

Lens and Retina Formation Require Expression of *Pitx3* in *Xenopus* Pre-lens Ectoderm

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Pitx3 is expressed in tissues fated to contribute to eye development, namely, neurula stage ectoderm and prechordal mesoderm, then presumptive lens ectoderm, placode, and finally lens. *Pitx3* overexpression alters lens, optic cup, optic nerve, and diencephalon development. Many of the induced anomalies are attributable to midline deficits; however, as assessed by molecular markers, ectopic *Pitx3* appears to temporarily enlarge the lens field. These changes are usually insufficient to generate either ectopic lenses to enlarge the eye that eventually differentiates. Conversely, use of a repressor chimera or of antisense morpholinos alters early expression of marker genes, and later inhibits lens development, thereby abrogating retinal induction. Reciprocal grafting experiments using wild-type and morpholino-treated tissues demonstrate that *Pitx3* expression in the presumptive lens ectoderm is required for lens formation. Contradictory to recent assertions that retina can form in the absence of a lens, the expression of *Pitx3* in the presumptive lens ectoderm is critical for retina development. *Developmental Dynamics* 234:577–589, 2005. © 2005 Wiley-Liss, Inc.

Key words: lens placode; *Pitx*; *Xenopus laevis*; pituitary gland; retina; *Pax6*; *Rx*; *Six3*; *Lens1*; β -crystallin; *Otx2*; laterality; morpholino oligonucleotide

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INTRODUCTION

The preliminary steps of eye development appear to involve the bifurcation of an anterior field (Li et al., 1997), and the subsequent definition of optic competence in the neural plate and lens ectoderm. These two tissues come in apposition, and the lens placode and optic cup are induced. This process is probably under the influence of genes such as *Otx2*, *Pax6*, *Six3*, *Rx*, and *Lens1* (see Chow and Lang, 2001). Although competence to form lens is elicited in ectoderm during early gas-

trulation (Servetnick and Grainger, 1991), the lens placode itself does not form until after the anterior neural tube bulges to form optic vesicles. When this neural tissue comes into close proximity to anterior ectoderm, a lens placode is induced, which in turn stimulates the optic vesicles to form cup-like structures—the precursors to the neural and pigmented layers of the retina. The lens placode dimples to form a pit, which subsequently invaginates, and the placode then rounds up to form a vesicle, which ultimately dif-

ferentiates into lens. Presumably, the proximodistal (brain vesicle to lens) specification of the eye occurs during this mutually inductive ectoderm/neuroectodermal interaction.

There is a long history of reciprocal grafting experiments that question the necessity of presumptive lens ectoderm to induce optic retina and vice versa in amphibians (Ogino and Yasuda, 2000; Okada, 2000). Ultimately, these disparities can be attributed to contamination of grafted tissues (Grainger et al., 1988), or to hetero-

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chronic competence to response in experiments using tissues from different species. Recently, the requirement of lens to induce retina has again come into question. Null mutant *Pax6* mice (Hill et al., 1991) and dominant-negative *Pax6* electroporated chicks (Reza et al., 2002) fail to develop lens and retina; however, *Pax6* conditionally mutant mice (where the gene is knocked out specifically in lens placode) fail to form lens and yet still retain an ability to differentiate neural retina (Ashery-Padan et al., 2000). In this latter study, although lens failed to form, nevertheless, some genes indicative of lens specification before differentiation were still expressed. Similarly, chick embryos in which lens placode has been excised also develop a differentiated retina (Hyer et al., 2003). In contradistinction to both of these results, excision of earlier, pre-placodal presumptive lens ectoderm precludes differentiation of either lens or retina (Hyer et al., 2003). The molecular steps that underpin eye development are now coming into focus, and many of the genes known to be involved in this process have been reviewed elsewhere (Jean et al., 1998; Chow and Lang, 2001).

The *Pitx* genes appear to play a role in eye development: they encode paired-like/K50 homeodomain proteins, and three paralogs of the family (*Pitx1*, *Pitx2*, and *Pitx3*) have been cloned in vertebrates. In contrast to *Pitx1* and *2*, murine *Pitx3* has not been detected in the mammalian Rathke's pouch or in pituitary adenomas, and it is expressed primarily in mesencephalic dopaminergic neurons of midbrain, in somites, lens placode, and forming lens pit (Smidt et al., 1997). In mice, *Pitx3* has been identified as the causative locus for aphakia, a recessive deletion mutation resulting in small eyes that lack lenses. *Pitx3* protein is undetectable in the brain of aphakia mice (Smidt et al., 2004); nevertheless, *Pitx3* transcript that is undetectable by in situ hybridization is nevertheless detectable at low levels by reverse transcriptase-polymerase chain reaction (RT-PCR) in these homozygous mutant mice, and the deletion does not encompass coding exons (Semina et al., 2000; Rieger et al., 2001). Additionally, mu-

tation of human *PITX3* results in anterior segment mesenchymal dysgenesis and autosomal-dominant congenital cataracts (Semina et al., 1997, 1998). In both of these studies, the mutations reside outside of the sequences encoding the homeodomain, and the resulting protein likely retains partial function.

We isolated a *Xenopus Pitx3* homolog, which plays a role in lens placode development. Expression patterns of the gene have been described previously (Pommereit et al., 2001); however, we have noted additional regions of expression. Overexpression or inhibition of *Pitx3* function early in development alters the size of the medial pre-optic field as assessed by the markers *Pax6* and *Six3*. Phenotypes include microcephaly and abnormal eye development and are indistinguishable from morphologies that might be expected to arise from midline disorders. Interference with endogenous *Pitx3* activity by injection of an in vitro-transcribed *engrailed* repressor/*Pitx3* chimera results in abrogation of lens formation, and in the absence of this critical cue, further prohibits induction of the retina. This outcome is phenocopied at higher penetrance by morpholino-mediated translational knockdown, suggesting that the normal role of *Pitx3* is that of a transcriptional activator. Reciprocal graft experiments demonstrate that *Pitx3* must be active in the presumptive lens ectoderm in order for lens to form and for retina to be induced. *Pitx3* is necessary but not sufficient to induce lens.

RESULTS

Molecular Cloning and cDNA Analysis

A clone containing the complete open reading frame of *xPitx3* (Genbank accession no. AF265671, 2000) was isolated and characterized. Although there are minor allelic differences in the nucleotide sequence, the conceptual protein is identical to a second *xPitx3* reported by another laboratory (Pommereit et al., 2001).

