Spatially distinct head and heart inducers within the *Xenopus* organizer region

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Background: The mouse anterior visceral endoderm, an extraembryonic tissue, expresses several genes essential for normal development of structures rostral to the anterior limit of the notochord and has been termed the head organizer. This tissue also has heart-inducing activity and expresses *mCer1* which, like its *Xenopus* homolog *cerberus*, can induce markers of cardiac specification and anterior neural tissue when ectopically expressed. We investigated the relationship between head and heart induction in *Xenopus* embryos, which lack extraembryonic tissues.

Results: We found three regions of gene expression in the *Xenopus* organizer: deep endoderm, which expressed *cerberus*; prechordal mesoderm, which showed overlapping but non-identical expression of genes characteristic of the murine head organizer, such as *XHex* and *XANF-1*; and leading-edge dorsoanterior endoderm, which expressed both *cerberus* and a subset of the genes expressed by the prechordal mesoderm. Microsurgical ablation of the *cerberus*-expressing endoderm decreased the incidence of heart, but not head, formation. Removal of prechordal mesoderm, in contrast, caused deficits of anterior head structures. Finally, although misexpression of *cerberus* induced ectopic heads, it was unable to induce genes thought to participate in head induction.

Conclusions: In *Xenopus*, the *cerberus*-expressing endoderm is required for heart, but not head, inducing activity. Therefore, this tissue is not the topological equivalent of the murine anterior visceral endoderm. We propose that, in *Xenopus*, *cerberus* is redundant to other bone morphogenetic protein (BMP) and Wnt antagonists located in prechordal mesoderm for head induction, but may be necessary for heart induction.

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Background

Organizing centers in vertebrate embryos function during gastrulation to pattern the anterior–posterior axis. Upon transplantation to the ventral side of a host, the amphibian organizer can induce a secondary body axis [1,2] in a process involving the conversion of host mesoderm and ectoderm to dorsal and neural fates, respectively (reviewed in [3,4]). The organizer is not homogeneous in its inductive potential; rather, it has regional differences in its ability to self-differentiate and induce structures such as head and trunk tissues [5,6]. Studies in avian embryos also suggest a spatial separation of the signals required for anterior and posterior neural patterning [7,8].

Recent experiments have suggested that signals involved in the patterning of neural ectoderm anterior to the hindbrain in mammals may also be spatially separate from those required for more posterior neural patterning. For instance, ectopic nodes cause the formation of secondary body axes that extend only to the level of the hindbrain, suggesting that more anterior ectodermal patterning requires signals outside the node [9]. Conversely, removal of the anterior visceral endoderm results in embryos with anterior neural deficits [10]. Similar deficits in anterior, but not posterior, neural pattern have been observed in mosaic mouse embryos carrying wild-type embryonic cells, but lacking expression of either *nodal* or *Otx2* in their visceral endoderm [11,12]. Anterior truncations have also been observed in embryos homozygous for deletions of either of two homeobox genes, *Lim1* and *Otx2*, which are likewise initially expressed in the anterior visceral endoderm [13–16]. Together, these studies provide compelling evidence that the visceral endoderm that underlies the future head region is an important signaling center responsible for the generation of pattern anterior to rhombomere 3.

Murine anterior visceral endoderm expresses several genes that are also active in the organizer region of *Xenopus*. *Hex* and *Hesx*, which encode homeobox-containing proteins, are initially expressed in the mouse anterior visceral endoderm at prestreak and early streak stages,

respectively. *Xenopus* homologs of these genes (*XHex* and *XANF-1*) are first expressed in the dorsal blastopore lip at stage 10, just prior to the onset of gastrulation movements [10,17–19]. Overlapping early expression patterns have also been described for *XOtx2* and *Xlim-1*, suggesting a model in which the Spemann organizer has some activities in common with the mouse anterior visceral endoderm.

In contrast, the expression patterns of *cerberus* and its homologs, which encode members of the DAN family of secreted proteins [16,20–23], suggest that some activities reside outside the Spemann organizer. Mouse *mCer-1*, like *Hex*, is first expressed in the visceral endoderm at embryonic day 5.5 (E5.5), prior to streak formation, and becomes specific to the anterior visceral endoderm by E6.0. Chick *cCer* is expressed analogously in the prestreak hypoblast (L. Zhu, M. Marvin, A. Gardiner, A. Lassar, M. M., C. Stern and M. Levin, unpublished observations; M. Marvin, A. Gardiner and A. Lassar, personal communication). Unlike the *Xenopus* homologs of other genes expressed in the mouse anterior visceral endoderm, *cerberus* is expressed in deep dorsoanterior endoderm which underlies the Spemann organizer [23]. When injected into *Xenopus* embryos, *cerberus* is capable of inducing the expression of anterior, but not posterior, neural markers in animal cap explants, and the formation of ectopic heads in whole embryos [23]. Although it does not induce heads when injected into *Xenopus*, *mCer-1* produces an enlarged head region and induces neural markers in isolated animal caps [20,22]. Although consistent with the suggestion that *cerberus* is a head-inducing factor [23], it is not known if the *cerberus*-expressing deep dorsoanterior endoderm is required for head induction. This region, however, is necessary for heart induction [24]. Furthermore, *cerberus* may be involved in heart induction, as ectopic expression of *cerberus* or *mCer-1* in isolated *Xenopus* animal cap tissue can induce *XNkx2.5*, a homolog of *Drosophila tinman* and an early marker of precardiac mesoderm and ventral foregut [25]. Yet its role, if any, is unclear as *XMLC2a*, a marker of later cardiac differentiation [26], is not induced [20]. Thus, whether *cerberus*, or the *cerberus*-expressing deep dorsoanterior tissue, is necessary for anterior neural patterning, heart induction or both is unknown.

