

## Bmp signaling is required for development of primary lens fiber cells

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### SUMMARY

We have investigated the role of Bmp signaling in development of the mouse lens using three experimental strategies. First, we have shown that the Bmp ligand inhibitor noggin can suppress the differentiation of primary lens fiber cells in explant culture. Second, we have expressed a dominant-negative form of the type 1 Bmp family receptor *Alk6* (*Bmpr1b* – Mouse Genome Informatics) in the lens in transgenic mice and shown that an inhibition of primary fiber cell differentiation can be detected at E13.5. Interestingly, the observed inhibition of primary fiber cell development was asymmetrical and appeared only on the nasal side of the lens in the ventral half. Expression of the inhibitory form of *Alk6* was driven either by the  $\alpha$ A-crystallin promoter or the ectoderm enhancer from the *Pax6* gene in two different transgenes. These expression units drive transgene expression in distinct patterns that overlap in the equatorial cells of the

lens vesicle at E12.5. Despite the distinctions between the transgenes, they caused primary fiber cell differentiation defects that were essentially identical, which implied that the equatorial lens vesicle cells were responding to Bmp signals in permitting primary fiber cells to develop. Importantly, E12.5 equatorial lens vesicle cells showed cell-surface immunoreactivity for bone-morphogenetic protein receptor type 2 and nuclear immunoreactivity for the active, phosphorylated form of the Bmp responsive Smads. This indicated that these cells had the machinery for Bmp signaling and were responding to Bmp signals. We conclude that Bmp signaling is required for primary lens fiber cell differentiation and, given the asymmetry of the differentiation inhibition, that distinct differentiation stimuli may be active in different quadrants of the eye.

Key words: Bmp, Mouse, Lens, *Alk6*

### INTRODUCTION

The mature lens is a polarized structure consisting of a mitotic epithelial layer that covers the anterior surface and terminally differentiated lens fiber cells that occupy the interior volume and the posterior surface. In 1963, it was shown that if the lens of a chick embryo was removed and rotated 180° so that the epithelial layer faced the retina, it would completely repolarize over a few days (Coulombre and Coulombre, 1963). This implied that all of the signals necessary for the establishment of lens polarity are present in the ocular media. Since this experiment, the identity of lens polarization signals has been of interest.

One element of lens polarization is the differentiation of the lens fiber cells that express crystallin proteins and impart the features of transparency and refractivity (McAvoy, 1978). Evidence that fiber cell differentiation signals have their origin in the retinal cup comes from experiments showing that rotated mouse lenses are only able to repolarize in the presence of retinal tissue (Yamamoto, 1976). Several signaling pathways have been implicated in the regulation of lens polarity. These include the Fgf signaling pathway, which is important for fiber cell differentiation (Chow et al., 1995; McAvoy and

Chamberlain, 1989; Robinson et al., 1995a; Robinson et al., 1995b; Stolen and Griep, 2000), and the Igf1 pathway that can regulate fiber cell differentiation in the chick (Beebe et al., 1980; Beebe et al., 1987) and epithelial cell proliferation in the mouse (Shirke et al., 2001). Involvement of TGF $\beta$  ligands in lens development has recently been uncovered by the attenuated fiber cell differentiation phenotype observed when a dominant-negative TGF $\beta$  receptor is expressed in the lens (de Jongh et al., 2001).

Several bone morphogenetic protein (Bmp) family ligand genes are expressed during early development of the eye. These include *Bmp4* and *Bmp7* (Furuta and Hogan, 1998; Wawersik et al., 1999), each of which is believed to play an important early role. *Bmp7* has a role in lens induction (Wawersik et al., 1999) where it cooperates with Fgf receptor signaling (Faber et al., 2001). *Bmp4* has also been implicated in lens development and differentiation as it is genetically upstream of *Sox2* (Wawersik et al., 1999), a transcription factor that regulates expression of crystallin genes (Kamachi et al., 2001). The *Bmp4* transcript is present in both the presumptive lens and presumptive retina but is expressed predominantly in the dorsal optic cup as primary fiber cell differentiation begins at E11.5 (Furuta and Hogan, 1998). Within the lens lineage, *Bmp7* is

expressed in the presumptive lens ectoderm, lens pit and lens vesicle but is downregulated thereafter (Wawersik et al., 1999). Based on characterized expression patterns (of *Gdf6*, for example) (Chang and Hemmati-Brivanlou, 1999), it might be expected that many other Bmp family ligands will have a role in different aspects of eye development. Bmp signaling occurs by means of ligand binding a primary (type 2) receptor, a complex formation with a transducer, a (type 1) receptor and consequent phosphorylation of Smad proteins (Massagué, 1998; Piek et al., 1999). Three known type 2 receptors [*Bmpr2*, *Actr2a* (*Acvr2* – Mouse Genome Informatics) and *Actr2b* (*Acvr2b* – Mouse Genome Informatics)] and four known type 1 receptors [*Actr1* (*Acvr1* – Mouse Genome Informatics; also known as *Alk2*), *Bmpr1a* (also known as *Alk3*), *Bmpr1b* (also known as *Alk6*), *Actr1b* (*Acvr1b* – Mouse Genome Informatics)] are expressed in the developing eye (Dewulf et al., 1995; Feijen et al., 1994; Furuta and Hogan, 1998; Obata et al., 1999; Verschuere, 1995; Yoshikawa et al., 2000).

In this study, we have investigated the involvement of Bmp signaling in development of the lens. We show that the Bmp binding and inhibition protein noggin can suppress primary fiber cell elongation and lens size in explant culture. We also expressed a dominant negative Bmp type 1 receptor (*Bmpr1b*; also known as *Alk6*) in the developing lens in transgenic mice. Both the *Pax6* ectoderm enhancer (Kammandel et al., 1999; Williams et al., 1998; Xu et al., 1999) and the  $\alpha A$  crystallin promoter (Chepelinsky et al., 1985) were used to drive transgene expression. These mice show defects in the differentiation of primary lens fiber cells, suggesting that Bmp ligands are important for this aspect of lens development. Importantly, we show using anti-Bmpr2 (Gilboa et al., 2000) and anti-phospho-Smad (Itoh et al., 2001) antibodies that at embryonic day (E) 12.5, when primary fiber cell differentiation is beginning, equatorial lens cells have Bmp signaling machinery and are responding to Bmp signals. Finally, we show that the primary lens fiber cell differentiation defect is radially asymmetrical, perhaps implying that there are distinct differentiation stimuli active in different quadrants of the eye.

