Cell-autonomous involvement of Mab21l1 is essential for lens placode development

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SUMMARY

The mab-21 gene was first identified because of its requirement for ray identity specification in Caenorhabditis elegans. It is now known to constitute a family of genes that are highly conserved from vertebrates to invertebrates, and two homologs, Mab21l1 and Mab21l2, have been identified in many species. We describe the generation of Mab21l1-deficient mice with defects in eye and preputial gland formation. The mutant mouse eye has a rudimentary lens resulting from insufficient invagination of the lens placode caused by deficient proliferation. Chimera analyses suggest that the lens placode is affected in a cell-autonomous manner, although Mab21l1 is expressed in both the lens placode and the optic vesicle. The defects in lens placode development correlate with delayed and insufficient expression of Foxe3, which is also required for lens development, while Maf, Sox2, Six3 and PAX6 levels are not significantly affected. Significant reduction of Mab21l1 expression in the optic vesicle and overlying surface ectoderm in Sey homozygotes indicates that Mab21l1 expression in the developing eye is dependent upon the functions of Pax6 gene products. We conclude that Mab21l1 expression dependent on Pax6 is essential for lens placode growth and for formation of the lens vesicle; lack of Mab21l1 expression causes reduced expression of Foxe3 in a cell-autonomous manner.

Key words: Lens placode development, Mab21l1, Mouse, PAX6, optic vesicle

INTRODUCTION

The mab-21 gene was first identified because of its role in cell fate determination in C. elegans (Baird et al., 1991). Hypomorphic alleles prevent development of the male tail, which includes a set of nine bilaterally symmetrical pairs of sense organs known as rays. The identity of ray 4 is transformed to ray 6 in mab-21 mutants, which results in ray fusion (Chow et al., 1995). Interestingly, such defects in ray identity specification are also observed in mutants for egl-5, the Antennapedia-class homeodomain transcription factor and the C. elegans ortholog of PAX6, respectively. A genetic study has shown that egl-5, mab-18 and mab-21 lie on a common genetic pathway that is required for normal ray development (Chow and Emmons, 1994). Another mab-21 mutant phenotype, short and fat body with uncoordinated movement, implies a role in development of the hypodermis and the nervous system (Chow et al., 1995). The null allele of mab-21 is lethal prior to hatching and mosaic analysis has shown that MAB-21 functions both cell-autonomously and -nonautonomously with respect to cell fate choice (Chow et al., 1995). However, many biochemical properties remain unclear.

Previous studies have shown that mab-21 is highly conserved in various animal species, from vertebrates to invertebrates (Mariani et al., 1999; Wong and Chow, 2002b). The human ortholog of mab-21 has been isolated from a retinal cDNA library (Margolis et al., 1996) and the first murine ortholog of mab-21 was cloned from an embryonic mouse brain cDNA library after a search for novel genes (Mariani et al., 1998). We have independently immunopurified the mouse mab-21 gene Mab21l1 as a target candidate for HOXC4 by a method described previously (Tomotsune et al., 1993). Briefly, a DNA fragment, located ~2 kb upstream of the putative Mab21l1 transcription start site, was cloned through a search for DNA sequences binding to the HOXC4 protein in native chromatin (D. Tomotsune and N.T., unpublished).

Two mab-21 orthologs, designated Mab21l1 and Mab21l2, are known to exist in Drosophila, zebrafish, Xenopus, chicken, mouse and human, whereas only one is present in two closely related nematode species, C. elegans and C. briggsae (Ho et al., 2001; Lau et al., 2001; Mariani et al., 1999; Mariani et al.,
MATERIALS AND METHODS

**Mab21l1 targeting vector**

We isolated a Mab21l1 genomic clone from a 129 mouse genomic library. The targeting vector consisted of a 4.9 kb 5′ homologous region (EcoRI–EcoRII fragment) and a 2.3 kb 3′ homologous region (EcoRI–BamHI fragment) as shown in Fig. 1A. The coding region was replaced with a Neo' gene cassette from the pMC1neo poly(A) vector (Stratagene). For negative selection, a MC1-DTA (diphtheria toxin A) cassette (Palmiter et al., 1987; Thomas and Capecchi, 1987) was placed at the end of the 3′ homology region.