We undertook a series of microsurgical extirpation and explantation experiments to resolve the question of whether head-inducing and heart-inducing activities are co-localized in *Xenopus*. We found that *cerberus*-expressing tissue is not required for the formation of anterior neural structures, but is required for induction of precardiac mesoderm. In contrast, the mesodermal organizer tissue expressing *XHex* and *XANF-1*/*Hesx* was found to be necessary for the formation of anterior neural structures, but not for induction of trunk neural tissue. The region of the embryo with heart-inducing activity is deeper and substantially broader than that responsible for head induction and correlates well with the domain of *cerberus* expression. The observation that head and heart induction can be resolved spatially in *Xenopus* suggests that they are mediated by distinct molecules. Nevertheless, co-expression of *mCer-1* with *Hex* and *Hesx* in the mouse embryo supports a model in which these two distinct activities coincide spatially in the anterior visceral endoderm.

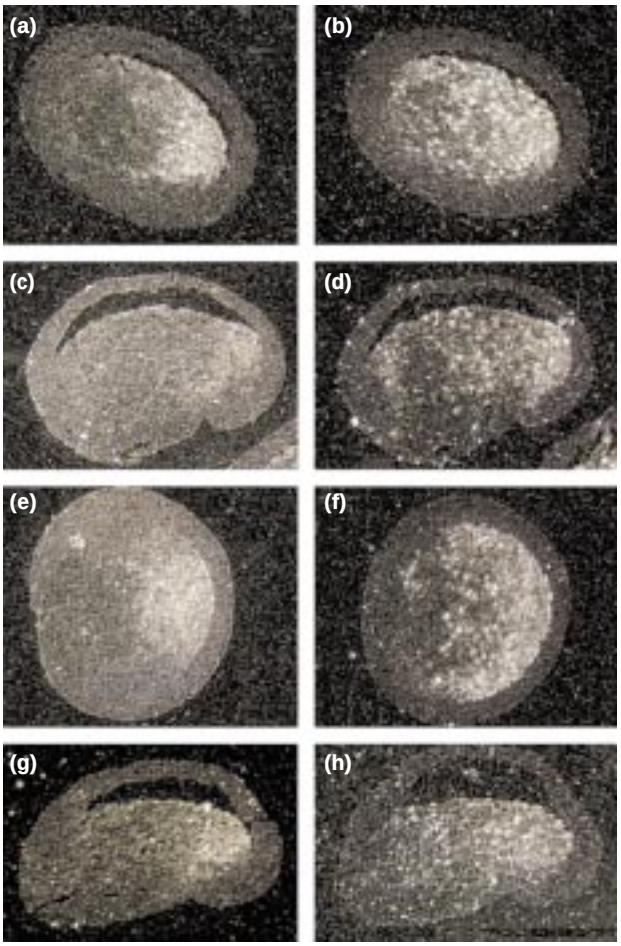
Results

Genes co-expressed in the murine anterior visceral endoderm mark different regions in the *Xenopus* embryo

In the mouse, several genes apparently involved in head and/or heart induction (*Hesx*, *Hex*, *Otx2*, *Lim1* and *cerberus*) are co-expressed in the anterior visceral endoderm. Coincident expression patterns, as well as documented involvement of this tissue in head and heart induction, suggested that these two activities might be linked. To test whether *Xenopus*, which lacks extraembryonic tissue, has an equivalent signaling center, we evaluated early gastrula-stage embryos for regions where expression of these genes might overlap. Figure 1 compares the expression patterns of *cerberus* and *XHex*. Whereas expression of these genes overlapped in a narrow domain of endoderm at the onset of gastrulation (stage 10), sagittal and horizontal sections demonstrated that *cerberus* expression penetrated substantially further into the endoderm and spanned a much broader dorsolateral arc (Figure 1a–d). The expression differences became more striking at stage 10.5 (Figure 1e–h). The regions of overlap and non-overlap suggested the existence of distinct signaling centers in the embryo.

Figure 2 shows the expression of additional markers in stage 10 embryos, bisected along the dorsoventral axis. Three regions were identified by these data (Figure 2i): a deep and broad region of endoderm defined by expression of *cerberus* alone; a small region of leading-edge dorsoanterior endoderm in which *XHex*, *XOtx2* and *Xlim-1* expression overlapped that of *cerberus*; and prechordal mesoderm, which lacked *cerberus* expression, had overlapping but non-identical patterns of *XANF-1* and *chordin* expression, and also expressed markers of the leading-edge endoderm. In agreement with previous studies, *chordin*, *XOtx2*, *XANF-1* and *XHex* transcripts were found confined to a 60–90° arc of dorsal mesoderm (data not shown; see [18,19,27,28]), which corresponds well with the location of organizer activity [5,29]. *Xlim-1* expression spanned a broader arc overlying that of *cerberus* expression (as in [30]). Instead of identifying a single signaling equivalent of the murine anterior visceral endoderm, the marker analysis suggested a model in which at least some activities of the murine extraembryonic tissue might be spatially separable in *Xenopus*. To test this possibility, we examined the effects of removing individually, by microsurgery, either the *cerberus*-expressing endoderm or the overlying prechordal mesoderm.

Figure 1



In the *Xenopus* embryo, *cerberus* is expressed in a broader and deeper region than *XHex*. Antisense **(a,c,e,g)** *XHex* and **(b,d,f,h)** *cerberus* cRNA probes were hybridized to (a–d) stage 10 and (e–h) stage 10.5 sectioned albino embryos. (a,b,e,f) Horizontal sections; (c,d,g,h) sagittal sections. At stage 10, expression of *cerberus* penetrated deeply into the endoderm in the interior of the embryo and extended over a broad arc on the dorsal side, whereas *XHex* expression was confined to a more shallow and narrow dorsal mesendodermal region. The differences in expression pattern were more pronounced at mid-gastrulation. In all panels, embryos are oriented with the dorsal side to the right.