## MATERIALS AND METHODS

### Generation of transgenic mice

A restriction fragment encoding a dominant negative mutant form (a point mutation resulting in K231R) (Zou and Niswander, 1996) of the rat *Alk6* cDNA (*Bmpr1b*) was sub-cloned into the polylinker of the plasmid *p* $\alpha$ *SPLONO* (Chow et al., 1995) to generate the transgene  $\alpha A$ -*Alk6*<sup>DN</sup> (Fig. 2A). A second transgene, designated *EE-1.0-K-Alk6*<sup>DN</sup> (Fig. 2A) was generated in *pSPLONO* (Chow et al., 1995) by subcloning a 340 bp, PCR amplified fragment encoding the *Pax6* ectoderm enhancer (Kammandel et al., 1999; Williams et al., 1998) adjacent to a 1 kb, *SpeI-XhoI* fragment containing the P0 promoter of *Pax6*. Subsequently, the cDNA encoding dominant-negative *Alk6* and containing a Kozak consensus (Kozak, 1986) translational start codon was subcloned downstream of the P0 promoter. The oligonucleotide used to add the efficient start codon was of the sequence ACTAGT-GGATCC-TACGTA-CCACCATGG. Transgenic FVB/N (Taketo et al., 1991) mice were generated and screened according to established methods (Hogan et al., 1986).

### In situ hybridization

Whole-mount in situ hybridization was performed as described (Nieto et al., 1996). All embryos were washed in PBS and fixed in 4%

paraformaldehyde at 4°C. Antisense RNA probes were labeled with digoxigenin during in vitro transcription and whole-mount in situ hybridization performed as described previously (Nieto et al., 1996). Section in situ hybridization using radioactively labeled SV40 sequence probes was performed as described previously (Robinson et al., 1995b).

### Histological analysis

Tissues for histological analysis included staged mouse embryos of whole eyes from postnatal animals. Tissue samples were prepared and stained either with Hematoxylin and Eosin (H/E) using conventional methods (Culling et al., 1985). Figures in this paper were prepared digitally using a Sony DKC1000 digital camera and Adobe Photoshop software.

### Immunohistochemistry

Staged embryos fixed were fixed in 4% PFA in PBS and immunofluorescently labeled according to conventional methods (Harlow and Lane, 1988). Polyclonal rabbit antisera for MIP26 (Horwitz and Bok, 1987) was used at a dilution of 1:200. Polyclonal rabbit antisera for  $\gamma$ -crystallin (Zigler and Sidbury, 1976) were both used at a dilution of 1:500. The anti-Bmpr2 (Gilboa et al., 2000) and anti-phospho-Smad (Itoh et al., 2001) antibody are affinity-purified rabbit polyclonals from ten Dijke and P. Knaus that were used at a 1:500 and 1:1000, respectively.

### 3D reconstructions

Reconstructions were created by tracing digital images of 15–20 H/E stained histological sections of wild-type and transgenic lenses in the program Canvas. Canvas tracings were then converted to simple line drawings and imported into the 3-D program FormZ. Individual section tracings were stacked along a z-axis and carefully aligned in both the x and y dimensions. The 'skin' command was then used to mold a skin over the aligned sections. The resulting lenses were then identified as whole objects that could then be rotated in three dimensions so that all sides of the lens could be observed.

### Explant culture

Explants of wild-type E10.5 lenses were made following the protocol described elsewhere (Wawersik et al., 1999). Briefly, E10.5 embryos were dissected under a dissecting microscope in PBS at 4°C and the eyes placed in DMEM at 4°C. When eyes from an entire litter had been collected, the presumptive retinal pigmented epithelium was carefully removed with needles. Eyes were then placed in 33% rat-tail collagen (two-thirds DMEM pH 4.0, one third rat-tail collagen, made basic with 0.8M NaCO<sub>3</sub> – 16  $\mu$ l/200  $\mu$ l 33% collagen), and left to gel for about 10 minutes. After hardening, the explant collagen gel was immersed in DMEM + 10% FCS with or without human recombinant Noggin (Regeneron Pharmaceuticals) at 300 ng/ml. Cultures were left to grow for 48 hours then fixed with quick-fix (Culling et al., 1995) and processed for paraffin wax sectioning.

## RESULTS

### Ex vivo inhibition of Bmp activity results in smaller lenses

As an initial test of the involvement of Bmp signaling in primary lens fiber cell differentiation, we performed a series of explant experiments where Bmp activity was inhibited by the antagonist noggin (Zimmerman et al., 1996). This general strategy has previously been used to assess lens responses (Wawersik et al., 1999). We performed this analysis with E10.5 tissues that have already been through the induction phases of

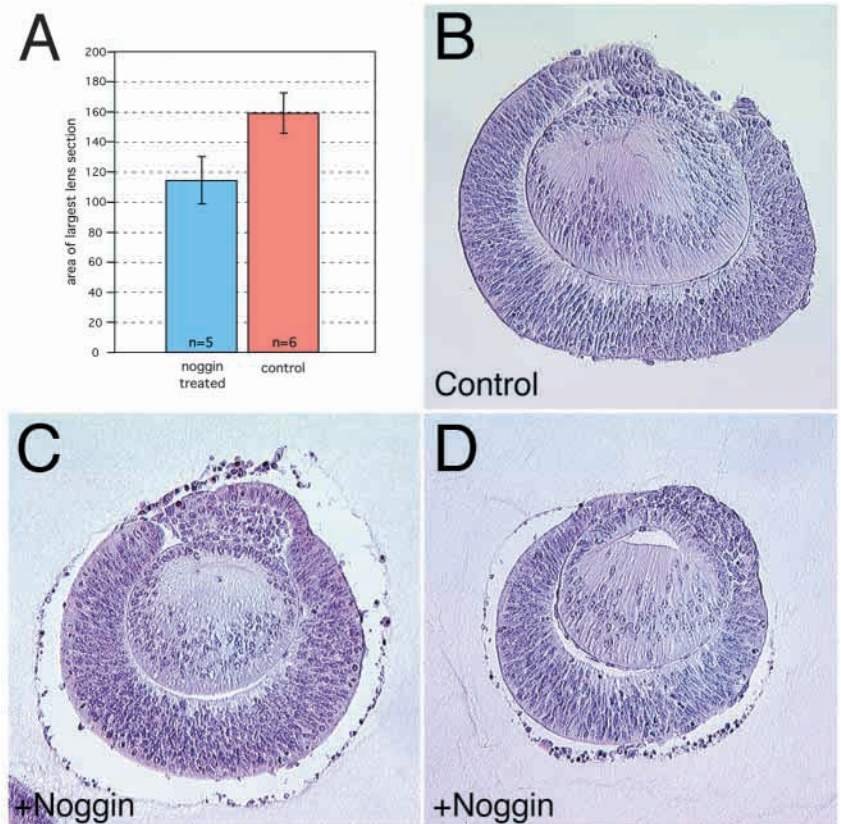
lens development. E10.5 mouse eye primordia were dissected, the retinal pigment epithelium removed (to allow good survival of retinal cells), and the tissue placed in rat-tail collagen gel submerged in defined media. After incubating at 37°C for 48 hours, spherical lenses with elongated and histologically identifiable lens-fiber cells develop.

