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A novel *fork head* gene mediates early steps during *Xenopus* lens formation

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SUMMARY

***Xlens1* is a novel *Xenopus* member of the *fork head* gene family, named for its nearly restricted expression in the anterior ectodermal placode, presumptive lens ectoderm (PLE), and anterior epithelium of the differentiated lens. The temporal and spatial restriction of its expression suggests that: (1) *Xlens1* is transcribed initially at neural plate stages in response to putative signals from the anterior neural plate that transform lens-competent ectoderm to lens-biased ectoderm; (2) further steps in the process of lens-forming bias restrict *Xlens1* expression to the presumptive lens ectoderm (PLE) during later neural plate stages; (3) interactions with the optic vesicle maintain *Xlens1* expression in the lens placode; and (4) *Xlens1* expression is downregulated as committed lens cells undergo terminal differentiation. Induction assays demonstrate that *pax6* induces *Xlens1* expression, but unlike *pax6*, *Xlens1* cannot induce the expression of the lens differentiation marker *β-crystallin*. In the whole embryo, overexpression of *Xlens1* in the lens ectoderm causes it to thicken and maintain gene expression characteristics of the PLE. Also, this overexpression suppresses differentiation in the lens ectoderm, suggesting that *Xlens1* functions to maintain specified lens ectoderm in an undifferentiated state. Misexpression of *Xlens1* in other regions causes hypertrophy of restricted tissues but only occasionally leads ectopic sites of γ-crystallin protein expression in select anterior head regions. These results indicate that *Xlens1* expression alone does not specify lens ectoderm. Lens specification and differentiation likely depends on a combination of other gene products and an appropriate level of *Xlens1* activity.**

Key words: Winged helix, Epidermis, *fork head*, Crystallin, Pax6, *Xenopus*

INTRODUCTION

The currently favored model for lens determination in *Xenopus* includes four stages: competence, bias, specification and differentiation (Grainger, 1996). Competence refers to a period during mid to late gastrulation when an extensive region of the anterior (including ventral) ectoderm becomes responsive (competent) to inductive signals for lens formation. Bias refers to the interactions between the anterior neural plate and the adjacent lateral ectoderm that establish the domain of presumptive lens ectoderm (PLE) (Henry and Grainger, 1987; Servetnick and Grainger, 1991). The PLE acquires lens-forming bias during neural plate stages (Grainger et al., 1997). Specification refers to the autonomous ability of PLE explants to synthesize crystallins, the major proteins of the differentiated lens (Henry and Grainger, 1990). Differentiation begins several hours after the evaginated optic vesicle contacts the overlying PLE. During this phase, the PLE undergoes morphogenetic changes including thickening into the lens placode, invagination of the placode into a vesicle, and full differentiation into three cellular layers: the primary lens fibers, the secondary lens fibers, and the anterior lens epithelium (Nieuwkoop and Faber, 1994). The primary and secondary lens fibers terminally differentiate, whereas the anterior epithelial cells remain proliferative and continue to generate new lens fiber cells.

Lens development provides an ideal model system for elucidating the cellular and molecular mechanisms for each step in the determination process. Although the exact nature of the lens inducing factor(s) is not known, there is evidence that the coordinated expression of several transcription factors is necessary at each step of lens formation. The homeobox gene *otx2* and the paired homeobox-containing gene *pax6* are expressed in anterior ectoderm including the PLE during the process of lens-forming bias (Hirsch and Harris, 1997; Zygar et al., 1998). Mice mutant for *pax6* either lack lenses or have abnormalities in lens morphogenesis (Hill et al., 1991; Grindley et al., 1995), and ectopic expression of *pax6* induces the formation of ectopic lenses in *Xenopus* (Altmann et al., 1997). A murine homolog of the *Drosophila sine oculis* gene, *six3*, is expressed in developing lens (Oliver et al., 1995) and its ectopic expression in the killifish induces a fully differentiated lens (Oliver et al., 1996). Numerous other gene products are expressed in the lens and lens anlage (Cvekl and Piatigorsky, 1996), but their involvement in the steps of lens formation are not known.

We have identified a novel transcription factor, *Xlens1*, that likely functions in early stages of lens development. It belongs to the *fork head* gene family, whose members contain a 110 amino acid binding domain that allows these proteins to bind DNA as monomers (Kaufmann and Knochel, 1996). Many *fork head* genes have roles in directing events in pattern formation, cell determination and tissue specification (Dirksen and Jamrich, 1992, 1995; Sasaki and Hogan, 1994; Kaufmann and Knochel, 1996; Mariani and Harland, 1998; Bourguignon et al., 1998). In this study, we show that *Xlens1* expression is confined to the lens anlage during the process of lens bias (Grainger et al., 1997; Zygar et al., 1998) and to the proliferating cells of the anterior lens epithelium during lens differentiation. In explants, *pax6* can induce the expression of *Xlens1*; however, *Xlens1* does not induce expression of either *pax6* or *β-crystallin*. Directed overexpression of *Xlens1* represses the differentiation of the lens and appears to maintain the lens ectoderm in a specified, yet undifferentiated state. Unlike *pax6*, ectopic expression of *Xlens1* outside the lens ectoderm rarely initiates ectopic sites of γ-crystallin expression, and only in restricted regions of the head. Normally, these regions also express the *pax6* and *six3-like* genes, suggesting that multiple gene products, including *Xlens1*, must be expressed at appropriate levels for lens differentiation to occur. These data indicate that *Xlens1* expression results from the initial steps in the process of lens-forming bias. As a result of subsequent steps in lens biasing and specification, *Xlens1* likely functions to maintain a determined, yet undifferentiated lens ectodermal population.

MATERIALS AND METHODS

Identification and characterization of *Xlens1*

cDNA fragments were amplified from a stage 30 *Xenopus* head cDNA library using fully degenerative primers to the regions KPPYSY and EPGNPG of the *fork head* domain. Sequence analysis of these amplicons established that one contained a novel *fork head* box. This amplicon was used to screen approximately 2×10^6 clones of the same library. The isolated cDNA clone contained a 1.5 kb insert, which was sequenced in both directions to confirm accuracy of the code. Several putative translational start sites were identified upstream of the *fork head* box, and the presence of a poly(A) region at the 3′ end indicated that this insert contained the entire coding region.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed according to Harland et al. (1991). Some embryos were frozen in OCT medium, and sectioned with a cryostat.

Targeted misexpression

Using PCR, an amplicon was produced that contained the open reading frame of *Xlens1* starting from the second putative translational start site and two sites for the restriction enzymes *Kpn*I and *Xho*I. This amplicon was subcloned into the SP64R1 vector. Capped mRNA was synthesized from this construct using the mMessage mMachine Kit (Ambion Inc). Green Fluorescent Protein (GFP) was also synthesized and served as a lineage tracer to identify those cells that inherited *Xlens1* mRNA. GFP mRNA alone was used as an injection control.