Extirpation of *cerberus*-expressing endoderm decreases heart, but not head, formation

Dorsal marginal zone (DMZ) tissue was dissected from stage 10 embryos and the deep, large cells were removed (Figure 2h, green region; Figure 2i, to the left of the dashed line). Explants were allowed to heal for 15–20 minutes and then assayed for marker expression by *in situ* hybridization (Figure 2a′–f′,g). Endodermal expression of *Xsox17β* [31] was eliminated in these explants, leaving only a small patch of expression in an external layer of suprablastoporal (non-deep endodermal) cells in all explants examined (Figure 2g), demonstrating that this operation effectively removed the deep and leading-edge endoderm. Even after overnight development of the *in situ* hybridization chromogenic reaction, 83% of the explants (n = 23) either completely lacked *cerberus* mRNA or expression was confined to a small cluster of cells (similar to the pattern seen in Figure 2a′). In contrast, we observed strong expression of *chordin*, *XOtx2*, *XHex*, *XANF-1* and *Xlim-1* in such explants, indicating retention of prechordal mesoderm (Figure 2b′–f′).

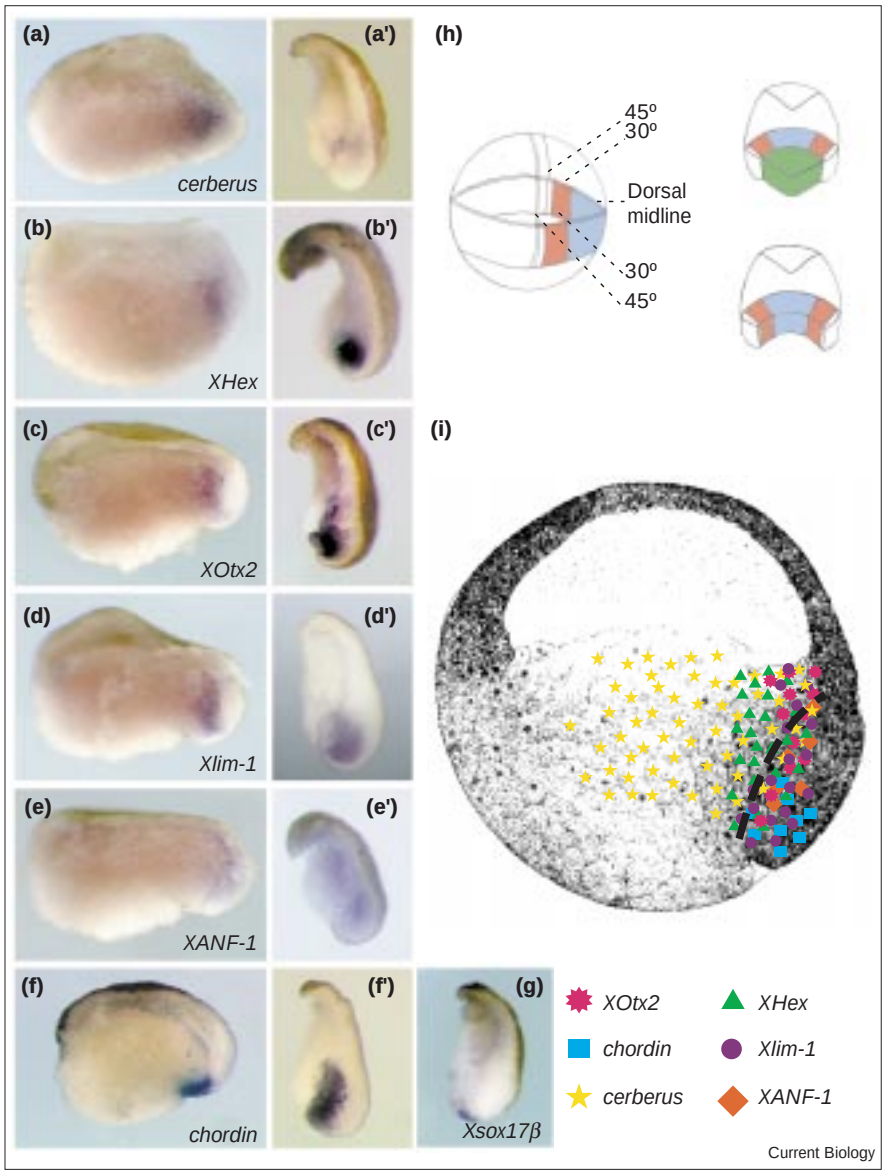
Having established the microsurgical techniques to selectively remove the *cerberus*-expressing cells (deep and leading-edge endoderm), we then assayed the effect of extirpating these regions from the developing explants and embryos. DMZ explants at stage 10 were cultured to tadpole stages, at which time they were examined for a head and a beating heart (Figure 3). Removal of *cerberus*-expressing endoderm from explants and whole embryos reduced the formation of beating hearts from 100% when endoderm was present (n = 41 and 45, respectively) to 15.1% (n = 97) and 7.1% (n = 85), respectively, consistent with a requirement for this tissue in heart induction [24]. Importantly, head formation was largely unaffected by removal of *cerberus*-expressing tissue (91.7%, n = 97 for explants; and 100%, n = 85 for whole embryos).

An examination of region-specific genes confirmed that anterior neural ectoderm of endoderm-deficient explants and embryos was properly patterned (Figure 3d–i). Expression of *XOtx2* and *XEn2* at stage 25 (which mark the forebrain and the hindbrain–midbrain border, respectively) was observed in 100% of explants (n = 13 and 11, respectively) and embryos (n = 11 and 11, respectively). *Krox20*, which at this stage is expressed strongly in rhombomere 5 and weakly in rhombomere 3 of the hindbrain, was observed in 92% of explants (n = 13) and 100% of embryos examined (n = 13).

The lack of heart differentiation in the absence of *cerberus*-expressing endoderm suggests that *cerberus* could be required to specify precardiac mesoderm or is acting later in heart differentiation. Consistent with the former possibility, *XNkx2.5*, an early marker of the *Xenopus* cardiogenic mesoderm [25,32] was observed in only 13% (n = 23) of the DMZ explants that lacked endoderm but in 100% (n = 21) of explants with endoderm (Figure 3j,k).