The addition of the Bmp antagonist noggin to whole eye explants at E10.5 resulted in the development of smaller lenses after 48 hours of culture. We measured the total area (in arbitrary units) of the largest lens section of five noggin-treated and six control explants and showed that there was a statistically significant difference (Fig. 1A). The smaller size of the lenses could also be seen in histological sections of control (Fig. 1B) and noggin-treated (Fig. 1C,D) explants. As primary fiber cells make up most of the area of a lens section at the stage the explant assay was performed, these data indicate that directly or indirectly, Bmps are required for primary fiber cell development. Difficulty in orienting explants of this size precluded a meaningful analysis of primary fiber cell development asymmetries.

#### Generation of transgenic mice expressing *Alk6<sup>DN</sup>* in the lens

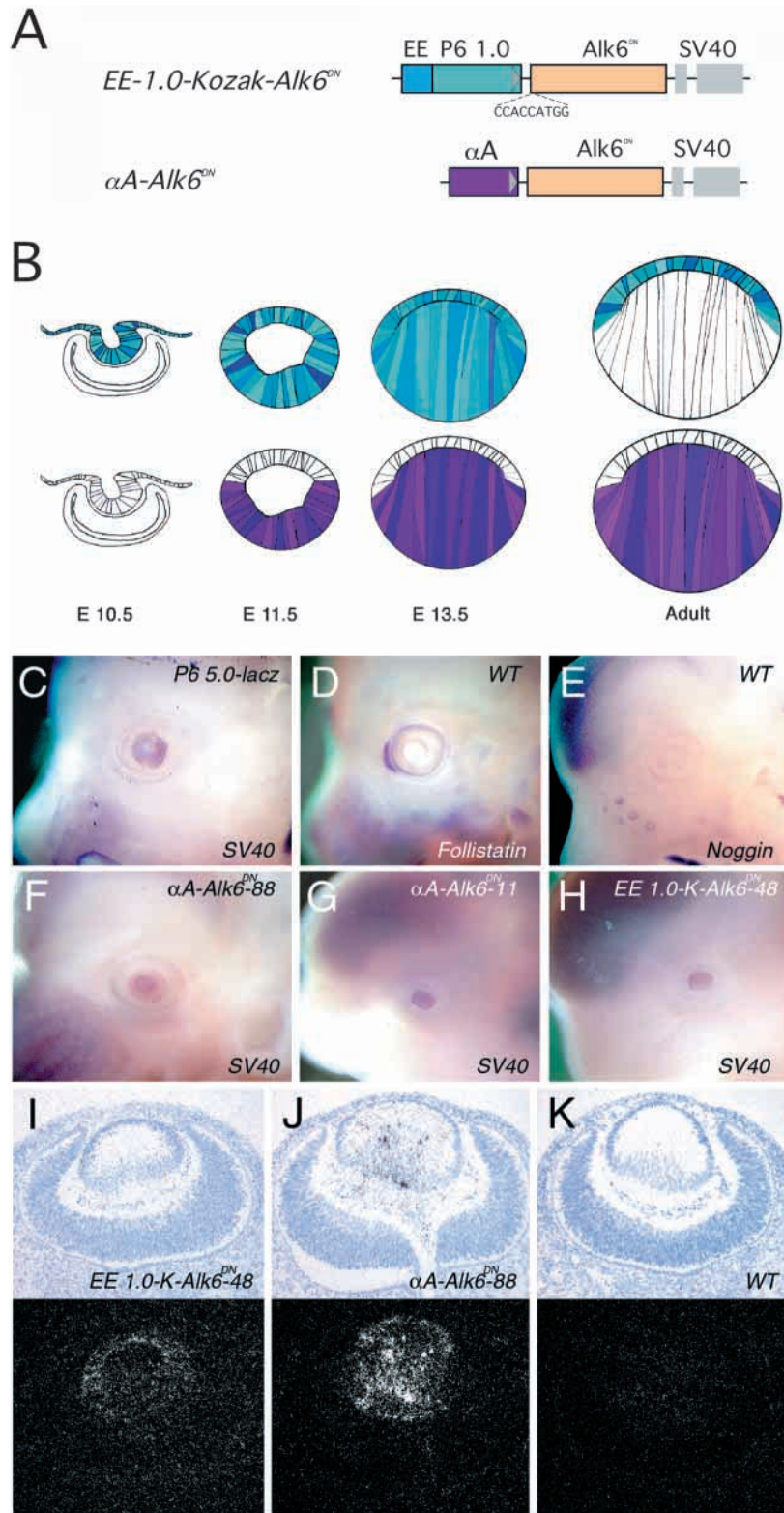
To address the question of whether Bmp receptor activity was important for lens development using a second experimental strategy, we generated transgenic mice that expressed a dominant-negative form of the type 1 receptor *Alk6*. *Alk6* is normally expressed only at low levels in the developing eye (Furuta and Hogan, 1998) but would be expected to inhibit the activity of some Bmp ligands (Massagué, 1998). Transgenic mice were generated on the FVB/N background (Taketo et al., 1991) using the constructs designated *EE-1.0-K-Alk6<sup>DN</sup>* and *αA-Alk6<sup>DN</sup>* (Fig. 2A). The *EE-1.0-K-Alk6<sup>DN</sup>* construct takes advantage of the ectoderm enhancer from *Pax6* that has activity in the presumptive lens ectoderm, primary lens fiber cells as well as the early and mature lens epithelium (Williams et al., 1998) (Fig. 2B). By contrast, the *αA-Alk6<sup>DN</sup>* construct carries a portion of the *αA*-crystallin regulatory region that has activity only in primary and secondary lens fiber cells from about E11.5 (Chepelinsky et al., 1985) (Fig. 2B). Thus, these constructs provide distinct patterns of transgene expression and were designed to indicate whether different lens regions were responding to Bmp signals.