Expression Pattern of *xPitx3* During Early Development

Spatial and temporal expression of *xPitx3* was examined by whole-mount

in situ hybridization and by RT-PCR. Although expression in the pituitary and lens primordia has been described previously in *Xenopus* (Pommereit et al., 2001), we found that the gene was also expressed in additional tissues in a pattern more consistent with the activity of *Pitx3* seen in rodents (Semina et al., 1997; Smidt et al., 1997). Analysis by RT-PCR indicates that the gene is expressed slightly before gastrulation and then appears to substantially up-regulate at mid-neurula stages (Fig. 1). The earliest expression of *xPitx3* mRNA detected by whole-mount in situ hybridization was evident at the late neurula stage in the sensorial layer of anterior ectoderm. Figure 2A,B shows the early expression of *xPitx3* in the stomodeal-hypophyseal and the eye field, as well as below the margin where the cement gland will form. In cross-section, it is apparent that *xPitx3* is expressed in the eye field, prechordal plate, and ventral anterior ectoderm (Fig. 3A). Prechordal plate contributes to the extraocular tissues described as *Pitx3* expressing in mice (Semina et al., 1997). Expression in the developing pituitary persists (Figs. 2C,D, 3B), and *xPitx3* expression becomes increasingly prominent during the development of the lens (Fig. 2D,E). In the mid- to late neurula stages, *Pitx3* is faintly detected in presumptive oral plate (Fig. 2B and data not shown), which is distinct from the patterns of expression for either *Pitx1* or *Pitx2* (Campione et al., 1999). Because this pattern is distinct from the other two *Pitx* genes, and *Pitx3* does not express in cement gland, cross-hybridization is not a concern. During the late phase of lens induction, *xPitx3* is detected in the presumptive lens ectoderm, the part of the head ectoderm that has come into close apposition with the optic vesicle (Fig. 3C). We do not see expression on the archenteron roof and speculate that this observation could have been an artifact of trapping in a previous study (Pommereit et al., 2001). Expression continues in the lens ectoderm as it thickens and gives rise to the lens placodes. By stage 30, *xPitx3* is highly expressed in the developing lens and pituitary (Figs. 2D, 3D,E), and later, *xPitx3* is expressed in the otic vesicles, somites, branchial

arches, and lower jaw region (Fig. 2E,F). Up to this point, the expression of the gene is similar to the patterns of *Pitx3* expression described in mouse (Semina et al., 1997; Smidt et al., 1997) but for one respect: *Xenopus Pitx3* transcripts are detectable in the pituitary. When the head expands and the lenses have formed, *Pitx3* expression disappears from the lens and becomes asymmetrically expressed in the looping gut and heart. Sense controls demonstrate little in the way of background staining (Fig. 2G); however, by stage 43, *xPitx3* is expressed in the heart (Fig. 2H,I), the most distal region of the stomach and strongly on the right side of the growing intestine (Fig. 2H). Finally, as the intestine forms a double-coiled structure, the expression *xPitx3* is restricted to the end of the second coil as it loops clockwise and forms a S-shaped structure (Fig. 2I).

Overexpression of *xPitx3* and Its Effects on Eye Development

The early expression pattern of *xPitx3* and its persistence throughout lens development suggests a role for the gene in lens induction. To study the role of *xPitx3* during eye development, we performed gain- and loss-of-function experiments by overexpressing or inhibiting *xPitx3* by mRNA injection (10–300 pg) and morpholino knockdown in *Xenopus* embryos (Fig. 4). Ectopic expression resulted in developmental defects in the eye and anterior brain regions leading to craniofacial malformation (compare control Fig. 4A with 4B–E). The severity of defects elicited was dose-dependent until the amounts of RNA injected reached 100 pg or more. Beyond these levels, anterior structures were hard to interpret because of the severity and complexity of phenotypes observed. These high-dose embryos frequently displayed extreme underdevelopment of anterior head and bent body axes (59%; n = 200). Occasionally, misexpression of *xPitx3* induced lobulate structures attached to the eye (Fig. 4B; 1–2% of the time). The most common phenotype observed in embryos injected at the one-cell stage was a diminished diencephalon that was frequently associated with an extension of retinal pigmented epi-

thelium (RPE) toward the midline (Fig. 4C,D; for example, at 50 pg, 39.6%; n = 225). In more severe cases, the retinas formed more medially and even fused to each other at the expense of extensive reduction of the diencephalon (Fig. 4E). At 50 pg injected, this midline reduction was so extreme that cyclopia developed 15.6% (n = 225) of the time. We were surprised to find that *xPitx3* also induces the formation of ectopic cement glands (24%, n = 180; 35%, n = 225; and 78%, n = 212 of the time with 25, 50, and 100 pg *xPitx3* injected, respectively).

Inhibition of normal *xPitx3* activity by injection of a dominant-negative repressor chimera (150 pg *engrailed-Pitx3*) or by morpholino-mediated translational knockdown resulted in diminished eye structures and, in extreme cases, inhibition of eye development (Fig. 4F). To determine whether *xPitx3-EnR* injection specifically interferes with endogenous *xPitx3* function, we coinjected both transcripts into one-cell stage embryos. Eye defects caused by the mutant construct can be rescued by overexpression of the wild type transcripts. For example, in chimera-injected embryos (150 pg of *xPitx3-EnR*), 32% developed hypomorphic eyes and 14% lacked eyes entirely. When complemented with wild-type RNA (150 pg of *xPitx3-EnR* and 100 pg of *xPitx3* RNA), these deficits fell to 23% hypomorphic and 8% lacking. In terms of all deficits including axial perturbations, craniofacial anomalies and so on, wild-type RNA improved the proportion of embryos that developed normally from 20% to 37%. These results support the specificity of the chimeric protein.

Morphologies of control morpholino-injected embryos were normal insofar as head and eye morphology were concerned; however, *Pitx3*-directed morpholino-injected embryos displayed morphologies identical to those seen after injection of the *xPitx3-EnR* repressor construct: inhibited lens and retina development ensued, but at higher frequency. Morpholino action appears specific as assessed by in vitro assays (Fig. 5).

Sectioned specimens revealed an absence of differentiated retina either morphologically (Fig. 6A) or using markers likely to indicate the presence

of retina such as *Pax6* (Fig. 6B). The neural tube tended to look larger and was often irregularly shaped on the eyeless side, and this finding was reflected also in sections stained with riboprobe for *Pax6* and neural β tubulin (Fig. 6C).

Effects of *Pitx3* Manipulation Upon Expression of Eye Genes

Ectopic *xPitx3* (50–150 pg) results in ectopic expression of the medial stripe and a broadening of the *Pax6* expression domain in the anterior crescent at the early stages of development (Fig. 7A). At later stages, *Pax6* expression was up-regulated in the forebrain and was expanded in developing eye toward the midline (Fig. 7A'). Conversely, *Pitx3*/*engrailed* repressor (150 pg) or morpholino (18 pg) injection resulted in inhibited expression of *Pax6*—more markedly so with morpholino (Fig. 7B,B',C,C').

Ectopic *xPitx3* changes the early expression domain of *Six3*, where the transcripts appear to be enhanced medially and diminished distally (Fig. 7D). Nevertheless, in later stages of development, *Six3* expression in the eye was reduced but not abolished (Fig. 7D'). The chimeric construct and morpholino caused *Six3* expression to expand dorsally at both early and late stages (Fig. 7E,E',F,F').

Ectopic *xPitx3* has disparate effects on the expression of *Rx1* expression: it was reduced in presumptive retina regions (Fig. 7G) at neurula stages, however, its expression was expanded at the later stages when the optic vesicles were formed (Fig. 7G'). Repressor- and morpholino-mediated inhibition have the reverse effects (Fig. 7H,H',I,I').

Ectopic *xPitx3* did not induce changes in *Lens1* expression at the early stages (Fig. 7J), but an increase in intensity of transcript was observed in the developing eye at later stages (Fig. 7J'). Inhibition resulted after both repressor and morpholino treatment (Fig. 7K,K',L,L').

Otx2 expression in *xPitx3*-injected embryos was only marginally perturbed: *Otx2* expression appeared somewhat dispersed in neural tissues

while expression in the cement gland anlage was slightly enhanced (Fig. 7M). These early perturbations are consistent with the pattern of enlarged and ectopic cement gland inductions: *Otx2* itself induces cement gland. By early tail bud stages, *Otx2* expression normalized (Fig. 7M'). Repressor and morpholino inhibited *Otx2* but appeared to inhibit and induce diffuse expression dorsally (Fig. 7N,N',O,O'). Curiously, this inhibition was seen to a lesser degree on the control side as well (compare Fig. 7N, O to M).