**Targeting of ES cells and generation of Mab21l1−/− mutant**

The linearized targeting vector (30 μg) was electroporated into ES cells. Genomic DNA from 400 G418-resistant clones was digested with KpnI and SalI, and Southern blotted using 5′ and 3′ probes. Eight homologous recombinants were obtained, and cells from two of these clones were subjected to morula aggregation to generate chimeric mice as described earlier (Nagy et al., 1993). Chimeric males were mated with C57BL/6 females, and transmission of the targeted allele was confirmed by Southern blotting of tail tip DNA from the resultant progeny. Heterozygous mice were interbred to generate chimeric mice as described earlier (Nagy et al., 1993). From two of these clones were subjected to morula aggregation to generate chimeric mice as described earlier (Nagy et al., 1993).

Fig. 1. Generation of the Mab21l1-null allele. (A) Maps of the Mab21l1 locus, the targeting vector used for mutagenesis and the Mab21l1 targeted allele. White boxes represent the Mab21l1-coding region, the neo cassette and the DTA cassette. Black bars represent DNA fragments used as 5′ and 3′ probes for Southern blots; the sizes of restriction fragments detected are shown with lines. Arrowheads indicate the location of the primer pairs (primers m1F, m1R and neoR) used for PCR genotyping, with sizes of the PCR products given above. E, EcoRI; B, BamHI; K, KpnI; S, SalI. (B) Southern blots of tail DNA from progeny of heterozygous mice after KpnI and SalI digestion. Blots were probed with the 5′ probe, a 3.0 kb KpnI–EcoRI fragment, or the 3′ probe, a 1.3 kb BamHI–SpeI fragment. The endogenous KpnI fragment hybridizing with the 5′ and 3′ probes is 19 kb in length. Insertion of the neo cassette containing SalI sites reduced the KpnI fragments to 8.3 and 7.5 kb. (C) PCR genotyping of genomic DNA. Sizes of PCR products are indicated on the right. (D) Whole-mount in situ hybridization of Mab21l1 in E10.5 heterozygous and homozygous embryos. Note, Mab21l1 expression seen in the heterozygous embryo (left) is absent in the homozygote (right).
identical with both 129 and B6x129 hybrid backgrounds. The results described herein were obtained by analyzing mutant mice with a B6x129 hybrid genetic background.

**In situ hybridization**
Whole-mount in situ hybridization was performed as described (Nieto et al., 1996) at 65°C in 50% formamide containing 5× SSC. Paraffin and frozen sections were hybridized in situ at 65°C in 50% formamide containing 10% dextran sulfate. The Mab21l1 and Mab21l2 probes were derived from cDNA fragments, including the full-length coding region. Foxc3, Rx (Rax – Mouse Genome Informatics), Bmp4, Bmp7, Sox2, Maf, Chx10, Otx2, Six3, Hes1, Brn3b (Pou4f2 – Mouse Genome Informatics), α-crystallin (Cryaa – Mouse Genome Informatics), γ-crystallin (Cryga – Mouse Genome Informatics), Pax6 and Lhx2 probes have been described previously (Blixt et al., 2000; Furukawa et al., 1997; Furuta et al., 1997; Kamachi et al., 1998; Kawauchi et al., 1999; Liu et al., 1994; Matsuo et al., 1995; Oliver et al., 1995; Sasai et al., 1992; Turner et al., 1994; van Leen et al., 1987; Walther and Gruss, 1991; Wistow and Piattigorsky, 1988; Xu et al., 1993). Some probes were derived from mouse embryo RNA by RT-PCR amplification using primers based on published cDNA sequences. All were labeled with digoxigenin using standard procedures.

**Histology**
Embryos were dissected in phosphate-buffered saline (PBS) and fixed in 4% paraformaldehyde in PBS overnight at 4°C. The fixed embryos were dehydrated through graded alcohols and embedded in paraffin wax, sectioned at 8 μm, and stained with Hematoxylin and Eosin.

**Immunohistochemistry**
Paraffin wax-embedded and frozen sections were microwave-irradiated for 5 minutes in 10 mM citrate buffer (pH 6.0), incubated in 3% H2O2 for 10 minutes and then incubated with primary antibodies overnight at 4°C. The anti-PAX6 antibody was diluted to 1:1000 (Inoue et al., 2000). Antigen-antibody complexes were detected by incubation with HRP-conjugated goat anti-rabbit IgG (Medical and Biological Laboratories, Japan) diluted 1:100 for 1 hour at 37°C, followed by visualization with diaminobenzidine hydrochloride (DAB).