To test the consequences for lens development of *Alk6<sup>DN</sup>* expression, we generated transgenic mice with both the *EE-1.0-K-Alk6<sup>DN</sup>* and *αA-Alk6<sup>DN</sup>* constructs. As phenotypes were mild in heterozygous mice, we bred a number of lines to homozygosity. The most severe defects were observed in *αA-A6<sup>DN</sup>* line 11 and of *EE-A6<sup>DN</sup>* line 48 homozygous animals. Homozygous mice from these two lines were used in all subsequent analysis. For brevity, homozygous mice are referred to by the transgene designation. A summary of the phenotypes in transgenic lines is given in Table 1.



**Fig. 1.** Noggin-treated lens explants have reduced development of primary lens fiber cells. E10.5 mouse eye primordia were explanted into collagen gel in culture either in the absence [A (red bar) and B] or presence [A (blue bar), C,D] of the Bmp inhibitor noggin at 300 ng/ml. Quantification of the area of the largest lens section (in a series) in control and experimental explants indicated that noggin treatment reduced lens growth (A). At this stage of development, primary lens fiber cells make up most of the area in a lens section. The larger size and improved development of primary lens fiber cells in untreated explants (B) can be observed by comparison with those that were noggin-treated (C,D).

In order to examine transgene expression, in situ hybridization analysis was performed (Fig. 2C-K). Whole-mount *P6 5.0-lacZ* embryos (Williams et al., 1998) provided a positive control for lens hybridization signal at E13.5 using a probe to the SV40 sequences included in the *Alk6* transgenes (Fig. 2C). Negative controls included wild-type whole-mount embryos hybridized with follistatin (Fig. 2D) or noggin (Fig. 2E) probes and these provided contrasting patterns of hybridization where the lens was negative. An SV40 hybridization signal was detectable in the lens of whole-mount *αA-Alk6<sup>DN</sup>* transgenics from E11.5 and later [Fig. 2F (E13.5) and Fig. 2G (E11.5)] and in *EE-1.0-K-Alk6<sup>DN</sup>* transgenics from E9.5 (for example, E11.5, Fig. 2H). Section in situ hybridization of E12.5 control and transgenic tissues were also performed using radioactively labeled SV40 sequence antisense probes (Fig. 2I-K). Matched brightfield and darkfield micrographs indicate that, as would be anticipated, *EE-1.0-K-Alk6<sup>DN</sup>* transgenics show hybridization signal throughout the lens but primarily in the developing lens epithelium (Fig. 2I). By contrast, *αA-Alk6<sup>DN</sup>* transgenics show transgene expression in the differentiating primary lens fiber cells (Fig. 2J). Importantly, there is no indication of differences in



**Fig. 2.** *Alk6<sup>DN</sup>* transgene constructs and lens expression. (A) The *EE-1.0-K-Alk6<sup>DN</sup>* and *αA-Alk6<sup>DN</sup>* transgene constructs. The positions of the *Pax6* ectoderm enhancer (EE, blue box) and the *Pax6* P0 promoter region (P6 1.0, green box) in *EE-1.0-K-Alk6<sup>DN</sup>* are shown. In *αA-Alk6<sup>DN</sup>* the promoter region is indicated by the purple box. In both constructs, the dominant-negative *Alk6*-coding region (containing a point mutation giving K231R) is indicated in yellow. Included in both constructs is the SV40 virus small t antigen gene region (gray) that contains splicing and polyadenylation signals. In the *EE-1.0-K-Alk6<sup>DN</sup>* construct, the translation start codon was engineered to the most efficient consensus sequence as defined by Kozak (Kozak, 1986). (B) Schematic (not to scale) of the expression pattern of the EE containing promoters (B top, blue) and the *αA* promoter (B bottom, purple). The EE promoter begins expression at E9.5 in the surface ectoderm, continues to be expressed at E10.5 in the lens pit. By E11.5 and through E13.5 it is expressed in all of the cells of the lens, but in adulthood, expression is restricted to the lens epithelial layer. Expression of transgenes driven by the *αA* promoter begins at E11.0 in differentiating primary fiber cells and continues in all lens fiber cells into adulthood. (C–H) Whole-mount in situ hybridization. Hybridization of an antisense SV40 probe to a positive control, the *P6 5.0-lacZ* reporter line at E13.5 is shown in C. Two negative controls for the E13.5 lens, follistatin (D) and noggin (E) are shown adjacent. Follistatin labels nasal periocular mesenchyme at E13.5. An SV40 probe hybridization signal is also observed in the lens of three separate lines of transgenic mice including *αA-Alk6<sup>DN</sup>-88* at E13.5 (F) *αA-Alk6<sup>DN</sup>-11* at E11.5 (G) and *EE-1.0-K-Alk6<sup>DN</sup>-48* at E11.5 (H). (I–K) Matched brightfield (upper panel) and darkfield (lower panel) of section in situ hybridization with radioactively labeled antisense SV40 probe on E12.5 eye tissue from (I) *EE-1.0-K-Alk6<sup>DN</sup>-48*, (J) *αA-Alk6<sup>DN</sup>-88* and (K) wild-type control.

hybridization signal intensity on the nasal or temporal side of the lens.

#### Dominant-negative *Alk6* inhibits primary lens fiber cell differentiation

To permit a detailed assessment of the phenotype in *αA-Alk6<sup>DN</sup>*

and *EE-K-Alk6<sup>DN</sup>* transgenic mice, we performed a histological analysis from E9.5–E18.5. No phenotypic change was observed during the induction phases of lens development, but clear defects were observed in primary fiber cell elongation. Lenses of E13.5 mouse embryos from lines of both *EE-1.0-K-Alk6<sup>DN</sup>* and *αA-Alk6<sup>DN</sup>* transgenics show primary fiber cell elongation