Finally, we analyzed the effects of *xPitx3* misexpression on maturing lenses by using β B1-crystallin probe in whole-mount in situ hybridization. Ectopic *Pitx3* had little effect upon β B1-crystallin in lens- however, the chimeric construct and the morpholino diminish or abrogate expression of β B1-crystallin on the injected side of the embryos (Fig. 7P,Q).

Pitx3 Induces Eye Marker Genes in Ectoderm Cap Explants

To further investigate the role of *Pitx3* in altering expression of other genes important to eye development, embryos were injected with *Pitx3* or control mRNA near the animal pole at the one-cell stage. At blastula, the animal caps were removed and cultured for two days. Uninjected caps, or animal caps injected with β -galactosidase mRNA fail to exhibit expression of the eye marker genes *xPax2*, *xPax6*, β -B1-Crystallin, *xSix3*, or *xRx1*. Similarly, animal caps probed for expression of *Xbra* (*brachyury*) demonstrate an absence of mesoderm (Fig. 8A). When animal caps are derived from embryos first injected with *Pitx3* mRNA, several eye markers are elicited, including *Pax2*, *Rx1*, *Lens1*, *Crystallin*, and *Six3* (Figs. 8B–E), but not *Pax6* (Fig. 8F). Other markers of anterior and/or eye development such as the hatching gland markers *XCG* and *Otx2*, as well as lens differentiation marker β -crystallin are induced to express by *Pitx3* (Fig. 8G–I). *Pitx3* is in turn inducible by both *Lens1* and *Pax6* (Fig. 8J,K).

Morpholino-Mediated Knockdown of Pitx3 Inhibits Ectodermal Graft Competence to Induce Eye Structures

To define whether antisense morpholino inhibition of *Pitx3* was mediated specifically through effects on ectoderm, morpholino-injected and control embryos were used to provide reciprocal swaps of presumptive lens ectoderm. Fluorescein-tagged morpholino was used to provide a lineage marker to gauge the efficacy of the surgeries (Fig. 9). *Pitx3* morpholino-treated ectoderm is not competent to yield lens and induce retina when grafted into wild-type embryos. By comparison, wild-type ectoderm transplanted to morpholino-injected host gives rise to normal eye development. Finally, ectopic *Pitx3* is insufficient to induce flank ectoderm to achieve competence to form lens (Table 1). Grossly sectioned embryos demonstrate neither contamination nor mixing of grafted ectoderm with host tissues (Fig. 10).

DISCUSSION

The frog *Pitx3* gene encodes a conceptual amino acid sequence with high conservation to previously described mammalian homologs. Nevertheless, although the gene appears to be expressed in many of the same tissues

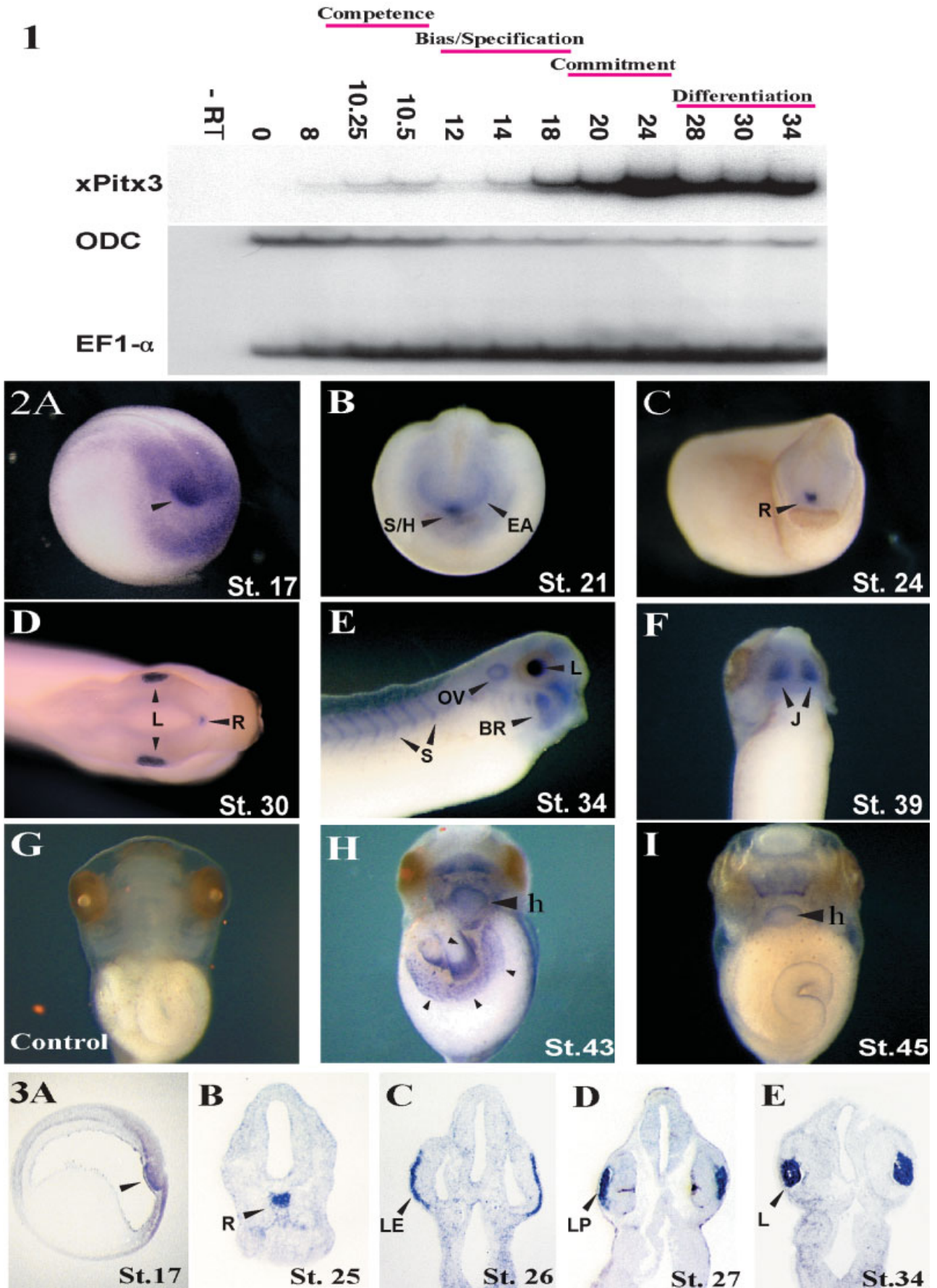
(lens, somites, and branchial arches; Semina et al., 1997; Smidt et al., 1997), there are some regions of expression unique to frog such as the anterior ectoderm which borders the folding neural plate, and the ectoderm which ultimately gives rise to the pituitary gland. We have discovered that the gene is expressed in a few regions additional to the pituitary primordium and lens placode reported by Pommereit et al. (2001): *Pitx3* also expresses in the early eye field, the lateral regions of prechordal plate, and ectoderm below the presumptive cement gland region during neurula stages. In addition, there is symmetrical low-level and general expression in the lateral plate mesoderm, at neurula stages, and a period of asymmetrical expression in looping heart and gut after stage 41. Riboprobe in situ hybridization patterns were identical irrespective of whether 5' *xPitx3* probe (excluding homeobox) or complete sequence were used. Furthermore, although *Pitx* gene family members overlap in the pituitary gland, *xPitx3* expression domains were distinct in other respects from the patterns revealed for *Pitx1* and *Pitx2*. For example, *xPitx3* probe was never found in the cement gland (*Pitx1*) or in a band of left lateral plate mesoderm (*Pitx2*).