Taken together, these studies indicate that head-inducing and heart-inducing activities are not co-localized in *Xenopus* embryos. The endoderm, characterized by *cerberus* expression, is not necessary for head formation *in vivo*, although misexpression of this gene induces ectopic heads. This tissue, however, appears to be essential for an early step in heart induction.

Figure 2



Genes co-expressed in the mouse anterior visceral endoderm identify different regions of the *Xenopus* embryo. The indicated antisense cRNA probes were hybridized to (a–f) stage 10 whole embryos bisected along the dorsoventral axis and to (a′–f′) dorsal marginal zone (DMZ) explants without endoderm encompassing an ~100° arc of tissue dissected at stage 10, from which endoderm was removed. Explants have been cut sagittally through the organizer to reveal internal staining. The dark regions seen on the surface of the embryos (panels c and f in particular) are pigmented cells of the animal hemisphere and do not represent staining. (g) A DMZ explant without endoderm showing lack of *Xsox17β* hybridization except at the suprablastoporal region (bottom of explant). This shows that the endoderm had been completely removed. (h) Diagram depicting embryo dissections. Left, whole embryo; top right, DMZ explant; bottom right, DMZ explant without endoderm. Precardiac mesoderm is located 30°–45° to either side of the dorsal midline. Red, precardiac mesoderm; blue, Spemann organizer; green, endoderm. (i) Schematic diagram of the three regions of gene expression observed. The dashed line approximates the boundary along which endodermal tissue was removed.

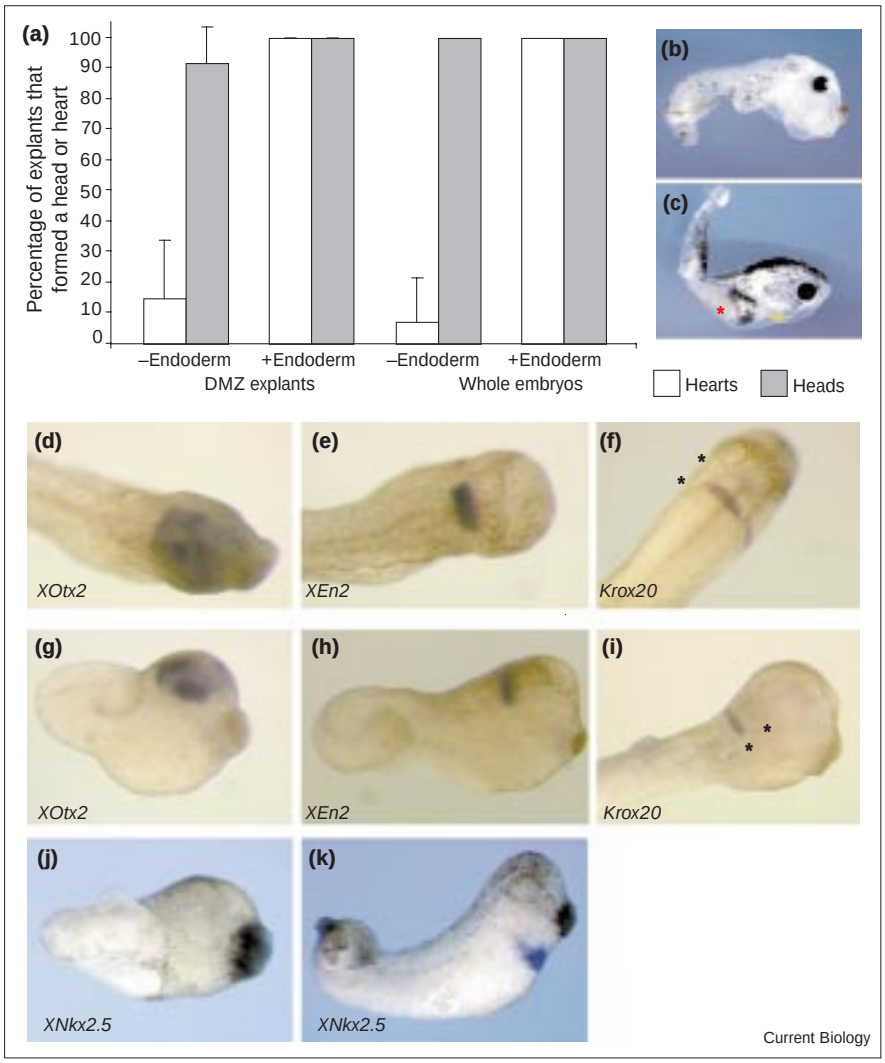
Small regions of the *cerberus*-positive domain are sufficient for heart induction

We next determined whether the entire region of *cerberus*-expressing tissue was needed for heart induction. When an ~60° arc of endodermal tissue, which underlies only the organizer, was removed (Class I explants), beating hearts were observed in 89.6% of DMZ explants (n = 140), suggesting that more lateral regions have heart-inducing activity (Figure 4). In the reciprocal experiment, 94.4% of explants in which endoderm was removed only from beneath the heart primordia (Class II explants) also formed beating hearts (n = 26). As expected, we observed head formation in nearly all of these explants (89.1% and 100%, respectively). These data demonstrate that as little as a third of the *cerberus*-expressing endoderm is sufficient for heart formation, although this activity appears to be distributed throughout the entire *cerberus* domain.

