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

Farhad Khosrowshahian,1 Marian Wolanski,1 Wing Y. Chang,2 Kazuhiro Fujiki,3 Larry Jacobs,1 and Michael J. Crawford1*

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 gastrulation (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 differentiates into lens. Presumably, the proximodistal (brain vesicle to lens) specification of the eye occurs during this mutually inductive ectoderm/neurectodermal 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-
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 (Semia et al., 2000; Rieger et al., 2001). Additionally, mutation of human PITX3 results in anterior segment mesenchymal dysgenesis and autosomal-dominant congenital cataracts (Semia 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 microophthalmia 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 engrafted 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 conserved 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 (Semia 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 sensory layer of anterior ectoderm. Figure 2A,B shows the early expression of Pitx3 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 extracocular tissues described as Pitx3 expressing in mice (Semia 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 (Semenia 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,1), 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 epithelium (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 engrafted-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 ecell less 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 βB1-crystallin probe in whole-mount in situ hybridization. Ectopic Pitx3 had little effect upon βB1-crystallin in lens; however, the chimeric construct and the morpholino diminish or abrogate expression of βB1-crystalline 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 were injected with 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 asymmetrical 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., 2001).

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**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 lens bias/specification events (St. 19–26). 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 lens bias/specification events (St. 19–26).

**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 the 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,J). BR, brachial arch; EA, eye anlage; J, jaw musculature; L, lens; OV, otic vesicle; R, Rathke’s pouch; S, somite; S/H, somodomea—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.

Pitx3 IS REQUIRED FOR EYE DEVELOPMENT
Fig. 4. B–F: Dorsal view of tadpoles injected with \( xPitx3 \) at the one-cell stage (B–E), and \( xPix3\)/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 \( \beta \) tubulin. C: Note the additional neural tissue projecting unilaterally (black arrow). Nt, neural tube. Scale bar = 200 \( \mu \)m.
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...
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-

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**Fig. 8.** Animal cap explant induction by *Pitx3, Lens1*, and *Pax6*. A: Un injected animal caps probed with *Xbra* demonstrate the absence of mesoderm, because the pan-mesodermal marker fails to hybridize. B–F: However, injection of *Pitx3* 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), *β1*-Crystallin (lens), and *Otx2* (retina, brain). J,K: Both *Lens1*- injected (J) and *Pax6*- injected (K) embryo explants induce *Pitx3*.

**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.
hhibit 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 was expressed more widely and more frequently with morpholino. 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

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**TABLE 1. Effects of Treatments**

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

*PLE, presumptive lens ectoderm; MO, morpholino.
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 morpholino 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 ectoderm 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 AGT 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 ATG CAT ACT GTG GCC CGA TCC A). A sequence immediately 5’ of the Pitx3 start codon was identified as ideal for morpholino-mediated translational knockdown, ATTCCTTTCAAC-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 previously 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 × 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 × 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-GCTAATCTTGTTGAGGGAT and the control sequence was CCTCTTAC-CTTACAGGTAACTTATA. 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 desired 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 RNa- sin (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-AAGTCCGTTGATCATACAA and CCTCTGAAAGTGAGGACA; EF1α-CAGATTGTTGCTGGATATG and ACTGCGTGTGACCTCTTA; ODC-GTCATATGGAGGTGATAG and TC-CTTTCCGCTTCCTCTGTA). 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 × 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 × 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 × 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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