Pitx3 expression is detectable by RT-PCR in late stage blastulae, earlier than *Pax6*, *Rx*, and *Lens1* but not *Six3* (Kenyon et al., 1999; Zuber et al.,

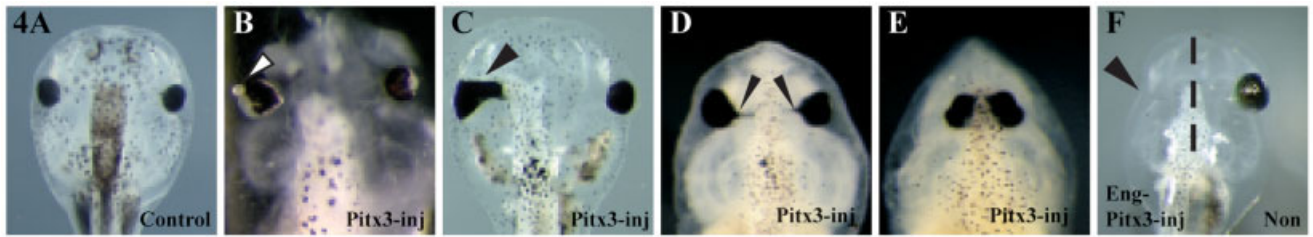
Fig. 1. Temporal expression of *xPitx3* analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR), and its correlation with different phases of lens induction. *xPitx3* mRNA is detected in the mid-blastula and early gastrula during the period of early lens competence (stage [St.] 9–11.5). *Pitx3* expression is sustained throughout the late gastrula and neural plate stage, concurrently with lens bias/specification events (St. 12–19). Its expression appears to be significantly up-regulated slightly before the neural tube stage (St. 19) and persists through lens commitment (St. 19–26) and differentiation (St. 26–34). Controls represent transcription detectable for ornithine decarboxylase (ODC) and elongation factor 1-alpha (EF1- α).

Fig. 2. Spatial expression pattern of *xPitx3* assayed by whole-mount in situ hybridization. **A–C:** Early stage embryos seen laterally from a slightly anterior perspective (right) in (A), head on (B), and from the right side with the head twisted to face out on the right (C). **D:** Anterior top view of a stage 30 embryo showing that *xPitx3* is highly expressed in the differentiating lens and Rathke's pouch (the cement gland is at the right). **E,F:** A lateral view (E) and a ventral view (F) of a tadpole at stage 39. *xPitx3* is expressed throughout heart and gut development. **G–I:** Sense control embryos (G) reveal no hybridization of probe, whereas both gut and heart display asymmetrical labeling during looping of both organs (H,I). BR, branchial arch; EA, eye anlage; J, jaw musculature; L, lens; OV, otic vesicle; R, Rathke's pouch; S, somite; S/H, stomodeal-hypophyseal anlage; h, heart.

Fig. 3. **A–E:** Parasagittal section (A) and anterior transversal sections (B–E) of whole-mount in situ hybridization of *xPitx3* during the pituitary and eye development. A: Anterior is oriented right, and the arrowhead indicates the prechordal mesoderm and eye anlage region. R, the pituitary primordium–Rathke's pouch; LE, lens ectoderm; LP, lens placode; L, lens.



Figs. 1-3.



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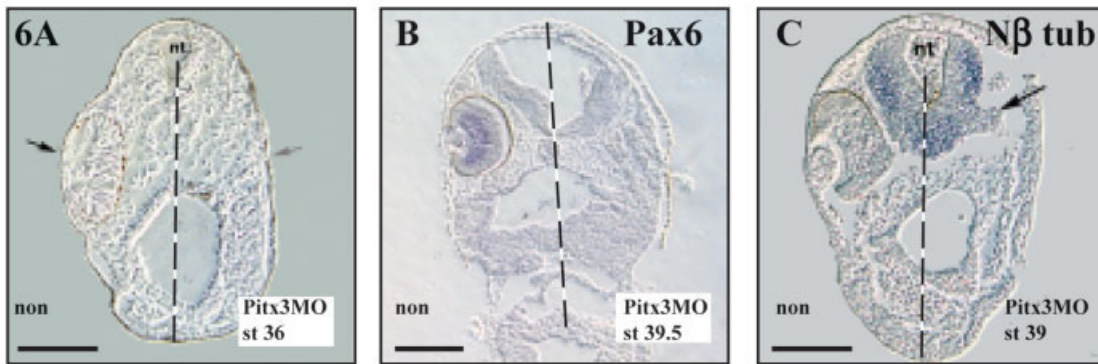
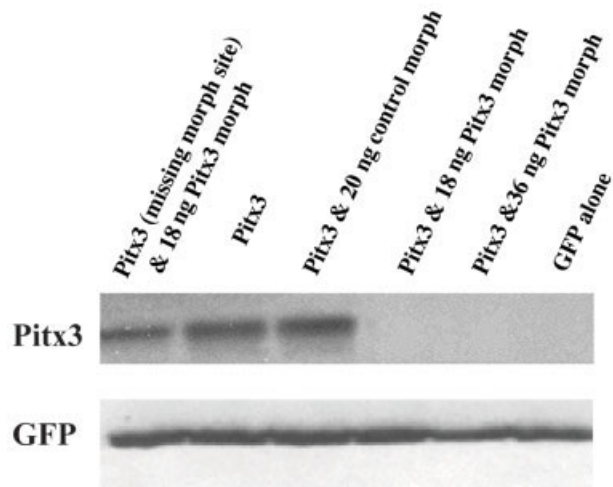


Fig. 4. B–F: Dorsal view of tadpoles injected with *xPitx3* at the one-cell stage (B–E), and *xPitx3*/engrailed repressor chimeric RNA at the two-cell stage embryos (F). Overexpression of *xPitx3* induced severe eye defects. **A:** Uninjected control tadpole at stage 46. **B:** Anterior head reduction is here associated with an enlarged and multilobed lens (arrowhead). **C:** Extension of retinal pigmented epithelium (RPE) toward the midline is shown by an arrow. **D,E:** Colobomas is associated with reduction of the anterior brain structures (D), which is even more pronounced in E. **F:** Dorsal view of a *Xenopus* tadpole injected with *xPitx3-EnR* in one blastomere at the two-cell stage. Interference with normal *xPitx3* activity resulted in the absence of an eye on the injected side.

Fig. 5. Antisense morpholino oligonucleotides inhibit in vitro translation of *xPitx3*. The specificity of Pitx3 morpholinos (Pitx3-Mo) was examined by Retic Lysate in vitro translation system (Ambion), and radiolabeled products were analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Lane 1: Pitx3-Mo did not affect the translation of *Pitx3* mRNA lacking the Pitx3-morpholino-equivalent site. Lane 2 revealed that the Pitx3 construct containing the Pitx3-morpholino-equivalent site was synthesized in the absence of Pitx3-Mo. Lanes 3–5: Translation of *Pitx3* was blocked in the presence of Pitx3-Mo at 18 ng (lane 4) and 36 ng (lane 5) but not by a standard control oligonucleotide (lane 3). *xPitx3* is synthesized in the absence of the Pitx3 morpholino (Pitx3-Mo). Cotranslation of green fluorescent protein (GFP) was unaffected by Pitx3 morpholino.