Head induction and organizer tissue

Removal of endoderm in the preceding experiments did not impair head induction, indicating that endoderm is not necessary for head induction *in vivo*. Recently, Zoltewicz and Gerhart [5] assayed head-inducing activity in portions of the gastrula-stage organizer by inserting tissue into the blastocoel of an early gastrula (the Einsteck procedure) or by conjugating it with responsive ectoderm. They concluded that the prechordal mesoderm located at the lower (vegetal) portion of the organizer is sufficient for head

Figure 3



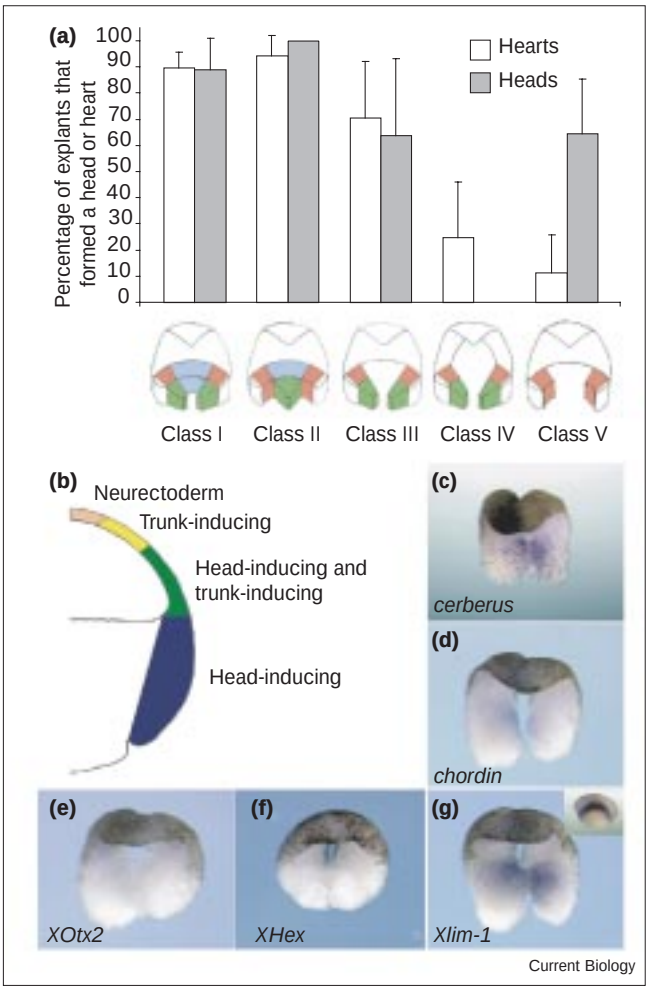
In *Xenopus*, *cerberus*-expressing endoderm is not required for head formation. Endoderm was removed from DMZ explants and whole embryos as described (see main text and Figure 2 legend). Explants were examined at stage 42 for the formation of a head and a beating heart. **(a)** Removal of endoderm decreased heart (white), but not head (grey), formation in both explants and whole embryos. Mock microsurgical manipulations on whole embryos, in which an incision was made to open the embryo vegetally, but not remove endoderm, did not impede head or heart formation (+endoderm histograms on the right of the panel). **(b,c)** DMZ explants (stage 42). In (b), endoderm was removed; note the absence of a heart and endodermally derived gut tissue. In (c), the endoderm was left intact, and both heart (arrow) and gut (asterisk) are visible. Both explants had well-formed heads. **(d–f)** Endoderm-deficient whole embryos and **(g–i)** DMZ explants were analyzed at stage 25 by *in situ* hybridization for expression of neural markers. At this stage, expression of *Krox20* was strong in rhombomere 5 and weaker in rhombomere 3 (both indicated by asterisks). Expression of all genes appeared normal. **(j)** Endoderm-deficient DMZ (stage 25) explant that did not stain with *XNkx2.5*. **(k)** Explant with intact endoderm showing expression of this gene.

induction. In light of the possibility that *cerberus* may be involved in head induction [23], we asked whether the prechordal mesoderm was necessary *in vivo*, or whether other embryonic regions, specifically the *cerberus*-positive endoderm, had this activity as well.

DMZ explants were prepared as before, but the endoderm beneath the heart primordia was left intact while a region of the organizer (spanning a 60° arc of prechordal mesoderm and underlying endoderm) was removed. The upper boundary of extirpation was marked by the location of the blastocoel floor (Class III explants). Head formation decreased on average to 63.7% ($n = 90$). Unlike our other explants, variation in head formation between experimental batches was substantial and ranged from 33.3% to 91.3% (Figure 4a). Such variation could be caused if head-inducing activity tapers off sharply towards the edges of these explants such that slight topological differences between batches of embryos would result in varying amounts of activity to be included in the explant. To resolve this question, we performed the same dissection, but expanded the ablated region to include more posterior organizer tissue located above the boundary of our previous extirpation (Class IV explants). This caused a complete loss of head formation ($n = 117$), consistent with activity in the upper (posterior) regions of the organizer (Figure 4a). Importantly, both classes of explants expressed *HoxB9*, a marker of spinal cord (Table 1). This indicates that the ablation did not remove future neural ectoderm, as trunk-inducing tissue, which at the stage of dissection is located more vegetally than the ectoderm, was still present (see Figure 4b).

To verify that head induction observed in Class III explants was not caused by the remaining *cerberus*-expressing endoderm, we examined similar explants

Figure 4



Spatially distinct head-inducing and heart-inducing regions in *Xenopus*. (a) The effect of removing small regions of tissue from DMZ explants on heart and head formation was examined at stage 42. The regions of tissue removed are depicted in the explant diagrams. Note that the entire dorsal 100° region of endoderm was observed to have heart-inducing activity, whereas head-inducing activity appeared to be restricted to the mesoderm of the Spemann organizer. Red, precardiac mesoderm; blue, Spemann organizer; green, endoderm. (b) Diagram depicting regions of head-inducing and trunk-inducing activity in the early *Xenopus* gastrula. Although clear distinctions between these regions are shown in this figure, gradients of activity are likely to exist *in vivo*. In addition, the limit of head-inducing activity may not extend as far in the animal direction as shown, but the exact limit could not be resolved from our explant dissections. (c–g) Class IV explants were dissected at stage 10, allowed to heal briefly, and analyzed by *in situ* hybridization for the expression of organizer markers. (g) Expression of *Xlim-1* in these explants is consistent with its broad domain of expression (see inset showing DMZ explant). Explants are shown with the interior of the embryo facing the reader. In several cases, healing has begun and the heart primordia are fusing along the dorsal midline.

lacking endoderm (Class V). Head formation (64.5%, $n = 88$) was identical to that in Class III explants. As expected, incidence of heart formation was substantially diminished (11.5%). We conclude that head-inducing activity resides within the mesodermal region of the organizer. Deep and leading-edge endoderm, in contrast, appear neither sufficient nor necessary for head differentiation, at least past stage 10.