**Table 1. Transgenic mice generated with *Alk6*<sup>DN</sup> constructs**

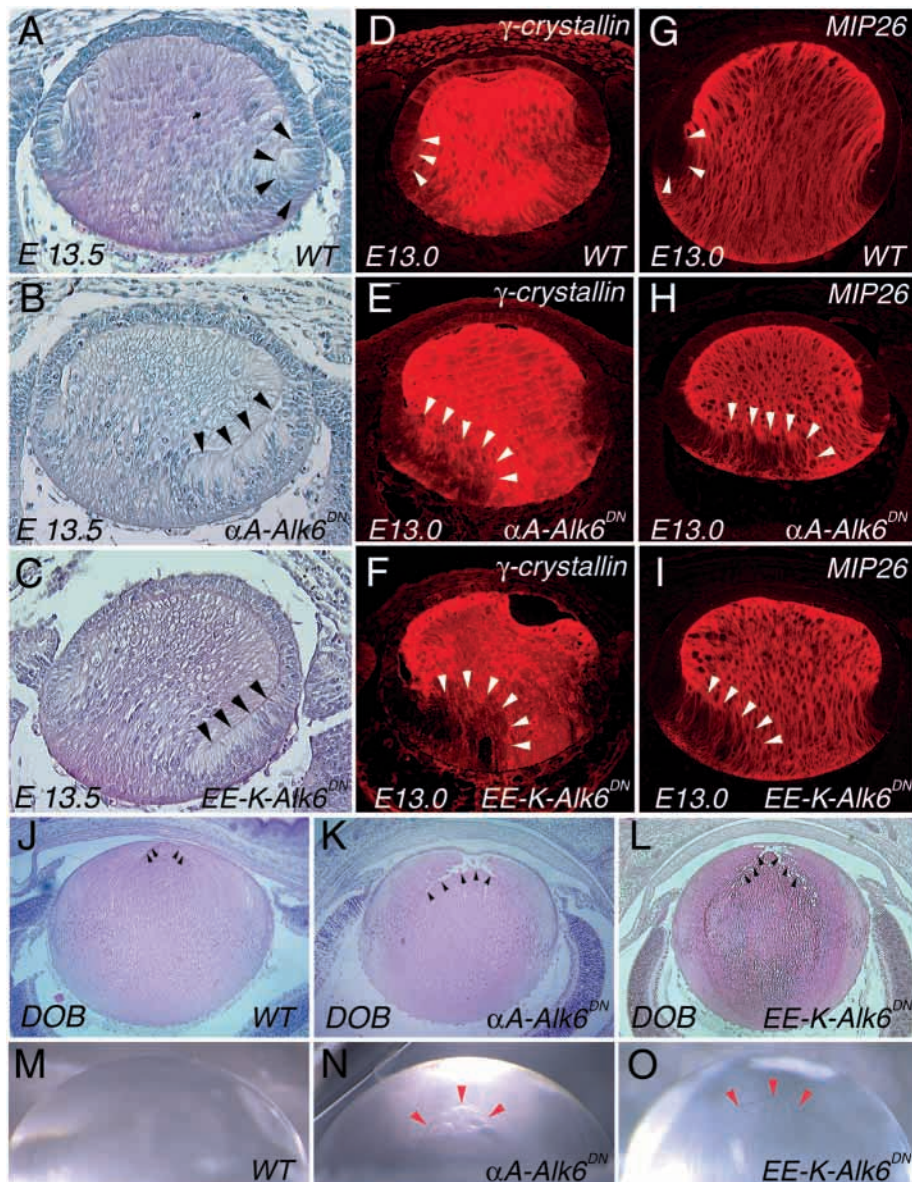
Construct	Founder	Germline transmission	In situ SV40	E13.5 defect*	Cataractous lens*
<i>EE-1.0-K-Alk6</i> <sup>DN</sup>	18	Yes	+++	Mild	Yes
	28	Yes	++	Mild	Yes
	31	Yes	++	Mild	Yes
	45	No	–	–	–
	48	Yes	++++	Yes	Yes
	59	Yes	ND	Yes	Yes
<i>αA-Alk6</i> <sup>DN</sup>	55	No	–	–	–
	11	Yes	++++	Yes	Yes
	26	Yes	ND	No	ND
	88	Yes	++++	Yes	Yes
	95	Yes	ND	No	ND

\*Phenotype assessed in homozygous transgenic mice.

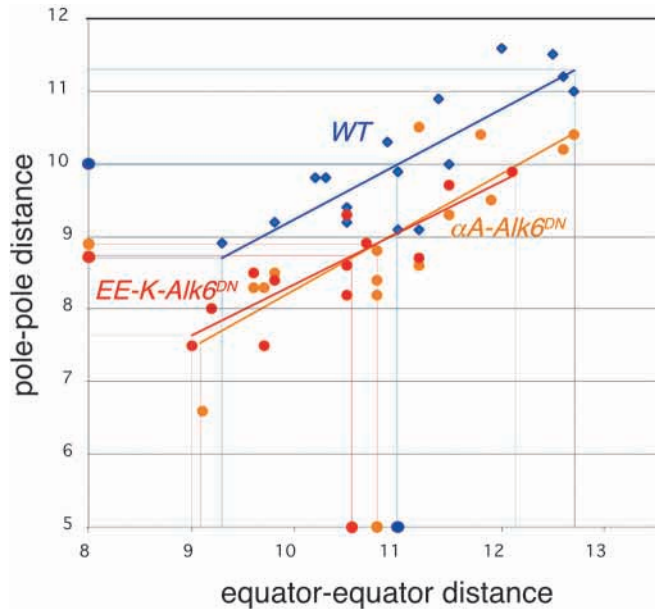
defects. Interestingly, however, the defect was not distributed evenly across the lens width. This was most obvious in

Hematoxylin stained sections (Fig. 3A-C) when examining the boundary between equatorial cells and the primary fiber cell mass. In wild-type lenses, this boundary exists only anterior to the equator (Fig. 3A) but in *EE-1.0-K-Alk6*<sup>DN</sup> line 48 and *αA-Alk6*<sup>DN</sup> line 11 transgenics, the boundary extends close to the posterior lens pole but only on the nasal side (Fig. 3B,C). The nature of the defect is emphasized by the distribution of the fiber cell differentiation markers  $\gamma$ -crystallin and MIP26 (Fig. 3D-I). In the E13.0 wild-type lens, both  $\gamma$ -crystallin and MIP26 are restricted to the fiber cells and are found at high levels of immunoreactivity along the posterior aspect of the lens from equator to pole (Fig. 3D,G). By contrast, E13.0 lenses from both *αA-Alk6*<sup>DN</sup> and *EE-1.0-K-Alk6*<sup>DN</sup> transgenics show nasal side domains that have dramatically reduced levels of immunoreactivity for both markers (Fig. 3E-I). This indicates that expression of the dominant-negative *Alk6* from either transgene construct inhibits primary fiber cell differentiation.