Fig. 6. Sectioned specimens reveal an absence of differentiated retinal structures. Embryos were injected with Pitx3 morpholino at the two-cell stage on the right-hand side. **A,B:** Retinal structures present on the control side (black arrow) are not visible on the injected side at a morphological level in cross-sections (gray arrow) (A), or in longitudinally sectioned specimens probed for *Pax6* (B) and neural β tubulin. **C:** Note the additional neural tissue projecting unilaterally (black arrow). Nt, neural tube. Scale bar = 200 μ m.

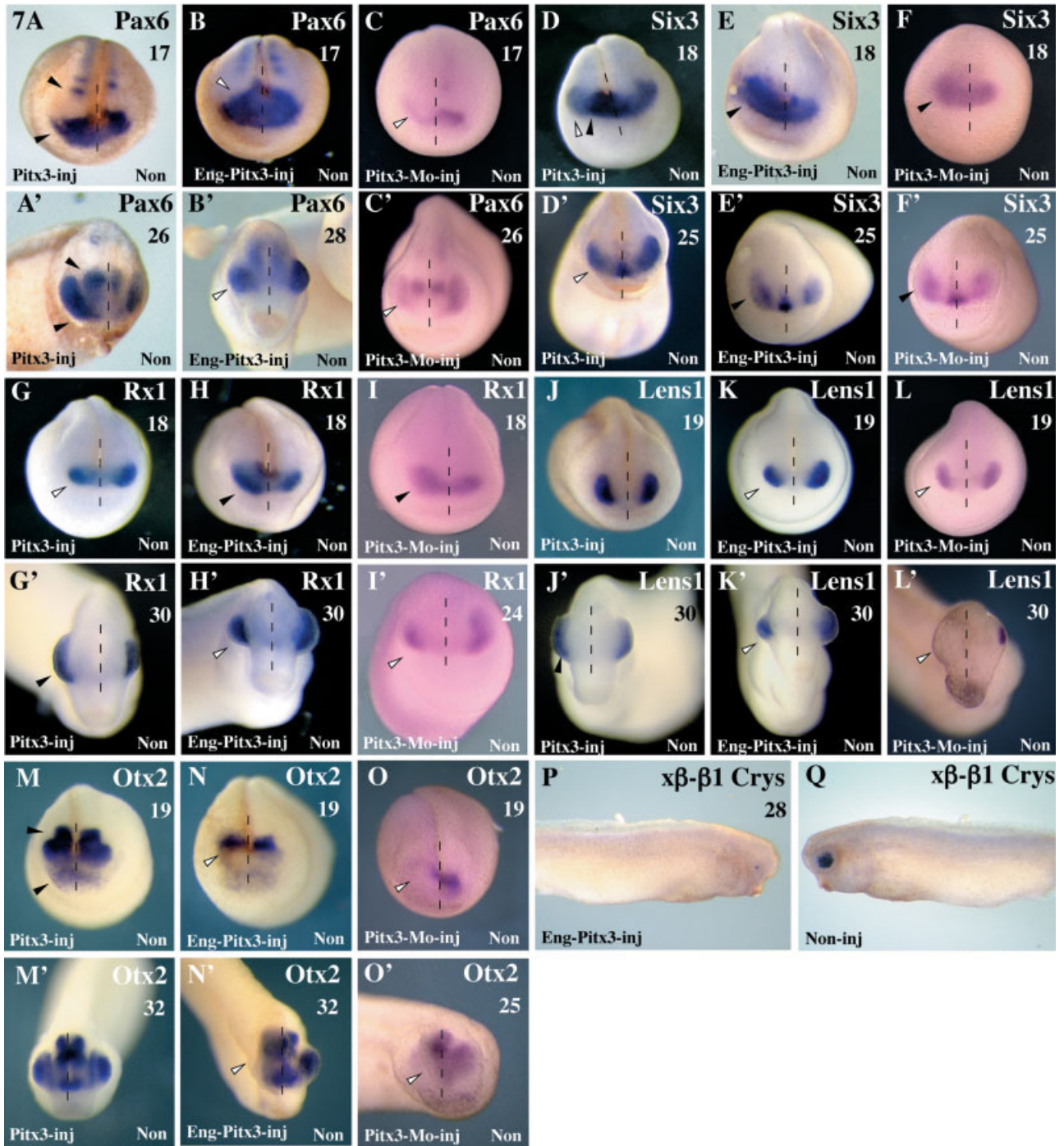


Fig. 7. Whole-mount in situ hybridization shows the effects of overexpression of *xPitx3* and *xPitx3/engrailed* repressor chimeric RNAs (Eng-Pitx3), as well as *Pitx3* morpholino oligonucleotides (Pitx3-Mo) on the expression of eye marker genes. The uninjected side of embryos (left side) was used as a contralateral control to compare the gene expression changes in the injected side. **A,D,G,J,M:** Anterior view of embryos microinjected with *xPitx3* wild-type RNA analyzed at the early (A,D,G,J,M) and later (A',D',G',J',M') stages of development. **B,E,H,K,N:** The expression patterns (anterior view) of these genes at both the early (B,E,H,K,N) and late stages (B',E',H',K',N') were examined upon overexpression of *Pitx3/engrailed* repressor chimera. **C,F,I,L,O:** Microinjection of Pitx3-Mo revealed similar effects on eye marker genes in their early (C,F,I,L,O) and later (C',F',I',L',O') expression as demonstrated by Eng-Pitx3 overexpression. **Q:** Lateral view of tail bud stage embryo showing the effect of the *xPitx3-EnR* overexpression on *Xenopus* β - β 1-Crystallin expression domain. **P:** The uninjected control side of the same embryo (lateral view). An increase and reduction of the expression domains of examined genes are shown with black and white arrowheads, respectively.

2003). Subsequently, the gene undergoes its most intense period of expression at the time that the lens placodes

begin to form. Even before the presumptive placodes thicken, *Pitx3* is expressed in ectoderm that overlies

the neural vesicles and in prechordal mesoderm. Expression continues through placode, lens pit, and lens