Frozen sections were prepared as follows. Embryos fixed in 4% paraformaldehyde in PBS overnight at 4°C were rinsed in PBS for 10 minutes then cryoprotected in a sequential series of 10, 20 and 30% sucrose in PBS. The embryos were oriented in OCT compound (Tissue-Tek), and rapidly frozen in liquid nitrogen. Sections cut at 14 μm were mounted on MAS-coated glass slides (Matsunami Glass, Japan).

**Detection of proliferating or apoptotic cells**
Pregnant mice were intraperitoneally injected with 3 mg of bromodeoxyuridine (BrdU). Embryos were sacrificed 1.5 hours later and fixed in 4% paraformaldehyde in PBS overnight at 4°C. Sections (6 μm) of embryos embedded in paraffin wax were microwaved for antigen retrieval as described above, then stained with an anti-BrdU antibody using a BrdU Labeling and Detection Kit II (alkaline phosphatase, Roche). Quantitation was performed using five sections from three embryos of each genotype. TdT-mediated dUTP nick end labeling (TUNEL) analysis was also performed using a In Situ Cell Death Detection Kit (HRP, Roche).

**Chimera analysis**
As most Mab21l1+/– mice proved to be sterile with natural mating, we obtained Mab21l1+/– embryos by in vitro fertilization based on the methods of Toyoda et al. (Toyoda et al., 1972a; Toyoda et al., 1972b). Briefly, female homozygotes were superovulated by intraperitoneal injections with 5 IU of pregnant mare serum gonadotropin (PMSG) and 5 IU of human chorionic gonadotropin (hCG) 48 hours later. Unfertilized eggs were collected from the ampullae of oviducts 16-17 hours after hCG injection and placed in TYH medium equilibrated under 5% CO2. Sperm were collected from the caudal epididymis of males and suspended in TYH medium (Toyoda et al., 1972a). After capacitation for 1-2 hours, ~5-10 μl aliquots of sperm suspension were added to 0.3 ml TYH medium containing eggs, so that the final density of spermatozoa was 150-300×106. The efficiency of in vitro fertilization was evaluated 6 hours after insemination by examining release of the second polar body and the formation of female and male pronuclei. Fertilized eggs were either incubated for another 24 hours under the same conditions or transferred to m-WM medium for further development (Witten, 1971). ROSA26 Tg/+ and wild-type embryos were also generated by in vitro fertilization.

Eight-cell embryos were aggregated as described to generate chimeras (Koizumi et al., 2001). Chimeric embryos were harvested at a stage equivalent to 12.5 dpc, fixed in 1% formaldehyde, 0.2% glutaraldehyde and 0.02% NP-40 in PBS for 30 minutes at 4°C, then washed twice with PBS for 30 minutes at room temperature. Fixed embryos were stained for 2 days at 37°C in PBS containing 1 mg/ml X-Gal, 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 2 mM MgCl2 and 0.02% NP-40 to detect β-galactosidase. The embryos were fixed again in 4% paraformaldehyde, embedded in paraffin wax and sectioned.

**RESULTS**

**Generation of the Mab21l1 mutant**
The targeted allele was detected in the progeny of heterozygous mice by Southern blotting using 5’ and 3’ external probes (Fig. 1B). The heterozygous mice were apparently normal and were interbred to produce homozygous mutants. The resulting progeny were classified according to genotype at 3 weeks of age (Fig. 1C). Frequencies of homozygotes, heterozygotes and wild type were 25%, 50% and 25%, respectively, implying that the mutation does not affect survival. Whole-mount in situ hybridization revealed the absence of Mab21l1 transcripts in E10.5 homozygous embryos (Fig. 1D).

**Eye and preputial gland defects in adult Mab21l1 deficient mice**
Visual inspection revealed obvious eye defects in all adult Mab21l1 homozygotes (Fig. 2A,B). Mab21l1+/– eyes were much smaller than in wild-type mice (Fig. 2C) and histological analysis indicated the presence of only a rudimentary lens and revealed the lack of the iris and ciliary body in adult homozygotes (Fig. 2D,E). The anterior eye chamber was not formed and the prospective chamber was occupied by pigmented cells (Fig. 2D, part a; E, part b). The cornea was also affected so that epithelial and mesenchymal cells were not clearly segregated (Fig. 2E, part b). Although the optic nerve and retinal pigmented epithelium were present, along with retinal lamination and the major class of retinal cells, the layer was much thinner than in the normal retina (Fig. 2E, part d).