Embryos were obtained by gonadotropin-induced natural matings of *Xenopus* adult frogs. Fertilized embryos were selected for targeted blastomere injection as previously described (Klein, 1987; Moody, 1999). Progenitor blastomeres of the lens were identified using *Xenopus* fate maps (Moody, 1987a,b; Moody and Kline, 1990), and injected unilaterally at the 4, 8 and 32-cell stages with *Xlens1* mRNA mixed with 200 pg of GFP mRNA. Injected embryos were fixed, cryoprotected and serially sectioned at 20 μm. To identify affected tissues, sections were submitted to immunostaining. The primary antibodies were: (1) *Xenopus* γ-crystallins (Henry and Grainger, 1990), (2) epidermis-specific cytokeratins (Jamrich et al., 1987), (3) somitic muscle (12/101, Developmental Hybridoma Bank; Kintner and Brockes, 1984); (4) neuronal surface glycoprotein (HNK-1, ATCC; Nordlander, 1993); (5) proliferating cell nuclear antigen (PCNA, Sigma). Fluorescent secondary antibodies or PAP protocols were applied as previously described (Huang and Moody, 1992). To determine the cellular thickness of affected ectoderm, cell nuclei were visualized with Hoechst reagent (Sigma; 100 μg/ml). Specimens were analyzed by epifluorescence microscopy and laser confocal microscopy. To determine if cell death was induced in the overexpression experiments, the presence of apoptotic nuclei was assayed by the TUNEL procedure (Gavrieli et al., 1992; Hensey and Gautier, 1998). Tissue sections were incubated with terminal deoxynucleotide transferase and labeling nucleotide mixture containing fluorescein-UTP (Boehringer-Mannheim). Positive cells were visualized using epifluorescence microscopy. The number of positive cells was counted in every section of the lens ectoderm, defined between the dorsal-ventral and anterior-posterior borders of each retina. Total numbers per retina were compared by paired and unpaired *t*-tests.

Animal cap assay and RT-PCR

Xlens1 mRNA and *pax6* mRNA (Hirsch and Harris, 1997) was synthesized as described above. Each mRNA was microinjected singly into both blastomeres of 2-cell embryos at the animal pole. Embryos were cultured until stage 8-8.5, at which time a small animal cap was dissected. Tissue explants were cultured until control siblings reached either stages 22-23 or 34. RNA was isolated according to the PURESCRIPT Kit protocol (Gentra Systems Inc.). For cDNA synthesis, 1 μg of RNA was reverse transcribed using Superscript II (LTI) according to the manufacturer's protocol. The following primer sets were using for detection of gene expression by PCR: *Xlens1* (U:CCTCTGGAGGCAGGAGAAGAAAACG, D:TCTGAGGGTT-ATATCCAGAGCCAA); *β-crystallin* (U:TGCCTGGAGTGGAA-CAATGC, D:TGTTGAACCATCCCATAGCC); *pax6* (U:GGGCA-ACAATCTACCTATGC, D:TGGCCAGTACTGAGACATGT); *H4* (U:CGGGATAACATTCAGGGTA, D:TCCATGGCGGTAACTGTC). Primer sets were tested at a series of cycle numbers to ensure that amplification occurred within a linear range. PCR products were separated on a thin 2% agarose gel, which was stained with Syber Green I (Molecular Probes). Fluorescence was detected using a FluorImager (Molecular Dynamics).

RESULTS

Identification and characterization of *Xlens1*

Sequence analysis revealed that the isolated insert contains several putative translation initiation sites 5′ to the *fork head* box as well as a polyadenylation signal with a poly(A) region in the 3′ UTR (Fig. 1A,B). Analysis of this open reading frame using the Netstart 1.0 program (Pedersen and Nielsen, 1997) predicted that the putative translational start site is at position 169 (Fig. 1A,B), which suggests a protein size of 365 amino acids. Results from a 5′ RACE experiment using stage 25 *Xenopus* head mRNA supported the theory that this construct contains the entire coding region (data not shown).

Xlens1 is not identical to any previously reported *fork head* gene. However, the *fork head* domain of *Xlens1* is highly conserved (Fig. 1C), as shown in the protein alignments of

A.

B.

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GATATCCTGGCTGGAGGGATCCCAGATGACTTTTGCTGTGTTGGGACTGAGAGGGTTTTGTTTATGAATTTGGCAAGTTTCCCTT     85
ATTATTCAGGCATGTGCACCATGACTGCAGATTCCCAGCACTCACCCACTGAAGCTGCCAGCCCCATGCCAAGCTCGCCCAGCAT    170
                                                                                    M      1

GGACTCCCCTGACTCCGTCAGAGTCAAATGTGAATCCAAGGGGAGCTGCAGTCCAGAGGAGGGTGTGAATAATGGGCTACCAGAA    255
 D  S  P  D  S  V  R  V  K  C  E  S  K  G  S  C  S  P  E  E  G  V  N  N  G  L  P  E      29

GAACACAACCAGGCCTCTGGAGGCAGGAGAAGAAAACGTCCAGTCCAAAGGGGAAAGCCACCCTACAGCTATATTGCCCTCATTG    340
 E  H  N  Q  A  S  G  G  R  R  R  K  R  P  V  Q  R  G  K  P  P  Y  S  Y  I  A  L  I      57

CAATGGCCATAGCCAATTCCCCAGAAAGGAAGCTGACACTAGGGGGCATTTACAAATTCATCATGGAAAGGTTCCCCTTCTATAG    425
A  M  A  I  A  N  S  P  E  R  K  L  T  L  G  G  I  Y  K  F  I  M  E  R  F  P  F  Y  R      86

GGAGAACTCCAAGAAATGGCAGAACTCCATCCGCCACAACCTAACGCTTAATGACTGCTTTGTAAAGATCCCCAGGGAACCTGGG    510
  E  N  S  K  K  W  Q  N  S  I  R  H  N  L  T  L  N  D  C  F  V  K  I  P  R  E  P  G     114

CATCCAGGCAAAGGCAACTACTGGACTCTGGATCCTGCAGCTGAGGATATGTTTGACAATGGCAGTTTCCTCAGAAGAAGGAAGA    595
 H  P  G  K  G  N  Y  W  T  L  D  P  A  A  E  D  M  F  D  N  G  S  F  L  R  R  R  K     142

GATTTAAGAGGACAGATATCACCACCTACCCTGGGTATATGCAGAACAGTAGTGCCTTCACCCCCACTCCAACTGGAAGGGCGTC    680
R  F  K  R  T  D  I  T  T  Y  P  G  Y  M  Q  N  S  S  A  F  T  P  T  P  T  G  R  A  S     171