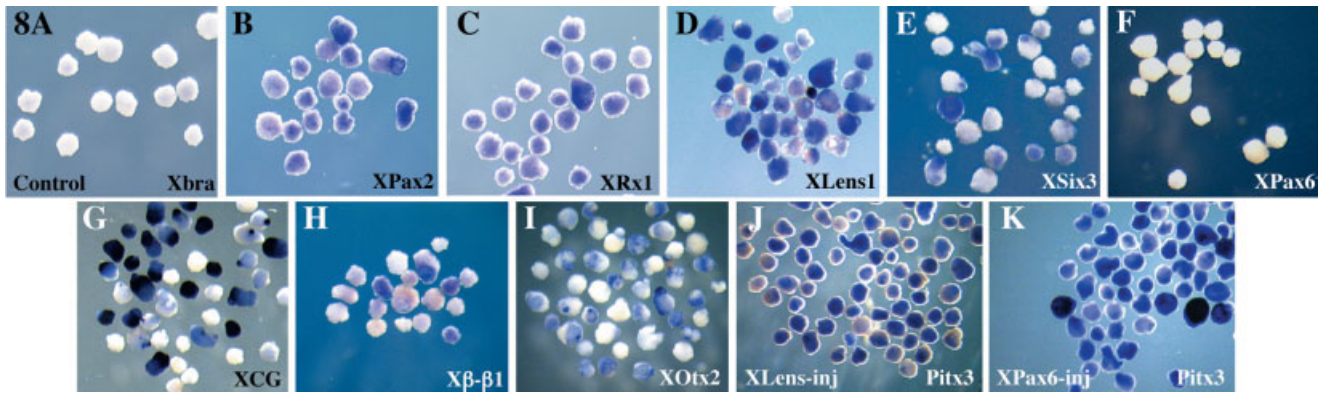


Fig. 8. Animal cap explant induction by *Pitx3*, *Lens1*, and *Pax6*. **A:** Uninjected animal caps probed with *Xbra* demonstrate the absence of mesoderm, because the pan-mesodermal marker fails to hybridize. **B–F:** However, injection of *xPitx3* transcript results in the induction of *Pax2*, *Rx1*, *Lens1*, and *Six3* but not *Pax6*. **G–I:** Similarly, *Pitx3*-injected embryo explants express the anterior markers *XCG* (cement gland), β -*B1-Crystallin* (lens), and *Otx2* (retina, brain). **J,K:** Both *Lens1*-injected (**J**) and *Pax6*-injected (**K**) embryo explants induce *Pitx3*.

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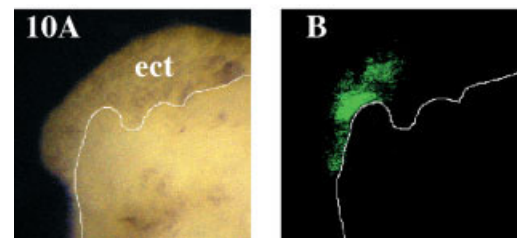
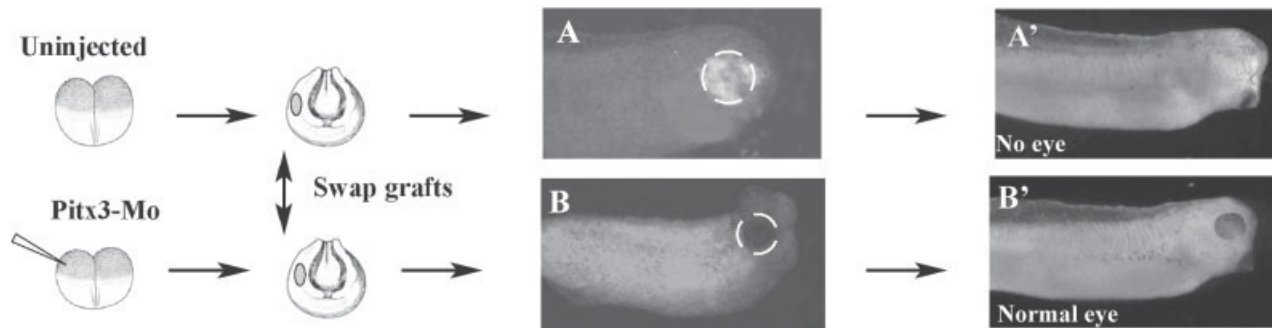


Fig. 9. Reciprocal transplantation of lens ectoderm verifies the significance of *xPitx3* during eye development. The schematic diagram illustrates the procedures involved in the transplantation of the presumptive lens ectoderm (PLE) between wild-type and *Pitx3* morpholino-injected embryos and vice versa. **A,A':** Inhibition of endogenous *Pitx3* prevented eye development in normal embryos grafted with PLE containing *Pitx3*-Mo. **B,B':** Wild-type PLE rescued eye formation in *Pitx3*-Mo injected embryos.

Fig. 10. Ectodermal grafts transplanted to presumptive eye regions show no evidence of contamination or mixing after transplantation. **A:** The white line demarcates the edge of ectoderm in grossly sectioned specimens. **B:** Ectoderm labeled with fluorescein isothiocyanate (FITC)-morpholino is visible under fluorescence microscopy. Ect, ectoderm.

vesicle stages of development but subsides shortly thereafter.

Ectopic *xPitx3* Induces Anomalous Eye Development

Overexpression of *Pitx3* transcript has profound effects upon craniofacial modeling, occasionally induces anomalous lens development, and

will also cause the formation of ectopic cement glands. *Pitx2* and *Pitx1* also induce supernumerary cement glands, and because the *Pitx3* gene is not normally transcribed in the cement gland, we hypothesize that an identical amino acid sequence in the homeodomain permits ectopic *Pitx3* to mimic *Pitx2* and *Pitx1* in this regard (Chang et al., 2001; Faucourt et

al., 2001). A common phenotype results in a diminished diencephalon, frequently in association with extension of the pigmented retina to the midline. In extreme cases, only a single anteriorly positioned eye forms. A similar morphological elongation of the pigmented retina at the expense of diencephalon has been seen in mutants of other genes that ex-

TABLE 1. Effects of Treatments^a

Treatment	n	Normal eye	Hypomorphic eye	Absent eye
A. Control embryos hosting with wild-type PLE	30	93.3%	6.7	0%
B. <i>Pitx3</i> -MO-injected embryos hosting wild-type PLE	32	43.8%	56.2%	0%
C. Control embryos hosting <i>Pitx3</i> -MO-injected PLE	45	11.1%	55.6%	33.3%
D. Control embryos hosting <i>Pitx3</i> -injected flank ectoderm	6	0%	0%	100%
E. <i>Pitx3</i> -MO injected embryos (18 ng, two-cell stage)	89	15.7%	45%	39.3%
F. Control MO-injected embryos (20 ng, two-cell stage)	114	97%	3%	0%

^aPLE, presumptive lens ectoderm; MO, morpholino.

hibit periods of expression in the developing eye such as *Pax2*, *BMP-4*, and *Pitx2* (Sanyanusin et al., 1995; Schimmenti et al., 1995; Torres et al., 1996; Gage et al., 1999). Significantly, similar retinal anomalies have also been described in animals that have experienced abnormal midline development (Macdonald et al., 1995; Chiang et al., 1996; Ahlgren and Bronner-Fraser, 1999; Agarwala et al., 2001). We note that no such similar anomalies have been reported after *Pitx2* or *Pitx1* overexpression; however, these defects likely reflect another function of *Pitx3* because they could also be ascribed to midline perturbations induced by ectopic expression.

Ectopic *xPitx3* Alters Expression of Eye Patterning Genes

When *Pitx3* is overexpressed in only one cell of a two-blastomere embryo, the uninjected blastomere serves as a contralateral control. When embryos were assessed for the activity of marker genes early during definition of the retinal and lens fields, it was apparent that there were, broadly speaking, two effects: those that reflected perturbation of midline specification and those that elicited a temporary enlargement of the lens field. For two of the markers, ectopic *Pitx3* had the effect of enlarging expression domains: *Pax6* expanded distally and medially and *Six3* medially. *Otx2* seems moderately up-regulated throughout. Additionally, although *Pax6* expression was possessed of the same number of bands in the developing brain, these bands were sometimes anteriorly shifted on the *Pitx3*-treated side (Fig.