We also found that the preputial glands displayed a marked reduction in overall size in Mab21l1+/– adult males, whereas other components of the urogenital system were normally developed (Fig. 2F-H). Histological analysis showed small intralumenal spaces when compared with the preputial glands of heterozygotes (Fig. 2H, inset). The cellular composition of epithelial cells and mesenchyme appeared normal (data not shown). These observations demonstrate that Mab21l1 is essential for the appropriate development of the preputial gland. Fertility determined by coitus was significantly affected in the homozygous mutants. As sperm derived from the homozygous mutants were capable of fertilization in vitro,
defects in the preputial gland could be the cause of infertility
of Mab21l1-mutant males. Although Mab21l1 is normally
expressed in various organs, such as the midbrain, spinal cord,
branchial arches and limb buds, we did not observe any
significant changes in these in Mab21l1–/– adult mice on
histological analysis. Mab21l1 is required for normal lens development
Eye defects in Mab21l1–/– were obvious at E12.5, as
represented by malformed pigmented epithelium on visual
inspection under a stereomicroscope, whereas no significant
difference was visible at E10.5 (Fig. 3A,B). A lack of any lens
was manifest at E16 (Fig. 3A,B). In addition, histological
analyses of E17 Mab21l1–/– fetuses revealed that the lens was
absent and that the prospective cornea and retina were
significantly thickened compared with Mab21l1+/– fetuses (Fig.
3C,D). At E13, the lens was absent and malformation of the
retina was observed, whereas the presumptive cornea and the
pseudostratification of retina were still morphologically normal
(Fig. 3E,F). At E10.5, the lens placode failed to form a lens
vesicle in the Mab21l1–/– eye, whereas the lens pit had already
separated from the surface ectoderm and the lens vesicle was
formed in the Mab21l1+/– eye (Fig. 3G,H). The earliest
morphological changes in Mab21l1–/– were seen at E9.5, when
the lens placode in the area in contact with the optic vesicle was
thickened to a lesser extent and narrower than in the Mab21l1+/–
embryo (Fig. 3I,J). At E9.0, we could not observe significant
differences in the prospective eye area between Mab21l1+/– and
Mab21l1–/– embryos. These results indicate that Mab21l1 has
an important role in the lens formation.

Mab21l1 is required for normal lens development
Defects in lens development in Mab21l1–/– embryos were
further investigated using molecular markers expressed in the
developing lens and neural retina. αA-crystallin is one of the
earliest genes to be expressed in the invaginating lens pit (van
Leen et al., 1987; Wistow and Piatigorsky, 1988). At E10.5,
αA-crystallin expression could be detected in the Mab21l1+/–
lens pit, whereas no signals were detected in Mab21l1–/–
embryos (data not shown). At E12.5 in the Mab21l1+/– embryo,
αA-crystallin transcripts were abundant in the lens fiber cells
with lesser amounts in the lens epithelial cells (Fig. 4A). In
Mab21l1–/– embryos, the expression of αA-crystallin was
restricted to the lens fiber cells in the Mab21l1+/– lens (Fig. 4C)
(Goring et al., 1992; van Leen et al., 1987). In the Mab21l1–/–
embryo, expression appeared to be affected but was still
detectable (Fig. 4D). Thus, it is likely that lens fiber cell
differentiation could occur in the Mab21l1–/– embryo, although
lens epithelial cells and lens fiber cells are indistinguishable by
histological analysis in the crystallin-positive domain (Fig.
4B,D). The expression of Chx10 and Brn3b, both of which are required for normal development of the neural retina (Burmeister et al., 1996; Ferda Percin et al., 2000; Gan et al., 1996; Liu et al., 1994; Liu et al., 2000; Turner et al., 1994), was similar in Mab21l1–/– and Mab21l1+/– retinas (Fig. 4E-H). In situ hybridization for Rx, Sox2, Otx2, Six3, Hes1, Pax6 and Lhx2 transcripts, the expression of each of which is known to partially overlap with Mab21l1 expression in the developing retina (Furukawa et al., 1997; Kamachi et al., 1998; Matsuo et al., 1995; Oliver et al., 1995; Sasai et al., 1992; Walther and Gruss, 1991; Xu et al., 1993), did not demonstrate any significant alteration (data not shown).

**Mab21l1 and Mab21l2 expression during eye development**

The lens defects of Mab21l1-null mutants prompted us to re-
ectoderm (Fig. 5C). By contrast, not restricted to the presumptive lens placode in the surface intense in the surface ectoderm and the optic vesicle, and was much lesser extent in the surface ectoderm (Fig. 5D). At the predominant seen in the dorsal optic vesicle and only to a extent in the surface ectoderm and the optic vesicle, and was not restricted to the presumptive lens placode in the surface ectoderm (Fig. 5C). By contrast, Mab21l2 expression was predominantly seen in the dorsal optic vesicle and only to a much lesser extent in the surface ectoderm (Fig. 5D).