TTACCCCAACAGCATATACTCTAGCGTTGGCTCTGGATATAACCCTCAGATACACCAGACTCACCACCCGGCTGTGGTACATCAG    765
   Y  P  N  S  I  Y  S  S  V  G  S  G  Y  N  P  Q  I  H  Q  T  H  H  P  A  V  V  H  Q     199

TACTACCAGTCTCCAGGTGAAGCTGGGCAAGGACAGCACAGGATGTTTAGTATAGACAGTTTAATTAACCAACAATCTTTGATGC    850
 Y  Y  Q  S  P  G  E  A  G  Q  G  Q  H  R  M  F  S  I  D  S  L  I  N  Q  Q  S  L  M      227

AACCATCCCCTGGTGCAGAGCTTACTCACCATTCCCTTGGCTTGAATGGGAACCTAGGGAACATGACCAACAGTTGTTCTGTAGG    935
Q  P  S  P  G  A  E  L  T  H  H  S  L  G  L  N  G  N  L  G  N  M  T  N  S  C  S  V  G     256

AGATCTTTCCTGTTTTCAGACTCAGTCAATCAGCCCCACAGGGGTAGGTTCATTGTTAAATCGGTCTTCTAATGCTGTGTCTTCC   1020
  D  L  S  C  F  Q  T  Q  S  I  S  P  T  G  V  G  S  L  L  N  R  S  S  N  A  V  S  S     284

AACCTAACCTATTCTTATTCCTCATCCCCACCTCACCTGCCAGTGCCCCCTGCCAGCTATTCCCCCAATAACTCACAGTTATATG   1105
 N  L  T  Y  S  Y  S  S  S  P  P  H  L  P  V  P  P  A  S  Y  S  P  N  N  S  Q  L  Y      312

GCTCCACTAGCAGGCTGGCTATGAGATCTGGACCTTGTGTAGATCACACAGACCAGCTCCTGTCTTTGCCCGGGACTCAAATAAA   1190
G  S  T  S  R  L  A  M  R  S  G  P  C  V  D  H  T  D  Q  L  L  S  L  P  G  T  Q  I  N     341

TGGGGTTTGTCAGTATAACAACAGTTCCTATATGAGACAAACACATTTTGCTTCTGGACTTGAAAGATATATGTAAGTGTCAAAG   1275
  G  V  C  Q  Y  N  N  S  S  Y  M  R  Q  T  H  F  A  S  G  L  E  R  Y  M                 365

ATCAATTATTGGGTTAGATTTCTTACAAACACAAATTCACTCTCCTATGGCTTTACTTGTGTAACTGTGCAAAGGTCACACAACA   1360
TACAACGCATAATTCACCTTGTTTTTTTTTTACTTGTGTTGTATAGTTTATTCAAGTTGAAATATTCACAACAGCCTTTTTTCT   1445
GTGCATTTTCATTTCAAAATAAACCTTGTTCACAAAAAAAAAAAAAAAAAAAAAAAAA                             1502
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C.

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                       H1          H2                   H3                        W1                  W2
Xlens1     RGKPPYSYIALIAMAIANSPERKLTLGGIYKFIMERFPFYRENSKKWQNSIRHNLTLNDCFVKIPREPGHPGKGNYWTLDPAAEDMFDNGSFLRRRKRFKR
TTF2       .................HA...R..........T.......D.P.................L.....A.R.......A...N.....ES............
FKHL15     .................HA...R..........T.......D.P.................L.....A.R.......A...N.....ES............
rFREAC8    ...............L.HA.G.R...AA..R..T...A...DSPR..................V.....N.............A.................
hFREAC8    ...............L.HA.G.R...AA..R..T...A...DSPR..................V.....N.............A.........P.......
Genesis      ..........T...LQ..QK....S..CE..SN...Y...KFPA.........S.............N...........QS..................
CWH3         ..........T...LQ..QK....S..CE..SN...Y...KFPA.........S.............N...........QS..................
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Fig. 1. Structure and sequence of the *Xlens1* cDNA. (A) Schematic representation of the *Xlens1* cDNA (1474 bases), including putative translational start sites (vertical lines) 5′ to the coding region (black box, 1093 bases), and the *fork head* domain within it (gray box, 303 bases). (B) The nucleotide sequence and translated coding region for *Xlens1*. The *fork head* domain is underlined and the polyadenylation signal site is bold.
(C) Comparison of the amino acid sequence of *Xlens1* and those members of the *fork head* family most closely related to *Xlens1*. Dots represent identity. RGKPPYSY is one of the conserved regions that indicate *Xlens1* and its four closest relatives may represent a new subfamily in the class 8 group. H1, H2, H3 indicate the three α-helical regions. W1 and W2 indicate the two wing-like regions. GenBank accession number for Xlens1 is AF186464.

Xlens1 with its closest relatives (Altschul et al., 1997). These include rat *TTF-2* (Zannini et al., 1997), human *FKHL15* (Chadwick et al., 1997), rat and human FREAC8 (Larsson et al., 1995), mouse *Genesis* (formerly HFH-2) (Sutton et al., 1996; Marsden et al., 1997) or its likely *Xenopus* homologue *XFKH6* (Dirksen and Jamrich, 1995) and chicken *CWH-3* (Freyaldenhoven et al., 1997a,b). Several of these genes function as regulators of stem cell populations or as growth stimulators (Sutton et al., 1996; Freyaldenhoven et al., 1997a), suggesting that *Xlens1* also may function in proliferative events during development. Using the classification system of Kaufmann and Knochel (1996), *Xlens1* best fits in the class 8 group of the *fork head* gene family, although *Xlens1*, *TTF-2* and *FKHL15* may represent a new subdivision within this group based upon a conserved RGKPPYSY within the *fork head* domain (Fig. 1C).