7A). (We have discounted the possibility that this finding is due to a suppression specifically of the extreme-most dorsoanterior regions using *noggin* as an indicator—ectopic *Pitx3* appears to have no effect on expression of this gene—data not shown.) *Rx1* is slightly down-regulated, but *Lens1* expression patterns were relatively unaffected. In contrast, during the later stages that encompass lens development, *Pax6*, *Lens1*, and *Rx1* were up-regulated, while *Six3* was somewhat suppressed, and *Otx2* was relatively unaffected. Generally, those genes that enjoy a period of medial expression in the unbifurcated lens field before stage 18 were up-regulated (Altmann et al., 1997). Later, in the period after eye field bifurcation, genes either went up or down depending upon whether or not they expressed predominantly in distal (lens and retina) or more medial structures (optic disc, stalk, and brain), respectively (Kenyon et al., 1999; Bernier et al., 2000; Zhou et al., 2000; Chuang and Raymond, 2001).

Manipulation of animal cap explants by means of *Pitx3* overexpression suggests that the gene can act to induce both early (*Rx*, *Pax2*) and late (*Lens1*, β -*B1-crystallin*) eye genes. The gene cannot induce activation of *Pax6*; however, *Pax6* can induce expression of *Pitx3*. Possibly, given its early expression pattern, *Pitx3* acts upstream of *Rx*, *Lens1*, and it may operate parallel to the *Pax6* inductive pathway. At later stages, it is likely that both *Lens1* and *Pax6* act to refine *Pitx3* expression.

We speculate that *Pitx3* mimics the role of other family members such as *Pitx1* or *Pitx2* in cement gland formation and consequently affects the expression levels of *XCG*, and *Otx2* in

induced tissues and in explants (Chang et al., 2001; Schweickert et al., 2001).

We wondered, given that the gene was sufficient to induce eye markers, whether it was also necessary. Therefore, *Pitx3* activity was inhibited either through activity of an *engrailed* repressor or by morpholino-mediated translational knockdown.

xPitx3/*engrailed* Repressor Construct and *Pitx3* Morpholino Abrogate Lens Development

Predictably, expression of an *engrailed* repressor/*Pitx3* chimera had the effect of inhibiting lens formation; however, it also removed the conditions necessary for formation of a retina: eye development can be completely inhibited. This effect is identical in phenotype but occurs more frequently with morpholino treatment, presumably reflecting a greater ease of diffusion and persistence in injected embryos. The repressor and morpholino appear to exert the reverse effect of wild-type *Pitx3*: *Pax6* expression is inhibited around the margins (especially so with the morpholino), whereas *Rx1* and *Six3* expression is elevated at early stages but diminished later. Both the morpholino and the repressor chimera have the longer term effect of inhibiting expression of lens and retinal markers on the injected side during the stages at which lens and retina commence differentiation. Late expression of *Pax6*, *Rx1*, *Lens1*, *Otx2*, and β -*B1-crystallin* defined diminished domains on the side of embryos injected with the morpholino or repressor construct. *Six3* expression

was slightly diffuse. Curiously, the repressor- and morpholino-treated embryos exhibited a slight down-regulation of *Otx2* on the control side relative to stage-matched ectopic-expressing and published expression patterns. We interpret this finding to reflect a midline patterning perturbation, which frequently resulted in anomalous brain, segmentation, and laterality development (data not shown).

Pitx3 overexpression might expand or prolong the contiguity of the anterior eye field with the result that midline structures fail to develop normally. This process could explain the development of abnormally extended retinas and of pin-headed cycloptic embryos. By contrast, in the instance of the morpholino and repressor chimera, suppression of lens development means that even though the other players in eye development might be expressed, appropriate structures cannot form because critical inductive interactions are lacking, i.e., the lens primordia fail to form. Reciprocal grafts between morpholino and wild-type embryos demonstrate that *Pitx3* activity in the pre-placodal lens ectoderm is critical to the competence of these cells, and consequently to the ensuing mutual differentiation of lens and retina: inhibition of normal *Pitx3* function at this juncture abrogates eye development. Conversely, competent pre-lens ectoderm can rescue lens and retina formation in morpholino-injected embryos. Pre-placodal expression of *Pitx3* in the ectoderm is therefore required for lens differentiation. By contrast, flank ectoderm-expressing ectopic *Pitx3* fails to form tissues that form lens or induce retina—clearly, *Pitx3* expression alone is insufficient to induce lens. Furthermore, even when wild-type presumptive lens ectoderm was grafted to morpholino-inhibited embryos, many of the eyes that formed were hypomorphic. Presumably, *Pitx3* expression in other regions such as the prechordal plate must exert an influence over the modeling of induced structures. These latter two observations are consonant with observations by others that several genes and tissues acting in concert are necessary for lens and retina induction (Zuber et al., 2003). Finally, because the mor-

pholino and repressor phenotypes represent merely different degrees of the same general phenotype, *Pitx3* must act as a transcriptional activator. This explanation is consistent with at least one documented instance showing that *Pitx3* activates mouse tyrosine hydroxylase promoter (Lebel et al., 2001).

Lens Differentiation Is Critical to Induction of the Retina

Recent studies have suggested that abrogation of *Pax6* expression in lens placodes prohibits lens differentiation, but nevertheless, retina can still form: lens is not necessary to induce retina (Ashery-Padan et al., 2000). It is worth noting that despite the absence of a differentiated lens in the aforementioned *Pax6* lens-mutant mice, surface ectodermal cells express pre-lens markers such as *Sox2*. By contrast, grafted *Pitx3*-deficient presumptive lens ectoderm also fails to form lens; nevertheless, differentiation of retina appears to be prohibited. Although lens placode and optic vesicle formation are the first visible sign of eye development, pre-placodal lens ectoderm already expresses factors critical to specification and induction. *Pax6*, *Lens1*, and *Pitx3* are expressed in pre-placodal lens ectoderm and *Six3* and *L-Maf* and three of the *Sox* genes are expressed shortly after as the placode begins to form (Ogino and Yasuda, 2000). Many of the transcription factors that regulate eye development are thought to both auto- and cross-regulate and to act in concert (Mathers et al., 1997; Zhang et al., 2000; Zuber et al., 2003). Because *Pitx3* does not induce *Pax6* in *Xenopus* explants, *Pitx3* is unlikely to be immediately upstream of this gene in the pathway leading to lens development. That said, *Pitx3* perturbation exerts an effect upon *Pax6*, *Six3*, *Lens1*, and *Rx* expression domains in vivo, presumably in the first case, at least, by indirect means.

Pax6 lens-mutant mice fail to form a placode but nevertheless form a retina (Ashery-Padan et al., 2000). Because *Pitx3* morpholino-treated embryos form neither lens nor retina, one of two circumstances must exist. Either *Pitx3* expression in pre-placodal ecto-

derm must be necessary and sufficient to stimulate retina formation, or *Pitx3* is nevertheless inducible by other factors in the *Pax6* lens-mutants despite the failure of lens placode formation (Ashery-Padan et al., 2000).