Fig. 5. Expression of Mab21l1 and Mab21l2 transcripts during eye development. In situ hybridization of coronal frozen (A-F) and paraffin wax (G,H) sections of wild-type eyes. Mab21l1 but not Mab21l2 transcripts are expressed in lens, whereas both are similarly present in retina. ip, lens placode; nr, neural retina; on, optic nerve; ov, optic vesicle; pe, retinal pigmented epithelium; pnr, presumptive neural retina; ppe, presumptive retinal pigmented epithelium; le, lens epithelium; se, surface ectoderm. Scale bars: 50 μm for A,B; 100 μm for C-H.

Mab21l1 acts cell autonomously for lens formation
The vertebrate lens arises from the facial surface ectoderm in response to inductive signals from the optic vesicle (Grainger, 1992). Impaired proliferation of the lens placode in Mab21l1–/– mice could be attributed to defects in either the lens placode or the underlying optic vesicles, or both, because Mab21l1 expression was seen in both (Fig. 5). To reveal whether the role of Mab21l1 is cell autonomous in lens development, we generated chimeric mice composed of wild-type and Mab21l1–/– embryonic cells. These Mab21l1–/– embryonic cells were aggregated to wild-type cells that were genetically labeled using lacZ expression derived from the ROSA26 locus (Zambrowicz et al., 1997). The chimeras were harvested at ~E12.5, and preparations were stained for β-galactosidase and analyzed histologically. In Mab21l1+/+ control chimeras, β-galactosidase-negative cells were distributed in the lens epithelium as well as in other tissues (Fig. 7A,A'). The contribution of Mab21l1–/– cells was low in 13 out of 24 Mab21l1–/– chimeras, and they
appeared histologically normal. The contribution of \textit{Mab21l1}^{-/-} cells was moderate in seven out of the remaining \textit{Mab21l1}^{-/-} chimeras (Fig. 7B,C) and, although lenses were present in all of these, they were significantly smaller than normal lenses (Fig. 7A’,B’,C’). Importantly, considerable contribution of \textit{Mab21l1}^{-/-} cells into this small lens was not seen in any of these moderate chimeras (Fig. 7b,c). In contrast to the lens itself, a variable contribution of \textit{Mab21l1}^{-/-} cells was observed in the retina, cornea and mesenchymal tissues (Fig. 7b,c). The contribution of \textit{Mab21l1}^{-/-} cells was high in four of the \textit{Mab21l1}^{-/-} chimeras (Fig. 7D), in which no lens was formed (as in the \textit{Mab21l1}^{-/-} mutants) (Fig. 7D’,d). The exclusion of \textit{Mab21l1}^{-/-} cells from the lens in chimeric mice implies that the \textit{Mab21l1} gene product acts in cell-autonomous manner during lens formation.

\textbf{Foxe3 expression is reduced in the \textit{Mab21l1}-mutant lens placode}

To gain insights into the molecular basis for lens defects in \textit{Mab21l1}^{-/-} embryos, we investigated the expression of \textit{Maf}, \textit{Foxe3}, \textit{Sox2}, \textit{Six3} and \textit{Pax6}, which are known to be involved in lens development (Ashery-Padan and Gruss, 2001; Chow and Lang, 2001; Kondoh, 1999). \textit{Foxe3}, encoding the forkhead domain, is first expressed in the presumptive lens placode; its expression is maintained in the lens pit and then restricted to the lens epithelium (Blixt et al., 2000; Brownell et al., 2000). Mutations in the forkhead domain of the \textit{Foxe3} co-segregate with dysgenetic lens (\textit{dyl}) phenotype, and the \textit{dyl}-mutant mice display defects in lens vesicle separation from the ectoderm, as well as a lack of proliferating cells in the lens epithelium (Blixt et al., 2000; Ormestad et al., 2002). Expression of \textit{Maf}, which encodes the basic-leucine zipper transcription factor, in the surface ectoderm demarcates the presumptive lens placode prior to invagination, and \textit{Maf} continues to be expressed in the lens fiber cells (Kawauchi et al., 1999). It has been shown to play an essential role in lens differentiation (Kawauchi et al., 1999; Ring et al., 2000). Upregulation of \textit{Sox2}, which encodes the