To understand better the inductive properties of the ablated tissue, we examined the stage 25 expression of several neural ectoderm genes in Class III and IV explants. Loss of heads correlated with the loss of *XEn2* and *XOtx2* (Table 1). In contrast, both sets of explants expressed *Krox20*, indicating that organizer mesoderm is needed for differentiation of neural ectoderm rostral to the level of the mid–hindbrain.

Importantly, Class IV explants retained high levels of *cerberus* expression, but lost expression of *chordin*, *XHex* and *XOtx2*, reinforcing the idea that these genes mark the tissue necessary for head induction (Figure 4c–f). Consistent with its broader pattern of expression, *Xlim-1* staining was also observed in these explants. Taken together, our data suggest that the *cerberus*-positive endoderm is insufficient to induce anterior ectodermal structures in the absence of head organizer mesoderm (consistent with results of [23]). Moreover, we find that it was not necessary when head organizer tissue was present.

Ectopic *cerberus* is unable to induce *Xenopus* homologs of genes expressed in the murine head organizer

Our conclusion that the *cerberus*-expressing tissue is not required for head induction contrasts with previous observations that overexpression of *cerberus* or *mCer-1* causes ectopic or enlarged heads. Thus, we tested the possibility that *cerberus* promotes head formation through an induction of the head organizer. We injected *cerberus* mRNA ventro-vegetally at the 8–16 cell stage, and explants of the ventral marginal zone (VMZ) were dissected at stage 10. Although these injections did result in the formation of ectopic heads in intact embryos, *in situ* hybridization analysis of the *cerberus*-injected VMZ tissue failed to demonstrate ectopic *XHex* (Figure 5b,d) or *XOtx2* (which at this stage is a marker of head mesoderm, rather than anterior neurectoderm; Figure 5f,h) expression. Similarly, animal caps dissected from embryos injected with *cerberus* mRNA into the animal region did not express ectopic *XHex*, *Xlim-1* or *XOtx2* (Figure 5i). We conclude that the induction of ectopic heads by injection of *cerberus* mRNA occurs without expression of genes suggested by mutational analysis to be essential for head induction in the mouse.

Discussion

We found that genes that are normally co-expressed in the mouse anterior visceral endoderm mark different regions of the *Xenopus* embryo. At the beginning of gastrulation, *cerberus* is expressed in the dorsoanterior endoderm (Figures 1,2; [23]). This observation has led to the suggestion that the anterior endoderm is involved in *Xenopus*

Table 1

Analysis of gene expression in explants lacking prechordal mesoderm.

Gene	Class III explants		Class IV explants	
	Explants expressing the gene (%)	*n*	Explants expressing the gene (%)	*n*
XOtx2	80	20	11	18
XEn2	78	9	5	21
Krox20	80	10	63	19
HoxB9	100	10	96	26
XNkx2.5	83	12	52	21

Explants were dissected at stage 10, as described (see text), and analyzed at stage 25 by *in situ* hybridization for the expression of neural-specific and heart-specific markers.

head induction and that this tissue may be the signaling equivalent of the murine anterior visceral endoderm [33]; however, *Xenopus* homologs of other genes expressed in the murine anterior visceral endoderm, such as *XHex*, *XOtx2* and *Xlim-1*, have overlapping but non-identical patterns of expression that together mark a subset of the *cerberus*-expressing endoderm and the prechordal mesoderm of the organizer region (see Figure 2i). Thus, in *Xenopus*, signaling activities found to be localized to the mouse anterior visceral endoderm appear distributed over different embryonic regions. Our results indicate that head and heart induction are separable, with heart-inducing activity localized in the *cerberus*-expressing endoderm and head-inducing activity in the organizer mesoderm.

Dorsoanterior endoderm is neither necessary nor sufficient for head formation

Removal of the *cerberus*-expressing endoderm caused a decrease in heart, but not head, formation in explants and whole embryos (Figure 3). Furthermore, this tissue was insufficient to induce heads in the absence of prechordal mesoderm (Figure 4). Although we conclude that this tissue is not needed for head induction, we cannot rule out the formal possibility that it acts prior to the onset of gastrulation. While other studies have suggested that the deep endoderm might be involved in head induction, none have shown this conclusively. The transcription factors Xsox17α and Xsox17β are expressed in the endoderm and are required for its formation [31]. Engrailed-repressor fusion constructs of these genes, which function in a dominant-negative fashion, block endodermal differentiation and cause variable head abnormalities when injected into embryos, suggesting that endoderm might play a role in head formation. These constructs were, however, also observed to interfere with the involution of endoderm, suggesting that the anterior deficits may be caused by a defect in gastrulation, rather than induction. In addition, it should be noted that several *Sox* genes are expressed in the central nervous system [34], raising the possibility that these dominant-negative constructs interfere with the functions of other *Sox* genes and disturb later steps in formation of anterior ectodermal structures.