Because *aphakia* mice exhibit at least detectable levels of *Pitx3* transcript despite deletion of promoter elements (Semina et al., 2000), we might speculate that even a diminished presence of *Pitx3* in ectoderm could be sufficient to elicit or sustain the expression of other eye transcription factors, thereby permitting the induction of neural retina in a manner similar to induction in *Pax6* lens-mutant mice (Ashery-Padan et al., 2000). Similarly, however impaired the rest of the translated product might be, extant human *Pitx3* mutants encode proteins with an intact homeodomain. Possibly, for induction of retina to be inhibited, *Pitx3* activity in lens-competent ectoderm must be completely abrogated—a circumstance achievable in morpholino-treated frogs but not in the naturally occurring mammalian mutants characterized thus far. Alternatively, the neurula stage expression of *Pitx3* in the eye field is unique, and signifies a mode of eye induction that is distinct from mammals. Discrimination between these two possibilities will have to await the description of a *Pitx3* knockout mouse.

Pax6 and *Six3* reciprocally regulate (Goudreau et al., 2002), and in frog, *Six3* expresses before *Pax* (Zuber et al., 2003). Additionally, *Pitx3* stimulates *Lens1*, *Rx1*, and *Six3* in animal cap explants. Moreover, *Pitx3* is expressed early during gastrulation, after *Six3* is detectable, coincident with *Pax6*, and before expression of *Rx1*. Taken in combination, it seems likely that *Pitx3* operates at a nexus in a network of reciprocally interacting and regulating genes where parallel pathways might operate (Zuber et al., 2003). Most models place *Six3* downstream of *Pax6* (Wawersik et al., 1999; Zuber et al., 2003); however, *Six3* may not merely be a downstream effector of *Pax6* activity, but a parallel and interacting signaler, and one to which *Pitx3* is likely to be more directly and reciprocally linked. *Pitx3* knockdown embryos, in losing retinal development, mimic *Six3* rather than *Pax6* mutant mice. In the coming months,

our agenda will be to identify the relationship between *Six3* and *Pitx3*.

EXPERIMENTAL PROCEDURES

Library Screen and Cloning

Following a preliminary screen of a *Xenopus* stage 28–30 head cDNA library (Hemmati-Brivanlou et al., 1991), a *Xenopus* head and heart cDNA library (stage 28–35) was constructed in commercially prepared vector (Stratagene) and screened by PCR with T3 and a forward internal primer to obtain the missing 5' end of the truncated clone. A 500-base pair fragment was amplified and sequenced, which showed a high similarity to human and murine *Pitx3* N-terminus. This fragment was used as a probe to screen the library for the full-length open reading frame of *xPitx3*. One positive clone was isolated and sequenced which contained the complete open reading frame of *xPitx3* (accession number AF265671).

An expression construct was derived using Vent polymerase (New England Biolabs) and primers (5' end TAG CCC AGG ATC CTT TTA ACA, 3' end GCT CTA GAT CAT ACT GGC CGA TCC), which bracketed the open reading frame and possessed restriction sites for *EcoRI* and *XbaI* at the 5' and 3' ends, respectively, and which facilitated insertion into pCS2-. A dominant repressor construct was made by cloning a Vent-amplified fragment into the *XhoI/XbaI* site of the engrailed repressor ENG-N plasmid (kind gift of M. Kessler; oligonucleotide sequences 5' ATT CCT CGA GCC ACA TGG ATT TCA ATC TTC, 3' GCT CTA GAT CAT ACT GGC CGA TCC A). A sequence immediately 5' of the *Pitx3* start codon was identified as ideal for morpholino-mediated translational knockdown, ATTCCCTCAAC-CAGGATTAGCCCA, and *Pitx3*-expressing clones were assembled in pCS2 for specificity tests. The clones were identical save for the presence or absence of the morpholino site. Constructs were verified by sequencing.

Embryos

Embryos were fertilized, dejellied in 2% cysteine, and cultured as previ-

ously described (Drysdale and Elinson, 1991). Developmental staging was according to Nieuwkoop and Faber (1967). Animals were reared and used in accordance with University, Provincial, and Federal regulations.

Microinjection

Synthetic capped mRNA of *Pitx3*, *Eng/Pitx3*, and/or *Green Fluorescent Protein (GFP)* transcript was made from linearized template using mMessage Machine (Ambion) driven by a SP6 promoter. Capped mRNA or morpholino was resuspended in water and injected into embryos with a Drummond nanoinjector. Injections were made into the animal pole of embryos at either the one-cell or two-cell stages. Concentrations of the capped mRNA injected ranged from 60 pg to 1.2 ng. Injection volumes never exceeded 9.2 nl. Injected embryos were cultured in $1.0 \times$ MBS and 2% Ficoll-400 (Sigma) at 13°C for at least 1 hr to allow healing before being removed and allowed to develop at room temperature. At this point, the solution was changed to $0.1 \times$ MBS. When injected embryos were intended for comparisons of one treatment to a control, the embryos were injected in one blastomere at the two-cell stage with the transcript of interest and GFP marker for identification and separation later. The contralateral side served as a control. For translation knockdown assays, the *Pitx3* antisense morpholino oligonucleotide sequence was TGG-GCTAATCCTGGTTGAAGGGAAT, and the control sequence was CCTCTTAC-CTCAGTTACAATTTATA. Morpholino specificity was assessed by analyzing in vitro translated [³⁵S]methionine labeled product (Retic Lystate, Ambion) that had been run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis apparatus according to standard protocols. *Pitx3* expression was assessed after addition of *Pitx3*-directed morpholino or control morpholino using constructs that either possessed or lacked the target sequence. In vitro transcribed/translated GFP served as an internal control for expression levels.

RT-PCR

Embryos were stored in batches of 10 in RNA later (Ambion) until all de-

sired stages had been collected. Purifications of RNA from each of the stages were done in parallel using oligo dT-polystyrene beads (Sigma DMN-10). From each of the sampled stages, mRNA equivalent to one embryo was withdrawn and cDNA was synthesized in the presence on RNasin (Promega) using reverse transcriptase according to the manufacturer's instructions (Omniscript, Qiagen). One-fifth volume of this reaction was used as template for amplification. PCR conditions were determined empirically to establish the linear range of amplification for *xPitx3*. Reactions were accomplished using a thermostable polymerase in 10 mM Tris (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 3 mM MgCl₂, 0.2 mM dNTPs, 0.1 mM [³²P]dCTP, and 1 μM of each primer (*Pitx3*-AAGTCCGTTGTCATCACA and CTTCTGGAAAGTGGAGCA; *EF1-α*-CAGATTGGTGTCTGGATATG and ACTGCCTTGATGACTCCTA; ODC-G-TCAATGATGGAGTGTATG and TC-CATTCCGCTCTCCTGA). Initial denaturation was for 3 minutes at 94°C, and cycling parameters were repeated 29 times at 94°C for 45 sec, 57°C for 1 min, and 74°C for 45 sec. One tenth of each reaction was run out on 4% polyacrylamide in $0.5 \times$ TBE and then monitored by autoradiography.

Ectodermal Cap Culture

Ectodermal explants were removed and cultured as previously described (Sive et al., 2000). Explants were then removed to $0.1 \times$ MMR and cultivated until they reached the stage at which sibling intact control embryos had developed prominent optic vesicles (stage 28 or later). Explants were then fixed and processed for in situ hybridization as described.

Whole-Mount In Situ Hybridization

In situ hybridizations were performed according to established protocols (Harland, 1991) using digoxigenin labeled riboprobes.

Grafting Presumptive Lens Ectoderm

Wild-type and *Pitx3* antisense morpholino-injected embryos were re-

moved to $0.5 \times$ MBS at stage 15, and ectoderm from the presumptive lens region was excised from adjacent embryos, and the grafts were exchanged. Embryos were then incubated at 17°C until scored.

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