\textbf{Fig. 6.} Analysis of cell proliferation and apoptotic cell death in \textit{Mab21l1}^{+/-} and \textit{Mab21l1}^{-/-} lens placodes. Immunohistochemical detection of BrdU incorporation in \textit{Mab21l1}^{+/-} and \textit{Mab21l1}^{-/-} embryos at various somite stages (A-H). Brackets (D,F) indicate reduced incorporation of BrdU in \textit{Mab21l1}^{-/-} small lens placodes. Red dotted lines indicate a \textit{Mab21l1}^{+/-} lens vesicle (G) and a \textit{Mab21l1}^{-/-} malformed lens placode (H). The inset shows a HE-stained serial section of the area within the box (H). TUNEL assay of 35-somite stage \textit{Mab21l1}^{+/-} (I) and \textit{Mab21l1}^{-/-} (J) embryos. Numbers of TUNEL-positive cells are slightly increased in the \textit{Mab21l1}^{+/-} lens pit (arrowheads). Histogram showing the percentage of BrdU-positive cells in \textit{Mab21l1}^{+/-} and \textit{Mab21l1}^{-/-} lens anlages (K). The ratio of BrdU-positive cells is reduced in the \textit{Mab21l1}^{+/-} mutants (\textit{t}-test; 27 and 32-somite stages, \(P<0.004\); other somite stages, \(P<0.0001\)). Vertical bars represent standard errors. lp, lens placode; lv, lens vesicle; ov, optic vesicle; pi, lens pit; se, surface ectoderm. Scale bars: 50 \(\mu\)m.
HMG box-containing transcription factor, is tightly associated with induction of the lens placode (Furuta and Hogan, 1998; Kamachi et al., 1998). Expression of Six3, the sine oculis related homeobox-containing gene, in the developing lens first appears around the time of lens placode formation (Lagutin et al., 2001). The homeodomain transcription factor PAX6 is expressed in both the surface ectoderm and the optic vesicle; it continues to be expressed in the lens and optic cup, and is essential for normal eye development (Walther and Gruss, 1991; Chow et al., 1999; Grindley et al., 1995; Hill et al., 1991).

We compared the expression of Maf, Foxe3, Sox2, Six3 and Pax6 in Mab21l1+/– and Mab21l1–/– embryos at the 22-somite (E9.5) to 40-somite stages (E10.5). At the 22-somite stage, prior to lens induction, expression of PAX6 and Six3 in the Mab21l1–/– surface ectoderm did not differ from that seen in Mab21l1+/– embryos. At the 24-somite stage, upregulation of Sox2 and the first expression of Maf in the surface ectoderm occurred simultaneously in the Mab21l1–/– and Mab21l1+/– embryos. At this stage, we did not observe any significant expression of Foxe3. At the 28-somite stage, Foxe3 was clearly expressed in the Mab21l1+/– presumptive lens placode but expression was not seen in the Mab21l1–/– embryos (Fig. 8A,B). We did not find any significant difference between PAX6 expression in the surface ectoderm and optic vesicles of Mab21l1+/– embryos and Mab21l1–/– embryos (Fig. 8C,D), or any significant differences of Maf, Sox2 and Six3 expression in the presumptive lens placode (data not shown). At the 35-somite stage, the lens placode failed to undergo sufficient invagination in the Mab21l1–/– embryo (Fig. 8E,H). Foxe3 expression was seen in the Mab21l1–/– lens placode, although its expression domain was much smaller than in Mab21l1+/– embryos and the expression level in each cell was significantly lower (Fig. 8E,F). By contrast, the level of Maf and PAX6 expression in the Mab21l1–/– lens placode was similar to that in the Mab21l1+/– developing lens pit (Fig. 8G,H; data not shown). At the 40-somite stage, expression of Foxe3 had totally disappeared in the Mab21l1–/– mutants, although a rudimentary lens placode was still present (Fig. 8I,J). Expression of Maf and PAX6 was maintained in the Mab21l1–/– lens placode as well as in the Mab21l1+/– lens vesicle. These results suggest that MAB21L1 function is required for the appropriate induction and subsequent maintenance of Foxe3 expression.