In the day-of-birth lenses, the lack of extension of the primary lens fiber cells leads to a break along the sutures (small



**Fig. 3.** Histological analysis of *αA-Alk6*<sup>DN</sup> and *EE-1.0-K-Alk6*<sup>DN</sup> transgenic lenses. E13.5 Hematoxylin and Eosin stained sections of wild-type mouse eyes (A), and homozygous mice of the *αA-Alk6*<sup>DN-11</sup> (B) and *EE-1.0-K-Alk6*<sup>DN-48</sup> (C) transgenic lines. The black arrowheads indicate the normal nasal side equatorial structure in wild-type mice (A) and the abnormal form (B,C) seen with both transgenic mouse constructs. (D-F)  $\gamma$ -crystallin immunolabeling of E13.0 lenses from wild-type mice (D) and from homozygous *αA-Alk6*<sup>DN-11</sup> (E) and *EE-1.0-K-Alk6*<sup>DN-48</sup> (F) transgenics. The white arrowheads indicate the nasal side domain in which primary fiber cells have low  $\gamma$ -crystallin levels. Similarly, MIP26 immunolabeling in wild-type E13.0 mouse lenses extends to the equator (G), while in homozygous *αA-Alk6*<sup>DN-11</sup> (H) and *EE-1.0-K-Alk6*<sup>DN-48</sup> (I) transgenics of the same age, there is a nasal side region in which labeling is absent or low. Sections in A-I are located in the ventral half of the lenses shown. (J-L) Day-of-birth Hematoxylin and Eosin stained sections of the eyes of wild-type mice (J), and homozygous mice of the *αA-Alk6*<sup>DN-11</sup> (K) and *EE-1.0-K-Alk6*<sup>DN-48</sup> (L) transgenic lines. This shows that the transgenic lenses are smaller and have emphasized suture spaces (J-L, arrowheads) owing to the lack of complete elongation of the primary fiber cells. (M-O) A comparison of whole-mount lenses dissected from wild-type (M) and homozygous *αA-Alk6*<sup>DN-11</sup> (N) and *EE-1.0-K-Alk6*<sup>DN-48</sup> (O) mice showing the refractile anomaly present in the transgenics (N,O, red arrowheads).



**Fig. 4.**  $\alpha A$ - $Alk6^{DN}$  and  $EE-1.0-K-Alk6^{DN}$  transgenic lenses have a reduced pole-pole dimension. Graph showing a linear regression analysis of the relationship between equator-equator (nasotemporal) and pole-pole dimensions in lens sections taken from wild-type (blue annotation), homozygous  $\alpha A$ - $Alk6^{DN}$  (orange annotation) and  $EE-1.0-K-Alk6^{DN}$  (red annotation) transgenic mice. Measurements taken for this analysis were made on the largest coronal lens sections in a given series. The average dimension for each data set is projected to the axes and marked by the colored dot. The equator-equator dimension in wild-type and transgenic mice is minimally different, but the pole-pole dimension between wild-type and either transgenic is. This indicates that the transgenic lenses are more ellipsoid in shape and that primary fiber cell differentiation is reduced.

arrowheads Fig. 3J-L). Secondary fiber cells have compensated for the short central cells by elongating around the edges (seen most clearly in Fig. 3K). Transgenic mice also have smaller lenses than wild type. This might be expected because the primary fiber cells make up a good proportion of the lens bulk at this stage. Adult mice of both lines (red arrowheads, side view, Fig. 3M-O) show cataracts. The appearance of this type of cataract seems to result from the growth of the secondary fiber cells elongating around the short primary fiber cells of the lens nucleus.

In histological sections taken for analysis, transgenic lenses often appeared flatter (a shorter pole-pole distance) than their wild-type littermates. To assess this, we measured the dimensions of E13.5 lenses from wild-type,  $EE-1.0-K-Alk6^{DN}$  line 48 homozygous and  $\alpha A$ - $Alk6^{DN}$  line 11 homozygous embryos. Transgenic lenses of both lines were not significantly smaller equator-to-equator, but the graph of lens shape (Fig. 4) shows that there are significant differences in pole-pole distance. This might be expected in lenses where the primary fiber cell development is perturbed as the elongation of these cells expands the lens along the pole-pole axis.

### Normal development of the mouse lens is asymmetrical

In order to understand the developmental basis of the

asymmetrical primary fiber cell elongation defect in  $\alpha A$ - $Alk6^{DN}$  and  $EE-1.0-K-Alk6^{DN}$  transgenics, we decided to assess carefully wild-type lens development. Histological sections of wild-type lenses from E10.5 to E13.5 indicate that asymmetries are apparent. At E10.5 and E11.5, the lens appears symmetric (data not shown) but by E12.5 it is clear that there is an asymmetric pattern of lens fiber cell elongation. Coronal sections taken from ventral to dorsal of wild-type E12.5 embryos (Fig. 5A-C) shows that in the ventral lens, fiber cells elongate first on the temporal side. Sections from the dorsal half of the lens do not show this asymmetrical distribution of primary fiber cell differentiation. In a second example (Fig. 5D-F), the differentiation marker MIP26 emphasizes that primary fiber cell differentiation begins on the temporal side of the lens vesicle.