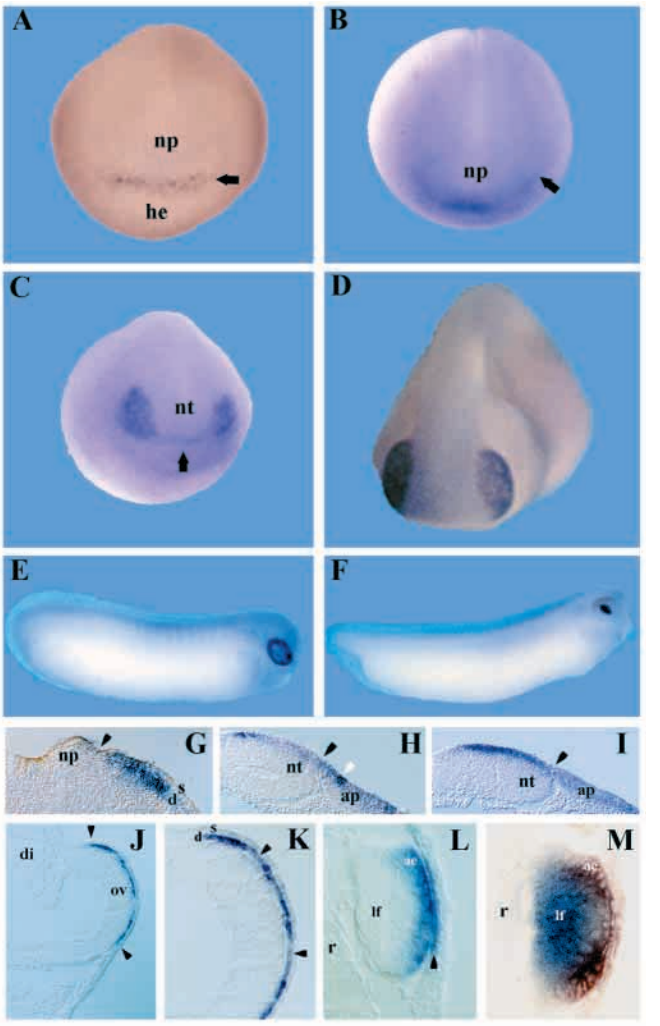

Fig. 2. Expression of *Xlens1* mRNA during development. (A-D) are anterior views of the head and (E-F) are lateral views. (A) *Xlens1* is first detected at stage 13 in a narrow field across the anterior midline (arrow), separating the anterior neural plate (np) from ventral head epidermis (he). (B) At stage 15 *Xlens1* expression has spread laterally to the anterior-lateral margins of the neural plate (np), reaching the limits of the trigeminal placode anlagen (arrow). (C) At stage 18 the midline expression of *Xlens1* is depleted (arrow), and the lateral patches adjacent to the anterior neural tube (nt) are prominent. (D) At stage 22 *Xlens1* expression is confined to the ectoderm overlying the optic vesicle. (E) At stage 28, *Xlens1* expression is confined to the ectoderm overlying the optic cup. (F) At stage 34, only the lens vesicle expresses *Xlens1*. (G) Sagittal section through the midline band at stage 15. *Xlens1*-expressing cells are in the superficial (s) and deep (d) layers of the ectoderm, just anterior to the anterior rim (arrowhead) of the neural plate (np). Anterior, right; dorsal top. (H) Sagittal section through the midline at stage 18. *Xlens1* expression is mostly depleted from the midline cells of the anterior ectodermal placode (ap), which will become the adenohypophyseal and olfactory placodes. A few *Xlens1*-expressing cells are still detected (white arrowhead) just anterior to the most rostral edge (black arrowhead) of the neural tube (nt). (I) Sagittal section through the lateral patch of *Xlens1* expression in the same stage 18 embryo as in H. *Xlens1* expression is mostly depleted from the placode (ap) anterior to the neural tube (nt), but is at high levels in both the superficial and deep (sensorial) layers of the ectoderm overlying the lateral aspects of the neural tube. Arrowhead delineates between the neural tube and the placode. (J) Transverse section through the optic vesicle (ov) at stage 22 embryo. *Xlens1* expression is confined to the ectoderm overlying the vesicle. There is a sharp depletion of *Xlens1* expression at dorsal and ventral points (arrowheads) where mesoderm intervenes between the optic vesicle and surface ectoderm. di, diencephalon. Dorsal, up; lateral, right. (K) Higher magnification of J, demonstrating that only the deep (d, sensorial) layer of cells in the PLE express *Xlens1*. Some cells (arrowheads) appear to have downregulated *Xlens1* expression. s, superficial layer. (L) Transverse section of a stage 31/32 lens vesicle. *Xlens1* is expressed only in the anterior epithelial cells of the lens (ae), adjacent to the surface ectoderm. The inner layer of the surface ectoderm (cornea) covering the lens vesicle also appears to express this gene (arrowhead). r, retina. (M) Transverse section of a stage 34 lens vesicle depicting both *β-crystallin* (blue) and *Xlens1* (brown) expression. The lens fiber cells (lf) express *β-crystallin*, but do not express *Xlens1*. Magnification, 70× (G-I), 75× (J), 170× (K), 250× (L,M).

Expression of *Xlens1* during embryogenesis

Xlens1 has a very distinct and restricted expression pattern during embryogenesis. It is first expressed at the end of gastrulation (stage 13) in a thin band of ectoderm adjacent to the anterior rim of the early neural plate (Fig. 2A). Both superficial and deep layers of the anterior midline ectoderm express *Xlens1*, which is best illustrated at stage 15 (Fig. 2G). This is the anterior placodal region that will give rise to the olfactory and lens placodes (Knouff, 1935). During intermediate neural plate stages (stages 14-16), the band of staining expands laterally along the rim of the anterior neural plate (Fig. 2B) to include the fate map of the presumptive lens region (Zygar et al., 1998). The confined expression of *Xlens1* in the ectoderm adjacent to the anterior neural plate indicates that the early signals necessary to initiate lens bias induce this gene. Furthermore, like *otx2* and *pax6* (Zygar et al., 1998) *Xlens1* is expressed in the presumptive nasal ectoderm (PNE) as well as the presumptive lens ectoderm (PLE) during the period when anterior ectoderm first acquires a lens-forming bias. By neural tube (stages 18-21), the midline expression in the PNE is depleted whereas its expression intensifies in the PLE (Fig. 2C,H,I). *Xlens1* is expressed by cells in both layers (deep and superficial) directly adjacent to the lateral neural tube (Fig. 2I). Thus, *Xlens1* expression becomes restricted to the PLE during later stages of lens forming bias and the early stages of lens specification (Zygar et al., 1998). At stage 22, when the optic vesicles evaginate from the diencephalon, *Xlens1* expression is restricted to the PLE, and only in the sensorial layer of the epidermis in contact with the optic vesicle (Fig. 2D,J,K). Through tail bud stages (stages 23-31), expression of *Xlens1* is maintained in the lens placode and lens vesicle (Fig. 2E,F). In the maturing lens (stage 32-onwards), *Xlens1* expression is restricted to the anterior lens epithelial cells, that population of the lens that remains proliferative (Fig. 2L,M). In some preparations, light staining is found in the deep sensorial layer adjacent to the lens (presumptive cornea) (Fig. 2L). *Xlens1* is not expressed in the differentiating fiber cells, and is excluded from those cells that express high levels of *β-crystallin* (Fig. 2L,M). At tadpole stages, *Xlens1* expression remains confined to the anterior lens epithelium, but there is additional expression in the ventral midline of the pharynx (data not shown). *Xlens1* continues to be expressed in the adult eye (Fig. 3A).