**Mab21l1 expression in the eye surface ectoderm and optic vesicles is dependent on PAX6**

PAX6 plays a central role in eye development as demonstrated by the allelic series of small eye (Sey) mutations in which the Pax6 gene is affected (Grindley et al., 1995; Hill et al., 1991; Ashery-Padan and Gruss, 2001). The requirement for Pax6 in lens development has been confirmed by analysis of conditional Pax6-null mutant mice (Le-mutant) in

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Fig. 7. Distribution of Mab21l1–/– cells in chimeric embryos. (A) Whole-mount view of an Mab21l1+/+ chimeric embryo. (B-D) Whole-mount views of Mab21l1–/– chimeric embryos. (A’-D’) Histological sections of embryos identical to those shown in A-D, respectively. (a-d) Higher magnification views of areas within the boxes in A’-D’. In the Mab21l1+/+ chimeric embryo, β-galactosidase negative cells are distributed in the retina (A’) and lens epithelium (a, arrowheads). No Mab21l1–/– cells (β-galactosidase negative cells are white) are present in the lens, whereas both wild-type (β-galactosidase positive cells are blue) and Mab21l1–/– cells are present in retina (B’-C’). (C’) Despite the high contribution of Mab21l1–/– cells in the retina, the lens is similar in size to that of the chimera in B’. A high contribution of Mab21l1–/– cells leads to absence of the lens (D’,d). Scale bars: 100 μm for A’-D’, 50 μm for a-d.
which PAX6 activity is specifically lost in the eye surface ectoderm, which results in the absence of lens placode formation (Ashery-Padan et al., 2000). The phenotype similarity of the Le-mutant to the Mab21l1–/– mutant and the preserved expression of PAX6 in the Mab21l1–/– mutant prompted us to examine the role of PAX6 in Mab21l1 expression (Fig. 8D,H), using Sey-homozygous mutant embryos.

At the 22-somite stage, Mab21l1 expression was hardly seen in the surface ectoderm and the optic vesicle in Sey homozygous embryos, when compared with expression in wild-type littermates (Fig. 9A,B). At the 32-somite stage, Mab21l1 expression was observed at low but significant levels in both degenerated optic vesicle and the surface ectoderm in Sey homozygotes (data not shown). At the 35-somite stage, Mab21l1 expression was greatly reduced in the degenerated optic vesicles in Sey/Sey embryos when compared with the wild-type embryos (Fig. 9D). At this stage, despite the lack of lens vesicles in Sey/Sey embryos, a few overlying surface ectodermal cells expressed small amounts of Mab21l1 transcripts (Fig. 9D). In wild-type embryos, Mab21l1 was strongly expressed in the lens and presumptive neural retina (Fig. 9C). In contrast to Mab21l1, the level of Mab21l2 expression was unperturbed in the Sey/Sey embryos at all stages examined (Fig. 9E,F; data not shown). These results suggest that expression of Mab21l1 is dependent on PAX6 both in the surface ectoderm and the optic vesicle.

DISCUSSION

In the present study, histological analyses of the crystallin-expressing rudimentary developing lens in Mab21l1–/– embryos revealed that invagination of the lens placode is primarily affected on the basis of proliferation, rather than on...
Role of Mab21l1 in lens formation

Inductive interactions between the optic vesicle and the surface ectoderm are known to be essential for induction and subsequent differentiation of the lens placode. Signals mediated by Bmp and Fgf family proteins are essential, converging on Pax6 expression in the lens placode (Faber et al., 2001; Furuta and Hogan, 1998; Wawersik et al., 1999). We found apparently normal expression of Pax6, Sox2, Six3 and Pax6 expression was not significantly affected. However, the significant reduction of Mab21l1 expression in the optic vesicle and the overlying surface ectoderm in the stages before induction of the lens placode in Sey homozygotes indicates that Mab21l1 expression in the developing eye is dependent upon the functions of Pax6 gene products. We conclude that Mab21l1 expression dependent on Pax6 is essential for lens placode growth and for formation of the lens vesicle through regulation of Foxe3 expression in a cell-autonomous manner.

Possible compensation of Mab21l1 loss by Mab21l2

It is interesting that defects in Mab21l1–/– mice are restricted to the eye and preputial gland even though the transcripts are also found in the midbrain, branchial arch, spinal cord and limb buds. It is presumed that this relatively mild phenotype in Mab21l1–/– mice might be caused by compensation by the closely related gene product MAB21L2, which exhibits very similar expression during development except in the developing lens and the primordium of reproductive organs (Mariani et al., 1999; Mariani et al., 1998; Wong et al., 1999) (R.Y. and N.T., unpublished). Intriguingly, Mab21l2–/– mutants exhibit much stronger defects in various tissues than Mab21l1–/– mutants, and die in the mid-gestational stage. It is presumed that MAB21L1 cannot fully compensate for Mab21l2 deficiency because the mutant embryos exhibit abnormalities in tissues where there is overlapping expression (R.Y. and N.T., unpublished). This could explain the phenotypical differences between our Mab21l1–/– mutants and previous loss-of-function embryos generated using an antisense ODN approach (Wong and Chow, 2002a). The latter mutants exhibit failure of embryonic turning and neural tube closure as well as eye defects. A slight reduction in MAB21L2 or an unpredicted depletion of other genes by Mab21l1–/– specific ODN could modify the phenotype caused by Mab21l1 depletion.