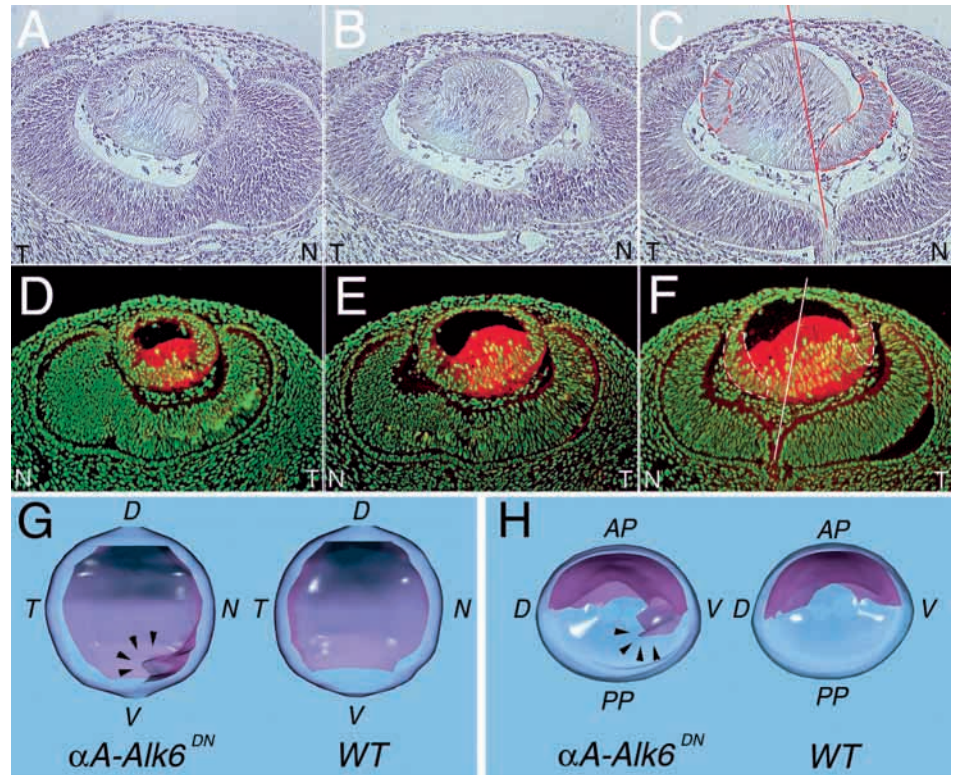
Because the  $\alpha A$ - $Alk6^{DN}$ ,  $EE-1.0-K-Alk6^{DN}$  defect at E13.5 was subtle, we decided to model the defect in three-dimensions (3D). The goal of the modeling was to show the boundary between epithelial cells and fiber cells as a 3D surface. In order to do this, serial lens sections from E13.5 wild-type and homozygote  $\alpha A$ - $Alk6^{DN-11}$  and  $EE-1.0-K-Alk6^{DN-48}$  animals were collected, and sections spaced about 20  $\mu\text{m}$  apart were traced. These tracings were stacked in a 3D drawing and rendering program, and one surface was extended over the spherical lens model. A second surface was extended over the arcs making up the epithelial-fiber cell boundary (Fig. 5G,H). The purple three-dimensional surface represents the boundary between the basal surface of the epithelial cells and the tips of the elongating fiber-cells. The glassy sphere represents the lens capsule. Homozygous  $EE-1.0-K-Alk6^{DN}$  (data not shown) and  $\alpha A$ - $Alk6^{DN}$  (Fig. 5G,H) lenses appeared similar. In the context of this analysis, the phenotype of the  $\alpha A$ - $Alk6^{DN}$  and of  $EE-K-Alk6^{DN}$  lenses at E13.5 is more easily understood. The implication is that the late differentiating ventronasal domain of lens vesicle epithelium has not elongated in  $\alpha A$ - $Alk6^{DN}$  and  $EE-1.0-K-Alk6^{DN}$  embryos, and results in the observed nasal side defect at E13.5. The further implication is that Bmp receptor activity is required for primary fiber cell differentiation in this discrete domain.

### Activated phospho-Smads are detected in the primary fiber cell precursors

The Smads are a family of signal transduction molecules active in the Bmp pathways and their phosphorylation indicates that a given cell is responding to Bmp signals (Miyazono et al., 2000). Taking advantage of recently generated antibodies specific for the type 1 receptor phosphorylated forms of Smad1, Smad5 and Smad8 (Korchynskiy et al., 1999), it was possible to directly examine whether Bmp signaling pathways were functional in the developing lens. This strategy allows the spatial pattern of Bmp responses to be examined. We also chose to use antibodies to the cytoplasmic domain of Bmpr2 (Gilboa et al., 2000) to assess its distribution. Though *Bmpr2* has been reported to be expressed in the adult rat eye (Obata et al., 1999) the expression pattern in the developing mouse eye has not so far been described.

To further validate the phospho-Smad and Bmpr2 antibodies in the mouse, we performed immunofluorescence labeling of embryonic regions where Bmps were known to be expressed. Specifically, we examined the surface ectoderm in the region of the E9.5 olfactory placode and first branchial arch as in these

**Fig. 5.** Asymmetry in normal lens development and in the phenotype of *Alk6<sup>DN</sup>* transgenic mice. (A–C) Hematoxylin and Eosin stained sections through the ventral eye region of the right eye of an E12.5 wild-type mouse. These are equally spaced serial sections located at 40  $\mu\text{m}$  (A), 80  $\mu\text{m}$  (B) 120  $\mu\text{m}$  (C) from the first ventral lens section. The reference proximodistal axis from the future optic nerve head to the mid point of the developing cornea is marked by the red line in C. These sections show that primary fiber cell elongation begins in a temporally located (T) domain of the posterior wall of the lens vesicle. The nasal (N) aspect of the posterior lens vesicle wall remains undifferentiated at this stage. The relative size of the undifferentiated domains is marked by the broken red lines. It is also observed that there is a larger domain of developing retina on the temporal side of the optic stalk. (D–F) Sections through the ventral eye region of the left eye of the same E12.5 wild-type mouse shown in A–C labeled for the differentiation marker MIP26 (red) and nuclei (green). These are also equally spaced serial sections located at 40  $\mu\text{m}$  (D), 80  $\mu\text{m}$  (E) and 120  $\mu\text{m}$  (F) from the first ventral lens section and serve to emphasize the temporal side (T) primary fiber cell differentiation asymmetry. The relative size of the undifferentiated domains is marked by the broken white lines in F. (G,H) Three dimensional renderings of E13.5 homozygous  $\alpha A$ -*Alk6<sup>DN</sup>*-11 and wild-type lenses in posterior pole (G) and nasal (H) views. The renderings were reconstructed from serial sections. The glassy sphere represents the lens capsule and the purple surface the boundary between epithelium and fiber cell mass. These renderings indicate clearly the ventronasal location of the primary fiber cell differentiation defect in the  $\alpha A$ -*Alk6<sup>DN</sup>*-11 homozygous transgenic lenses.



locations, there is strong expression of *Bmp4* (Fig. 6A) (Dudley and Robertson, 1997). With ligand expression, the *Bmp* signaling pathway might be expected to be active. Anti-phospho-Smad antibody labeling showed strong nuclear immunoreactivity in the epithelium of the olfactory placode and first branchial arch (Fig. 6B,C, red arrowheads) in a pattern that corresponded closely with the domain of *Bmp4* expression. The anti-Bmpr2 antibodies also labeled an adjacent section in the olfactory placode and first branchial arch epithelium but the labeling pattern appeared more cell surface than nuclear (Fig. 6D,E, red arrowheads). This indicated a strong spatial correlation between the *Bmp4* ligand, the *Bmp* type 2 receptor and nuclear phospho-Smad labeling suggesting that we are able to detect cells in which this pathway is active.