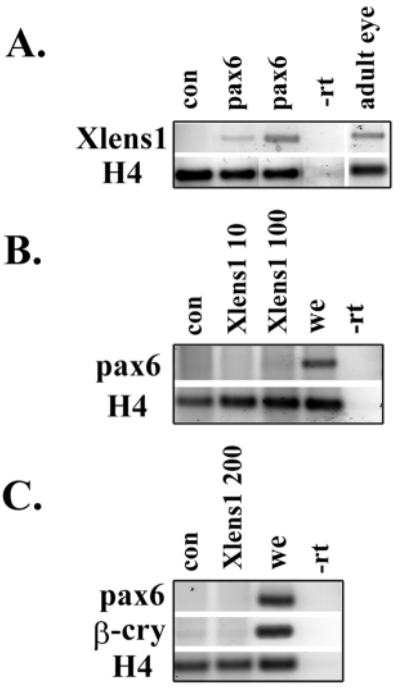

Fig. 3. Animal cap assays of pax6 and *Xlens1* injections. The horizontal axis denotes genes overexpressed in explants and the vertical axis genes tested for induction. Histone 4 (H4) demonstrates successful cDNA synthesis. con, control uninjected explants; we, whole embryo; –rt, minus reverse transcription product. (A) *pax6* (200 pg) overexpression induces expression of *Xlens1* in explants cultured until stages 22-24. This induction is detected at 28 cycles (lane 2) and there is an increase in amplified product at 30 cycles (lane 3) for the same amount of starting template, indicating that detection occurs within a linear range. *Xlens1* is still present in the adult eye (lane 5). (B) *Xlens1* does not induce expression of *pax6* in explants cultured until stages 22-24 (30 cycles). Two doses of *Xlens1* mRNA (10 pg, lane 2, and 100 pg, lane 3) were tested. (C) *Xlens1* (200 pg) expression does not induce either *pax6* or *B-crystallin* in explants cultured until stage 34 (30 cycles).

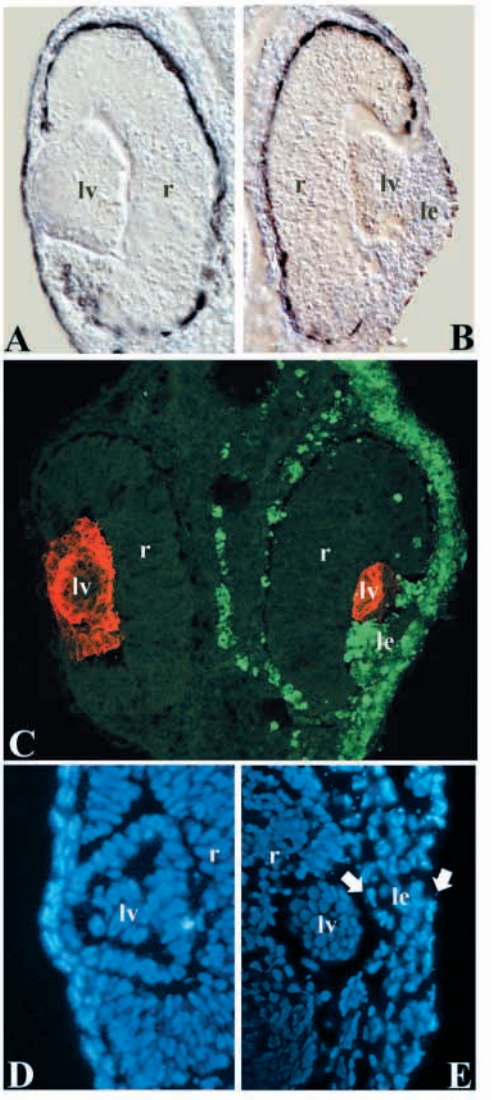

Fig. 4. Overexpression of *Xlens1* causes thickening in lens ectoderm and suppresses differentiation of lens fibers. (A) Differential interference contrast image of the uninjected side showing the normal histological relationship between the lens vesicle (lv) and the surface ectoderm, which is composed of two thin layers of cells. r, retina. 190×. (B) The injected side of the same embryo contains a smaller lens vesicle (lv) and a thickened lens ectoderm (le). r, retina. (C) Confocal image of a transverse section from stage 35. On the uninjected side (left) the expression of γ-crystallin protein (red) is comparable to that in normal, unperturbed embryos, with strong expression in the fibers of the lens vesicle (lv). On the injected side (right), γ-crystallin expression is confined to the fibers of a greatly reduced lens vesicle. The thickened lens ectoderm expresses high levels of *Xlens1* (green). 190×. (D,E) Cell nuclei stained with Hoechst. On control side (D), the ectoderm overlying the lens vesicle (lv) is 2 cell layers thick. On the injected side (E), the thickened ectoderm is 4-6 cell layers thick (between arrows). Magnification, 190× (A-C), 240× (D,E).

pax6 induces *Xlens1* expression in vitro

To determine some of the molecular events of *Xlens1* induction in the anterior placodal region and the stabilization of its expression in the PLE, we investigated the interactions of *Xlens1* with *pax6*. *Pax6* is expressed during lens formation (Hirsch and Harris, 1997; Zygar et al., 1998) and its overexpression in *Xenopus* induces the formation of ectopic lens tissue as defined by *β-crystallin* expression (Altmann et al., 1997). In animal cap explants, *pax6* overexpression results in the activation of *Xlens1* transcription (Fig. 3A). These results were confirmed in a minimum of eight independent assays. Since the number of PCR cycles was relatively high for detection, we confirmed that this result occurred within a linear range for the primer set and for the amount of starting template (Fig. 3A). In a reciprocal experiment, *Xlens1* alone is not sufficient to induce *pax6* expression or *β-crystallin* expression (Fig. 3B,C). Since gene induction could be dose-dependent, it was necessary to establish that this result was invariant and not dependent on the amount of *Xlens1* overexpression within the explant. In a minimum of four experiments, a range of doses (10–200 pg) was tested and at none did *Xlens1* induce either *pax6* or *β-crystallin* expression. Using doses higher than 250 pg resulted in abnormal cell division and explant death. These data indicate that *pax6* can activate gene expression characteristic of early (*Xlens1*) and late (*crystallin*; Altmann et

al., 1997) stages of lens development. Since *Xlens1* cannot induce lens differentiation products, it must have a function independent of *pax6* during the late events of lens formation.

Effects of *Xlens1* misexpression in intact embryos

Xlens1 was overexpressed in intact embryos by mRNA microinjection into specific cleavage stage blastomeres. Embryos injected with greater than 250 pg of *Xlens1* mRNA failed to gastrulate properly and most died. Specific phenotypes (described below) were produced with 100 pg, but labeled cells were often displaced into tissue lumens, which is an indicator of injection toxicity (Moody, 1999). In numerous independent experiments, doses between 25-50 pg produced consistent phenotypes without any signs of toxicity. These phenotypes were dose-dependent since they were not seen in embryos injected with 2.5-5 pg of *Xlens1* mRNA. Within the effective dose range, regardless of which blastomere was injected, the tissue distribution of *Xlens1*-expressing cells (GFP-positive cells) was comparable to that of the normal fate maps (Moody, 1987a,b; Moody and Kline, 1990) and GFP-injected controls. Therefore, overexpression of *Xlens1* does not change the fate per se of recipient cells.