Male and female sterility in Mab21l1 mutants

We also found a defect in the accessory reproductive organs, with malformation of the preputial gland in Mab21l1–/– adult males. Importantly, most Mab21l1–/– mice of both sexes were sterile; however, in vitro fertilization experiments revealed that the germ cells were normal (T.H. and H.K., unpublished). This suggests that infertility in Mab21l1–/– mice could be caused by defects in the preputial gland and/or by behavioral changes. Blindness per se is not responsible because another eye mutant, aphakia (Pitx3 – Mouse Genome Informatics), is fertile (Semina et al., 2000). By contrast, homozygous spdh-mutant male mice harboring a spontaneous mutation in the Hoxd13 gene lack preputial glands and are sterile, whereas the females exhibit normal fertility (Johnson et al., 1998). The difference between spdh/spdh males and females reflects the dispensable role of the preputial gland in females. The fact that Mab21l1–/– mice of both sexes are infertile with only incomplete loss of the preputial gland, indicates that some other factors may also be contributory. Sexual behavior could be affected in Mab21l1–/– mice for either of the following reasons. First, the preputial gland in male mice has been shown to contribute to pheromonal function: generating chemosignals that attract females and accelerating oestrus (Bronson and Caroom, 1971; Chipman and Albrecht, 1974). Second, the localization of Mab21l1 transcripts within the CNS is consistent with studies that link reproductive behavior to specific regions of the brain, collectively known as the vomeronasal system (VNS) (Segovia
and Guillamon, 1993). We identified Mab21I expression in the VNS, which includes the vomeronasal organ (VNO), accessory olfactory bulb, amygdala and hypothalamus (R.Y., unpublished). The VNO is a chemoreceptive organ that is thought to mediate pheromone effects (Halpern, 1987). Importantly, Mab21I2 appears not to be expressed in the VNS, except in the hypothalamus. Although we could not find any histological alterations in the VNS, it is possible that reproductive behavior could be profoundly affected in Mab21I+/– mice.

**Evolutional conservation of a genetic pathway including MAB21 family**

The MAB21 family is highly conserved and orthologs are found from *C. elegans* to humans (Mariani et al., 1999; Wong and Chow, 2002b). Several experiments have shown the importance of members during development in the nematode, zebrafish, *Xenopus* and mouse (Chow et al., 1995; Kawahara et al., 2002; Kudoh and Dawid, 2001; Lau et al., 2001; Wong and Chow, 2002a). In *C. elegans, mab-21* may modulate the functions of *egl-5*, an ortholog of *Drosophila Abdominal B* (*Abd-B*), and *mab-18*, a *Pax6* ortholog, during the specification of ray identity (Chow and Emmons, 1994; Chow et al., 1995). In addition, *mab-21* has been shown to act downstream of the TGF-β signaling pathway leading to the male tail morphogenesis (Morita et al., 1999). The present report provides evidence that Mab21I and Pax6 lie in the same genetic pathway involved in vertebrate eye development. The signaling axis TGF-β/mab-18/mab-21 in *C. elegans* is reminiscent of the Bmp7/Pax6/Mab21I axis implicated in lens development of mice. However, we cannot explain functional roles because of a lack of knowledge of common denominators in ray and eye morphogenesis.

However, the abnormal preputial gland formation in *Mab21I+/–* males is also seen in *spdh* homozygotes, a *Hoxd13* mutant (Johnston et al., 1998). As both *egl-5* and *Hoxd13* are *Drosophila Abd-B* orthologs (de Rosa et al., 1999), similar genetic cascades including *egl-5/mab-21* or *Hoxd13/Mab21I* could operate in ray identity specification and preputial gland formation, respectively. Therefore, during mammalian lens and preputial gland development, Mab21I, Hoxd13 and Pax6 may have a regulatory relationship that resembles that of the *mab-21, egl-5* and *mab-18* genetic pathway in *C. elegans* ray development. Further studies are required to understand the molecular functions of the MAB21 family members that contribute to the morphogenesis of various tissues across species.

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