this stage may therefore vary, depending on slight batch-to-batch differences in when inductive signals pass from the endoderm or the organizer, or both, to the primordia. We emphasize, however, that inclusion of deep endoderm invariably enhances the frequency of heart formation in our primordia explants, regardless of the stage, or the presence of organizer.

The role of the endoderm in heart induction thus appears to occur during early gastrulation and is completed during this time. When explanted at stage 10.5, explants of DMZ formed hearts at high frequency, with or without deep endoderm (Fig. 1). Likewise, the majority of whole embryos, whose endoderm was removed at stage 10.5, also developed beating hearts, though this manipulation dramatically decreased the incidence

of heart formation when performed earlier (Fig. 4). Thus it appears that, at least in the presence of the organizer, substantial amounts of deep endoderm are no longer required for heart formation by stage 10.5, well before the end of gastrulation. However, the presence of endoderm enhances heart formation in stage 10.5 explants of bridged primordia that lack organizer tissue, suggesting that competence to respond to endoderm signalling may actually persist through midgastrulation.

The inductive potency of the endoderm appears to be concentrated in the dorsoanterior regions of the early gastrula, as opposed to ventral (or posterior) endoderm. For example, we elicit significantly greater heart formation in VMZ recombinants by inclusion of dorsoanterior endoderm, than in the presence of ventral or no endoderm (Fig. 6). These findings are consistent with experiments performed in urodeles which demonstrated that heart-inducing signals are found mainly in anterior endoderm (Jacobson and Duncan, 1968). However, the presence of dorsoanterior endoderm alone seems insufficient to induce heart from ventral mesoderm, as suggested by our unsuccessful attempts to elicit heart formation in ventral marginal zones under the influence of dorsal endoderm alone (unpublished results). Some degree of dorsalization in the responding mesoderm may therefore be a prerequisite for effective endodermal signalling.

The organizer in *Xenopus* heart induction

Previous results suggest that heart induction in *Xenopus* is dependent upon the dorsalizing activity of the Spemann organizer (Sater and Jacobson, 1990). Our results are consistent with this conclusion, but indicate that the organizer role may be limited to only those signals that specify dorsoventral patterning in the adjacent mesoderm, while additional endoderm signals are required to complete the process of heart induction. For example, we have found that stage 10 DMZ explants, which retain organizer but lack deep endoderm, formed hearts at low frequency, while identical explants cultured with deep endoderm almost always underwent heart formation (Fig. 1). Moreover, we have shown that extirpation of the entire endoderm from whole gastrulae substantially decreased heart formation (Fig. 4), although ample organizer activity remained in these embryos, as shown by the development of normal axial organization (Fig. 5). Furthermore, through the use of VMZ recombinants, we have demonstrated that the presence of the organizer alone is insufficient to induce heart from isolated ventral tissues that have never been in contact with dorsoanterior endoderm (Fig. 6). We conclude, therefore, that signals derived from both the organizer and the dorsoanterior endoderm are essential for heart formation in *Xenopus*.

It is interesting, then, that transplants of organizer alone can result in the formation of a second heart when implanted into the ventral marginal zone of an intact host embryo, as in the classic Spemann graft (Spemann and Mangold, 1924; Sater and Jacobson, 1990). During the aberrant gastrulation of a secondary axis, however, it is possible that graft-dorsalized ventral mesoderm interacts with host anterior endoderm. A massive reorganization of mesoderm and endoderm results from the simultaneous gastrulation of both host and graft-induced tissues in transplanted embryos. Endoderm derivatives are often fused or shared where the two axes are joined ventrally, and the spatial restraints imposed by this orientation cause graft-induced hearts to develop adjacent to shared anterior endoderm. The occassional incidence of large fused hearts, shared between the two axes (Sater and Jacobson, 1990), is further indication that the respective heart-forming tissues from each axis may respond to common inductive influences.

Model of heart induction

Our results attest to a multistep process of heart induction in *Xenopus*, in which both the organizer and the endoderm participate. We suggest that the role of the organizer is to initiate the dorsalization events that specify heart-forming competency in the flanking marginal zone tissue. Furthermore, we propose that the deep dorsoanterior endoderm contributes immediate heart-inducing signals to the adjacent regions of dorsalized mesoderm. The combination of these influences establishes fields of cardiogenic potential in dorsolateral marginal zone, which become specified to form heart before the end of gastrulation. According to this model, embryos dorsalized by treatment with LiCl may form larger hearts due to the increased area of dorsalized mesoderm in these embryos. Likewise, UV-ventralized embryos may lack any mesoderm dorsal enough to participate in further induction by the endoderm. It is unclear whether such treatments may also affect the dorsoanterior endoderm (Kao and Elinson, 1988), and consequently affect potential inductive interactions. A testable prediction from our hypothesis is that treatment of ventral mesoderm with dorsalizing factors, such as noggin (Smith et al., 1993), may substitute for the organizer role in heart induction, and confer competency to respond to endoderm-derived signals.

Our model is also consistent with the known mechanisms of heart development in urodeles and other vertebrates (reviewed in DeHaan, 1965; Jacobson and Sater, 1988), in which heart induction occurs in dorsolateral or anterior mesoderm primordia and is attributed to interaction with endodermal tissues. In *Xenopus*, the timing of dorsalization and heart induction overlaps during gastrulation, presumably due to the shorter span of development in this species. We cannot, therefore, rule out the alternative possibility that the endoderm establishes competency in the primordia to respond to organizer signalling. We find this scenario unlikely, however, in light of the relative timing of dorsalizing and heart-inducing events observed in urodeles in which heart specification occurs subsequent to the establishment of the embryonic axes (Jacobson and Sater, 1988). A third possibility is that the organizer and endoderm signals synergize to establish fields of heart mesoderm. Although it is difficult to completely separate the individual roles of the inducing tissues in *Xenopus* heart development, the ability to distinguish endodermal effects in explant culture will nonetheless facilitate further characterization of the endodermal role, and provide an assay for the molecular nature of vertebrate heart-inducing signals.

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