Xlens1 misexpression caused focal tissue masses at the site of gene expression as indicated by GFP expression. It is interesting to note, however, that only specific tissues responded by hypertrophy, most notably ectodermal derivatives, suggesting a tissue-specific responsiveness to *Xlens1*. Injection in dorsal animal blastomeres resulted in a thickening of the tissue over the head on the injected side. These masses occurred in 64.8% of embryos (n=165) versus none in GFP-injected control embryos (n=88). Staining with tissue-specific markers showed the hypertrophied tissues were brain and epidermis (not shown). *Xlens1* misexpression in ventral animal blastomeres resulted in discrete masses on the ventral or lateral trunk in 72.2% of embryos (n=126) versus none in GFP-injected control embryos (n=48). Staining with tissue-specific markers and/or morphological characteristics indicated that these masses were composed of hypertrophied epidermis, lateral plate mesoderm and/or neural crest (not shown). The ectopic masses that resulted from the ventral overexpression of *Xlens1* are histologically similar to the epidermal tumors produced in *Xenopus* by the ectopic expression of the zinc-finger transcription factor *Gli1* (Dahmane et al., 1997). These data suggest that *Xlens1* shares with other *fork head* genes a common role in the regulation of cell proliferation (Brissette et al., 1996; Garry et al., 1997; Ye et al., 1997) and in non-lens tissue may substitute for other *fork head* genes.

To target *Xlens1* overexpression to the presumptive lens ectoderm, mRNA with lineage tracer was injected in a major 32-cell blastomere progenitor of the lens (D1.1.1; Moody, 1987b). 55% of the cases resulted in *Xlens1/GFP* expressing cells being located within or adjacent to the native lens (43/78). Of those cases identified by this criterion, overlying lens epidermis is thicker in 88% of injected embryos (Fig. 4B,C,E). On average, the hypertrophied lens ectoderm contains twice the normal number of layers of cells (Fig. 4D,E; control side=2.25±0.40, n=22; *Xlens1* side=4.58±0.40, n=13). These cell layers were delineated from the underlying mesenchymal cells by a basement membrane zone (DIC optics, not shown) and shown to be capable of proliferation by the expression of PCNA, an auxiliary protein of DNA polymerase specifically observed in proliferating cell nuclei (Hyde-Dunn and Jones, 1997; data not shown). Thus, the thickened tissue over the retina is likely the result of increased proliferation of the lens ectoderm and not of cell shape changes or alterations in cellular migration. When the GFP-positive cells were located within the thickened lens epidermis the expression of γ-crystallin protein is reduced or completely inhibited (n=31), the majority (n=16) presenting a phenotype identical to that shown in Fig. 4C. To ensure that the loss of crystallin expression was not due to cell death induced by *Xlens1* overexpression, tissue samples were assayed by the TUNEL method for apoptotic cell death. As previously noted (Hensey and Gautier, 1998) apoptotic cell death occurs naturally in *Xenopus* sensory structures. In control uninjected embryos (n=22) an average of 2.6±2.67 (±s.d.) dying cells were observed per eye. In *Xlens1*-injected embryos (n=8) presenting the phenotype shown in Fig. 4, the mean number of dying cells in the lens ectoderm per eye did not differ between injected (3.75±3.80) versus uninjected side (2.50±2.56) side (P>0.05, paired t-test) or between injected side and control embryos (P>0.05). Considering that there are more cells in the *Xlens1*-expressing lens ectoderm than control ectoderm (above), an increase in cell death cannot account for the loss of crystallin expression after *Xlens1* overexpression. These results suggest that *Xlens1* misexpression promotes the proliferation of the lens-forming ectoderm and suppresses its terminal differentiation.

To identify the state of determination of the thickened ectoderm, this phenotype was analyzed for three genes that are normally expressed at different points of lens development. *Pax6* is expressed in the PLE and lens vesicle (Fig. 5A,B; Hirsch and Harris, 1997; Zygar et al., 1998). A *six3-like* gene is expressed within the PLE (Fig. 5D; M. Dirksen and M. Jamrich, unpublished). We show that this gene is downregulated in the deep layers of the normal lens placode (Fig. 5E). *γ-crystallin* mRNA is not detectable in the PLE by in situ hybridization. However, it is expressed at high levels in the lens placode and vesicle (Fig. 5G,H; Smolich et al., 1994; Brunekreef et al., 1997). In *Xlens1*-injected embryos, the thickened lens ectoderm expresses *pax6* and the *six3-like* gene in both deep and superficial layers (Fig. 5C,F; n=5 cases for each marker), as is characteristic of the PLE (Fig. 5A,D) and not lens placode or vesicle (Fig. 5B,E). The *six3-like* expression is less intense than in control PLE, but its presence in the deep layers is indicative that the tissue is PLE and not lens placode. Furthermore, *γ-crystallin* expression is significantly reduced or absent from the thickened lens ectoderm (Fig. 5I), confirming that this tissue is PLE, not lens placode. Therefore, expression of those genes characteristic of the PLE but not of the lens placode indicate that *Xlens1* overexpression maintains the lens ectoderm in an undifferentiated state.

Xlens1 misexpression in non-lens tissue did cause ectoderm to thicken in regions other than the lens ectoderm (see above). These affected tissues did not express *pax6*, *six3-like* or *γ-crystallin* mRNA, indicating that *Xlens1* alone does not induce lens bias, but is a result of the process. However, misexpression of *Xlens1* in the anterior head on occasion (n=5/31) caused ectopic γ-crystallin protein expression (Fig. 6). This occurred only in regions anterior to the eye, either dorsal to the forebrain or in the maxillary branchial arch, which are regions that

normally express low levels of the *pax6* and *six3-like* genes (data not shown). The ectopic γ-crystallin-expressing cells were within a *Xlens1*-expressing mass, but did not achieve the highly elongated morphology of mature lens fibers (Fig. 6C). Although it has been reported that *γ-crystallin* mRNA is normally expressed in some non-lens tissue (Smolich et al., 1994), we never observed γ-crystallin protein in these regions of the anterior head (n=31). The fact that γ-crystallin protein is induced in ectopic fields where *pax6*, *Xlens1* and *six3-like* genes are coexpressed suggests that these sites contain the same combination of genes at their appropriate expression levels as in the endogenous lens. Because high levels of *Xlens1* expression in the lens ectoderm inhibit crystallin expression (Fig. 4), either an additional factor is present in these ectopic sites and/or the level of *Xlens1* activity is low enough in these cells that they can achieve one characteristic (γ-crystallin protein expression) of the differentiated lens.