Recent studies indicated that members of the Mix family of transcription factors are involved in endoderm formation [35,36]. Constructs encoding the Engrailed repressor (EnR) fused to Mix.1 or to Mixer have been shown to block endoderm formation, as assayed by a decrease in *Xsox17* and *cerberus* expression, when injected into embryos. Head defects in these embryos have been ascribed to the loss of the *cerberus*-expressing endoderm. Although the EnR–Mix.1 fusion did not affect expression of *chordin*, other genes normally found in the prechordal mesoderm were not examined. Thus, it is formally possible that these constructs might have interfered with expression of genes involved in head organizer function. Alternatively, anterior defects could be secondary to cell-movement abnormalities, possibly caused by a block in endoderm differentiation as observed with the EnR–Sox17 fusion. It should be noted that the loss of differentiated endoderm caused by the EnR–Mix.1 fusion protein also blocked heart formation (A.M. O'Reilly and P. Lemaire, personal communication), which does not depend on involution. This reinforces the idea that endoderm is required for heart induction. Thus, while there has been much speculation that the *cerberus*-expressing endoderm has the same head-inducing activities of the mouse anterior visceral endoderm, no studies have yet proven that this is the case. Our data, in contrast, suggest that *cerberus*-expressing endoderm is neither necessary nor sufficient for normal induction of head structures.

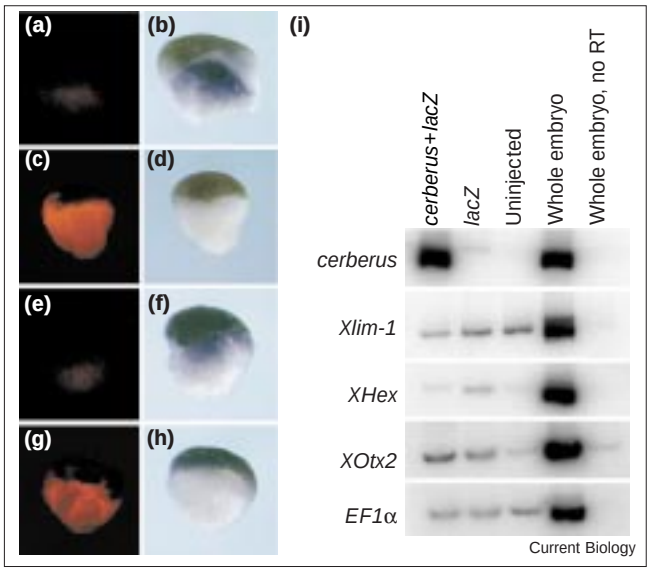
Organizer mesoderm is necessary for head induction

Our studies support the view that head-inducing activity lies within *Xenopus* prechordal mesoderm. Transplantation experiments demonstrate that this tissue is sufficient for

head induction [5] and our results indicate it is necessary as well (Figure 4). Similarly, the prechordal mesendoderm is required for the generation of anterior neural pattern in chicks [7,8]. In contrast, such activity appears to reside in the anterior visceral endoderm of mammalian embryos [11,12,37]. The differences are likely to reflect the variation in the timing of anterior patterning among these species. Whereas this process does not occur until gastrulation in avian and amphibian embryos, it begins prior to the formation/ingression of the prechordal mesendoderm in mammals. As a result, signals required for the differentiation of anterior structures must be expressed in a different tissue in mouse embryos than in amphibians or birds.

Our studies further demonstrated that removal of the deep endoderm at stage 10, in contrast to the prechordal mesoderm, did not impair head formation. This argues against a model in which the organizer serves to maintain head-inducing activity in the deep endoderm, although organizer factors upregulate *cerberus* mRNA [23] and may regulate heart-inducing activity as well (see below). Does the deep endoderm act on the anterior organizer to promote head-inducing activity? It is difficult to answer this question directly as grafted endoderm heals poorly and few secreted factors have been characterized in the deep dorsoanterior endoderm at these stages. Injections of *mCer1* and *cerberus* mRNAs into *Xenopus* embryos can produce enlarged and ectopic heads, respectively [20,22,23], providing evidence in support of such a model. Ectopic expression of *cerberus*, however, at concentrations and conditions capable of inducing expression of anterior neural markers and ectopic heads, was unable to induce the expression of *Xlim-1*, *XHex*, and *XOtx2* (Figure 5). These genes are expressed in the anterior organizer and their murine homologs have been shown by mutational and microsurgical analysis to be essential for normal anterior development [10,13–16].

Figure 5



Ectopic *cerberus* expression does not induce organizer genes. The two ventrovegetal blastomeres of each embryo were injected with 150 pg *cerberus* mRNA and rhodamine–lysinated dextran as a lineage label at the 8–16 cell stage. At stage 10, (a,b,e,f) control DMZ and (c,d,g,h) injected VMZ regions were dissected and examined for expression of (b,d) *XHex* and (f,h) *XOtx2* by *in situ* hybridization (purple; the dark color on the surface of the explants is pigmented cells of the animal hemisphere and does not represent hybridization signal). (a,c,e,g) Explants in (b,d,f,h) were viewed by epifluorescence optics to identify rhodamine-labeled cells. (i) Reverse transcriptase (RT)–PCR analysis of *cerberus*-injected animal caps using primers for the indicated genes. Embryos were injected animally with 150 pg *cerberus* and 500 pg *lacZ* mRNA, or 500 pg *lacZ* mRNA alone, into both blastomeres at the two-cell stage. Animal caps were dissected at stage 9 and cultured until stage 10.5. *EF1α* is shown as a positive control for the RT reaction. The right-hand lane shows the result of a negative control in which RT enzyme was omitted.

The *cerberus*-expressing deep endoderm is required to initiate cardiogenesis *in vivo*

Our finding that the *cerberus*-expressing dorsoanterior endoderm is necessary for heart induction is consistent with the recent suggestion that the anterior visceral endoderm in mice and the hypoblast in chick are both involved in an early aspect of heart induction [38–40]. Although all three of these tissues express *cerberus* homologs during the periods when they are thought to have heart-inducing activities, it remains uncertain whether *cerberus* is the heart inducer. While ectopic *cerberus* has been shown to induce *XNkx2.5*, an early marker, in animal caps and VMZ explants, later cardiac differentiation markers were not induced (our unpublished observations and [20]). Thus, *cerberus* may initiate a program of cardiogenesis in overlying precardiac mesoderm, and other signals may be required subsequently. It may be important that *cerberus* is not expressed after stage 13 in *Xenopus*, just prior to the appearance of bone morphogenetic proteins (BMPs) 2 and 4 in the heart region (our unpublished observations and [41]), suggesting a dependence on these factors after stage 14–15.

What is the role of *cerberus*?

If the *cerberus*-expressing endoderm is not required for head formation, why can ectopic expression of *cerberus* mimic this activity? Recently, Glinka *et al.* [42] argued that heads are induced when both ventralizing BMP and Wnt signals are blocked. Cerberus has been demonstrated to be an effective antagonist of these signaling pathways, binding BMPs 2 and 4, as well as XWnt8 [21,42,43]. Although it is not surprising, therefore, that ectopic expression of *cerberus* on the ventral side of *Xenopus* embryos can induce secondary heads, this may not be its normal function. Other BMP (Noggin, chordin) and Wnt (Dkk, FrzB) antagonists expressed in the prechordal mesoderm may fulfill this role [27,44–46]. Thus, while *cerberus* may be redundant for head induction, we suggest that it is involved in heart induction.

Conclusions

Whereas head-inducing and heart-inducing activities may co-localize in the murine anterior visceral endoderm, we found that these were spatially distinct activities within the *Xenopus* organizer region. Three regions of the *Xenopus* organizer were defined by gene expression. Organizer mesoderm was shown to be necessary for differentiation of anterior neural structures whereas the adjacent *cerberus*-expressing deep endodermal and leading-edge endodermal tissues were neither necessary nor sufficient. The *cerberus*-expressing endoderm was, however, essential for heart induction. The ability to spatially resolve head-inducing and heart-inducing activities suggests that they are mediated by different factors. Finally, we show that, although it does induce ectopic heads, misexpression of *cerberus* did not induce genes thought to be involved in head induction, suggesting that the induced heads may result from the inhibition of late blastula-stage ventralizing signals.

Materials and methods

Embryo and explant culture

Embryos, fertilized *in vitro*, were dejellied in 2% cysteine–HCl (pH 7.8) and maintained in 0.1 x Marc's modified Ringer's solution (MMR). Explant dissections were performed in 0.75 × MMR. Embryos were staged according to Nieuwkoop and Faber [47]. Animal caps were dissected at stage 9, and explants of the DMZ at stage 10. An eyelash knife was used to gently scrape away endoderm cells from DMZ explants and whole embryos, as described in Nascone and Mercola [24]. Explants to be examined for expression of organizer genes were allowed to heal for 15–20 min and then fixed. Explants to be examined for expression of neural-specific genes were cultured until sibling embryos were stage 25, whereas those to be scored for formation of heads and hearts were maintained until stage 42. Synthetic *cerberus* mRNA was transcribed from pCS2-cerberus.

In situ *hybridization*

Whole mount *in situ* hybridization was performed essentially according to the protocol of Harland [48]. Digoxygenin-labeled antisense cRNA probes were generated from the following linearized plasmids using the indicated RNA polymerase: pBSXHex (*Bam*HI, T7 polymerase), pBScerberus (*Eco*RI, T7), pGEM-XANF1 (*Not*I, SP6), pXH32-3 (Xlim-1; *Xho*I, T7), pGEM-HoxB9 (*Eco*RI, T7), pXOT30.1 (*Not*I, T7), pBS-XEn2 (*Not*I, T7), pGEMKrox20 (*Eco*RI, T7), pBSChd(59) (*Eco*RI, T7), Xsox17b (PCR product, T7) [18,19,23,27,30,31,45,49–51]. Radioactive *in situ* hybridization was performed as described [52]. Embryos were fixed in formalin (MEMFA; [48), embedded in paraffin and sectioned to a thickness of 8 μm prior to incubation with [^{35}S]UTP-labeled cRNA probe. Serial sections were hybridized to antisense *cerberus* and *XHex* probes, as well as sense *cerberus* probe. In no case was a signal detected with the sense probe.

RT–PCR

Whole embryos and explants were frozen on dry ice and stored at –80°C prior to mRNA isolation. RNA was isolated from embryos or pools of six animal caps with Trizol (Gibco BRL), using glycogen (1 μg/μl) as a carrier. RNA was treated with 2 U DNaseI (Promega) at 37°C for 30 min, which was then heat inactivated at 70°C for 10 min. Reverse transcription (RT) reactions were performed in a volume of 50 μl, using 0.4 μg oligo dT, MMRT buffer (Gibco BRL), 0.01 M DTT, 1 mM each dNTP and 200 U MMRT (Gibco BRL) at 42°C for 50 min. Reactions were then heat inactivated at 70°C for 10 min and stored at 4°C. PCR was carried out in a volume of 25 μl, using $MgCl_2$-free PCR buffer (Promega), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.15 μl [α–^{32}P]dATP, 1.25 U Taq DNA polymerase (Promega), and 5 μl RT reaction. Cycle parameters used were as follows: initial denaturation at 94°C for 2 min, followed by 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and a final elongation at 72°C for 5 min; 22 cycles were performed for EF1α and 27 for all others. Primer sequences: EF1α+, 5′-CAGATTGGTGCTGGATATGC-3′; EF1α–, 5′-ACTGCCTTGATGACTCCTAG-3′; XHex+, 5′-CCTCCCCTCTGTACCCCTTCTCC-3′; XHex–, 5′-CGGCGCTCAAACACCTCTCC-3′; Xlim-1+, 5′-CAAAACCGACCCGACACATAAGG-3′; Xlim-1–, 5′-TGCGGGCACAGAGGAAGGTA-3′; XOtx2+, 5′-AACACTGATCGCCCGACTTTG-3′; XOtx2-, 5′-GGTGCAACAAATCCATCCCG-3′. Primers used for *cerberus* were as described in Darras *et al.* [53]. PCR products were electrophoresed on a 6% acrylamide gel. Dried gels were imaged using a Molecular Dynamics PhosphorImager.

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