DISCUSSION

We have identified a novel *Xenopus* member of the *fork head* gene family, named *Xlens1* because of its nearly restricted expression in the anterior ectodermal placode, presumptive lens ectoderm (PLE) and anterior epithelium of the differentiated lens. The temporal and spatial restriction of its expression suggests that: (1) *Xlens1* is transcribed initially at early neural plate stages in response to putative signals from the anterior neural plate that transform lens-competent ectoderm to lens-biased ectoderm; (2) further steps in the process of lens-forming bias restrict *Xlens1* expression to the PLE during later neural plate stages; (3) interactions with the optic vesicle during neural tube stages maintain *Xlens1* expression in the lens placode; and (4) *Xlens1* expression is downregulated as committed lens cells undergo terminal differentiation. In vitro induction assays demonstrate that *pax6* induces *Xlens1* expression, but unlike *pax6*, *Xlens1* alone cannot induce the expression of the lens differentiation marker *β-crystallin*. In the whole embryo, overexpression of *Xlens1* causes the hypertrophy of restricted tissues, including the PLE, and suppresses the differentiation of lens fibers. *Xlens1* overexpression can lead to ectopic sites of crystallin protein expression rarely and only within specific anterior head regions, suggesting that multiple factors are necessary for full lens differentiation. Thus, the initial expression of *Xlens1* results from the earliest steps in the lens-forming bias. *Xlens1* expression is restricted to the PLE by the later stages of lens bias and early stages of lens specification. It likely functions in the maintainence of a committed, undifferentiated lens population.

Characterization of *Xlens1* as a novel member of the *fork head* gene family

Members of the *fork head* gene family encode transcription factors that bind to DNA as monomers. The DNA-binding domain was originally identified through homology comparison between the Drosophila *fork head* (*fkh*) gene (Weigel et al., 1989) and the rat *hepatocyte nuclear factor* (*HNF3*) gene (Lai et al., 1991). Numerous members of this family have been identified and categorized according to conserved regions within the DNA-binding domain (Kaufmann and Knochel, 1996). Based on protein alignments of the *fork head* domain, *Xlens1* most closely resembles members of the Class 8 group.

Several *fork head* genes regulate cell proliferation within embryonic and stem cell populations such as the *MNF* gene in muscle satellite cells (Garry et al., 1997), the *HFH-11* gene in the embryonic liver, lung, intestine and kidney (Ye at al., 1997), the *Genesis* gene in granulocytic stem cells (Xu et al., 1998) and the *whn* gene in keratinocytes (Brissette et al., 1996). The closest *fork head* gene related to *Xlens1*, *TTF-2*, can repress genes involved in terminal differentiation of the thyroid (Zannini et al., 1997). Similar to these genes, *Xlens1* is selectively expressed at high levels in the lens precursor population, but during differentiation the gene is downregulated until expression is completely restricted to the proliferative anterior lens population. We demonstrate experimentally that directed overexpression of *Xlens1* in the lens anlage expands the undifferentiated PLE and inhibits the terminal differentiation of lens fibers. Thus, *Xlens1* may share a function with other members of the *fork head* family as a tissue-specific growth regulator that prevents differentiation in a restricted, committed cell population. *Xlens1* does not cause cells to change fate, but may have a role in regulating proliferation in selected cell types. For example, affected lens ectoderm contains more layers of cells than normal and expresses PCNA, an auxiliary protein of DNA polymerase indicative of proliferating cells (Hyde-Dunn and Jones, 1997). Furthermore, tissue masses are produced when *Xlens1* is misexpressed in other, selected ectodermal derivatives. This latter effect may be the result of *Xlens1* mimicking the functions of other *fork head* genes that have sequence similarity and are normally expressed in these derivatives. For example, overexpression of two *Xenopus fork head* genes also causes expansion of neural ectoderm (Bourguignon et al., 1998; Mariani and Harland, 1998).

Xlens1 role in early lens determination

Lens formation is a multistep process that involves inductive interactions, spatially restricted cell proliferation, lens-specific gene expression and morphogenetic movements. Expression of the *Xlens1* gene is indicative of some of these processes. The initial phase of lens induction occurs during mid-gastrulation, when the ectoderm autonomously becomes competent to respond to lens-inducing signals (Servetnick and Grainger, 1991). The onset of *Xlens1* expression appears too late to be involved in the establishment of lens competence. During early neural plate stages, much of the lens-competent ectoderm loses its ability to form a lens, but restricted regions within the head ectoderm maintain the ability to respond to lens-inducing signals that originate from the anterior neural plate (Henry and Grainger, 1987, 1990; Grainger et al., 1997). This process of lens-forming bias appears to involve several progressive interactions, including the expression of multiple genes including *otx2, pax6* and *sox3,* and subsequently leads to the specification of lens ectoderm at neural tube stages (Grainger et al., 1997; Zygar et al., 1998). The spatial expression pattern of *Xlens1* appears to mark those lens-competent cells that have become biased for lens formation. The early, broad expression pattern of *Xlens1* in the anterior ectodermal placode, which will give rise to the anterior pituitary, olfactory sensory epithelium and lens, is consistent with the observation that the

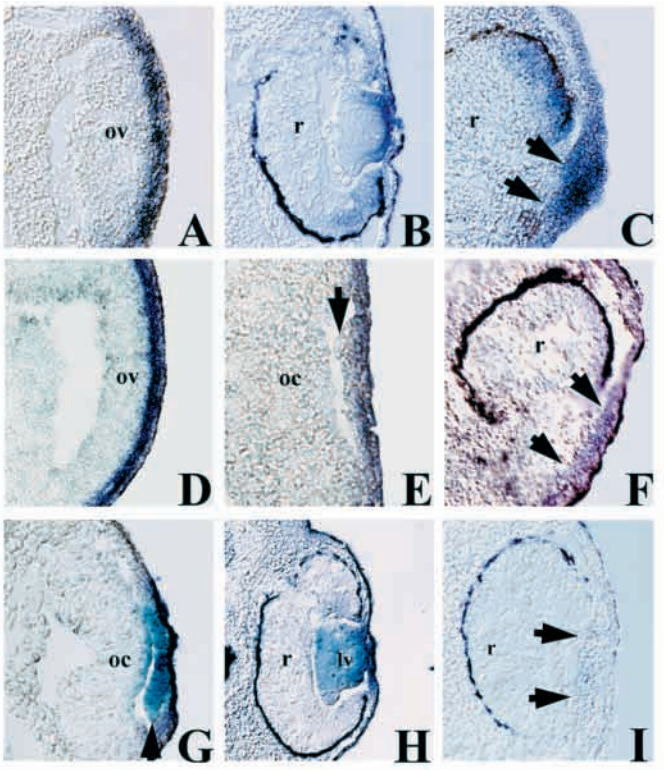

Fig. 5. In *Xlens1*-injected embryos, the thickened ectoderm expresses markers indicative of a PLE state of determination (A) In controls, *pax6* is expressed in the PLE. ov, optic vesicle. (B) In controls, *pax6* is expressed in the anterior lens epithelium of the lens vesicle. r, retina. (C) When *Xlens1* is overexpressed, *pax6* expression is expanded throughout the thickened ectoderm (arrows) overlying the retina in a pattern identical to control PLE (A). (D) In controls, *six3-like* is expressed in the deep layer of the PLE. (E) In controls, *six3-like* expression in the deep layers of lens ectoderm is downregulated when the lens placode begins to form (arrow). oc, optic cup. (F) When *Xlens1* is overexpressed, *six3-like* expression is maintained in the deep layers of the thickened lens ectoderm (arrows), similar to control PLE (D). (G) In controls, *γ-crystallin* is first expressed throughout lens placode. It is not expressed in the PLE. (H) In controls, *γ-crystallin* is expressed throughout the lens vesicle. (I) When *Xlens1* is overexpressed, *γ-crystallin* expression is absent from the thickened lens ectoderm (arrows), a pattern identical to control PLE (not shown). Magnification, 150× (A,C,D,G), 165× (H), 175× (B), 190× (E).

entire placodal region has a higher lens-forming potential than the rest of the embryo (Jacobson, 1966) and that lens can be induced in other placodal derivatives (Oliver et al., 1996). The intimate histological relationship between *Xlens1*-expressing placodal ectoderm and the anterior neural plate suggest that the lens inductive signals emanate from the latter. This early expression supports the idea that the initial stages of lens induction take place prior to optic vesicle formation (Grainger, 1996; Grainger et al., 1997). At later neural plate stages, lens-forming bias becomes restricted to the PLE (Grainger et al., 1997). Both *otx2* and *pax6* have been implicated in the process of lens bias (Zygar et al., 1998) and we show that *pax6* upregulates *Xlens1* expression. Thus, we believe that the initial step in *Xlens1* regulation is via the process of lens-forming bias.

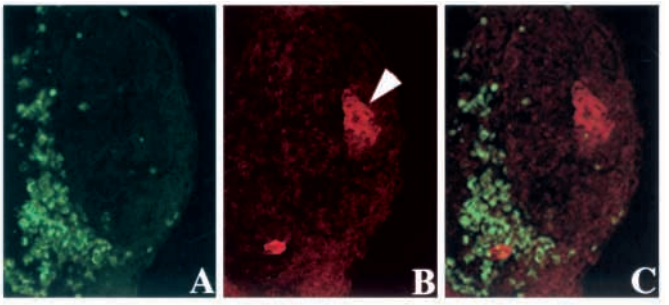

Fig. 6. Overexpression of *Xlens1* in selected regions of the anterior head of the embryo results in ectopic γ-crystallin protein expression. A duplicate set of confocal images acquired in a Z-series through 7 μm demonstrates that GFP-expression (A) and γ-crystallin expression (B) are in the same cells. In (C) the images are merged. Arrowhead in B indicates normal γ-crystallin staining in the native lens. 170×.

Expression of *Xlens1* during the process of biasing may prime those cells to be highly responsive to the weakly inductive signals from the optic vesicle that specify the PLE (Grainger et al., 1997). *Xlens1* expression appears to be upregulated during the early stages of lens specification. When the optic vesicles evaginate from the neural tube, the expression of *Xlens1* is restricted and maintained at high levels exclusively in the PLE that is in close proximity to the optic vesicle. This pattern of expression suggests that signaling from the optic vesicle is necessary for the maintenance of lens-specific expression and for the formation of the lens vesicle. This is supported by the observation that embryos carrying *Rx* mutations that eliminate either the early eye fields or the optic vesicles also lack lens structures (Mathers et al., 1997).

Xlens1 role in lens differentiation

Full differentiation of the lens involves the morphogenetic formation of a vesicle and the expression of the crystallin proteins characteristic of the fully differentiated lens fiber (Grainger, 1996). As discussed above, *pax6* initiates the lens pathway, including induction of *Xlens1* expression, but it also plays a later role in lens differentiation (Altmann et al., 1997). Numerous studies have shown that the lens-specific expression of several crystallin genes requires *pax6* (Cvekl and Piatigorsky, 1996; Altmann et al., 1997). The ectopic expression of mouse *six3* in medaka results in the formation of a fully differentiated lens (Oliver et al., 1996). The bZip transcription factor *L-maf* induces lens-specific genes in vitro and ectopic lens differentiation in vivo (Ogino and Yasuda, 1998). A disruption in the homeobox gene *Pitx3* is thought to be responsible for the *aphakia* mouse phenotype in which the formation of the lens placode appears normal but differentiation fails at the time of invagination (Semina et al., 1997). The HMG box-containing genes *Sox1*, *2*, and *3* are important for regulating crystallin gene expression. In homozygous *Sox1* mutants, the lens fibers fail to differentiate and γ-crystallin expression is significantly reduced (Nishiguchi et al., 1998). In the chick, *Sox 2/3* expression overlaps with *pax6* in lens ectoderm and ectopic sites of δ-crystallin expression concurrently express these two factors (Kamachi et al., 1998).

In contrast to these factors that positively regulate lens fiber

differentiation, *Xlens1* does not induce the expression of *β-crystallin* in vitro. Although overexpression of *Xlens1* in tissues outside the lens epidermis can cause rare ectopic sites of a lens protein marker, the morphology of these ectopic cells indicate that full differentiation of the mature lens phenotype is not achieved. Since *pax6*, *otx2* and *six3-like* are expressed in regions other than the developing lens, it is likely that these and perhaps other factors are required in combination with an appropriate level of *Xlens1* activity to produce a fully differentiated lens structure. The selective restriction of *Xlens1* expression to the developing lens suggests that this gene may mediate a critical component in the overlapping relationship of these factors.

The targeted overexpression of *Xlens1* in the lens ectoderm inhibits lens differentiation, as indicated by the loss of *γ-crystallin* mRNA and protein expression. Furthermore, the affected lens ectoderm expresses the *pax6* and *six3-like* genes in a spatial pattern indicative of the PLE rather than the differentiated lens placode. Additionally, normal *Xlens1* expression within the PLE appears mosaic at the stages when genes involved in terminal differentiation, such as *sox3* (stage 21; Zygar et al., 1998) and γ-crystallin, (stage 26/27; Smolich et al., 1994) are upregulated. This mosaicism suggests that *Xlens1* becomes downregulated in cells that are differentiating. The reduction of the *Xlens1* expression domain continues until only the anterior lens epithelial cells maintain expression in the mature lens. The observation that primary lens fiber cells do not express *Xlens1* suggests that *Xlens1* may function to delineate between the proliferating and differentiating lens cells after they are committed to a lens fate.

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