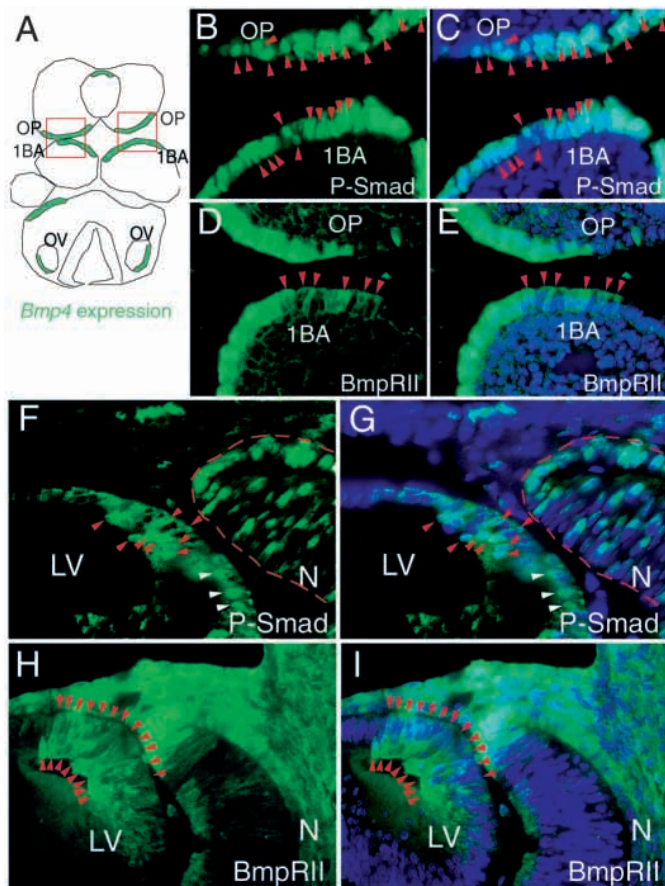
Interestingly, we could detect distinct patterns of anti-phospho-Smad and anti-Bmpr2 antibody immunoreactivity in the developing eye. Phospho-Smad immunoreactivity was detected in the lens vesicle at E12.5 in the equatorial cells adjacent to the anterior retinal rim (Fig. 6F, red arrowheads). The labeling was nuclear as indicated by the turquoise color obtained when merging phospho-Smad immunoreactivity with blue Hoechst nuclear staining (Fig. 6G, red arrowheads). Strong nuclear phospho-Smad immunoreactivity was also present in presumptive retinal and pigmented epithelial cells in anterior optic cup rim (Fig. 6F,G, broken red line). Importantly, there was also clear cell surface immunoreactivity for anti-Bmpr2 antibodies in cells of the lens vesicle equator (Fig. 6H,I,

red arrowheads) and the anterior optic cup rim. Anti-phospho-Smad and anti-Bmpr2 antibodies labeled lens vesicle cells on the nasal side (Fig. 6F–I, white N) but was also present on the temporal side (data not shown). Combined, these data indicated that *Bmp* signaling pathways are active in the equatorial lens vesicle cells that are the likely precursors of the primary lens fiber cells.

## DISCUSSION

We have used three distinct experimental strategies to address the issue of whether *Bmp* signaling is involved in development of the mouse lens. We have taken advantage of the *Bmp* inhibitor noggin (Zimmerman et al., 1996) to suppress *Bmp* activity in explant cultures of eye primordia. This results in attenuated differentiation of primary fiber cells as indicated by the smaller lenses that develop. In addition, we show that expression of a dominant-negative form of the type 1 *Bmp* receptor *Alk6* from two different lens-expressed transgenes has resulted in defects in primary lens fiber cell development. Interestingly, the primary fiber cell differentiation defects arise only in the ventronasal quadrant of the lens. Our analysis of wild-type lens development shows that the asymmetrical lens development defect in the transgenic animals has its origin in a transient phase of asymmetrical primary fiber cell differentiation in the normal lens. To our knowledge, this is the

first time this aspect of mouse lens development has been recognized. Finally, we have taken advantage of antibodies to



**Fig. 6.** Anti-Phospho-Smad and Bmpr2 labeling reveals Bmp responsive cells. (A) Schematic showing the pattern of *Bmp4* expression in olfactory placode (OP) first branchial arch (1BA) and otic vesicle (OV) of an E9.5 mouse embryo (Dudley and Robertson, 1997). The approximate region of tissue shown in B-E is indicated by the red boxes. (B) Anti-phospho-Smad labeling (green) of E9.5 mouse section in the region of the first branchial arch (1BA) and olfactory placode (OP). Intense labeling is observed in the epithelia of both structures. A merge of (B) with Hoechst nuclear labeling (blue) is shown in C. The turquoise color of nuclei in (C, red arrowheads) indicates nuclear phospho-Smad immunoreactivity. (D) Bmpr2 immunoreactivity in sections of E9.5 mouse first branchial arch (1BA) and olfactory placode (OP). Cell-surface labeling is apparent in the cells of the epithelia (red arrowheads) and more faintly in the underlying mesenchyme. A merge of (D) with Hoechst labeling (E) shows that, as would be expected for cell-surface labeling, the nuclei do not change color. (F) Anti-phospho-Smad labeling (green) of E12.5 mouse eye sections showing the lens vesicle (LV) and anterior optic cup rim (broken red line) on the nasal side (N). A nuclear pattern of labeling is observed in lens cells at the equator (red arrowheads) faintly in the more posterior presumptive primary lens fiber cells (white arrowheads) and in the optic cup rim. A merge of F with Hoechst nuclear labeling (G, blue) gives turquoise color nuclei thus confirming nuclear phospho-Smad labeling. (H) Bmpr2 immunoreactivity in sections of E11.5 mouse eye. Cell-surface labeling is apparent in the cells at the lens vesicle (LV) equator (red arrowheads) and in the cells of the developing retina and presumptive RPE. A merge of (H) with Hoechst labeling (I) shows that, as would be expected for cell-surface labeling, the nuclei do not change color.

*Bmpr2* (Gilboa et al., 2000) and Bmp-responsive phospho-Smads (Korchynskiy et al., 1999) to determine whether there is evidence for Bmp signaling machinery and responsiveness in cells of the early lens. This has shown that the equatorial cells of the lens vesicle express *Bmpr2*, but most important, also show nuclear immunoreactivity with antibodies raised to phospho-Smad1. This indicates that at the inception of primary fiber cell differentiation, the precursors are responding to Bmp signals. These data strongly suggest that Bmp signaling is important for primary fiber cell development but also raise a number of interesting issues.

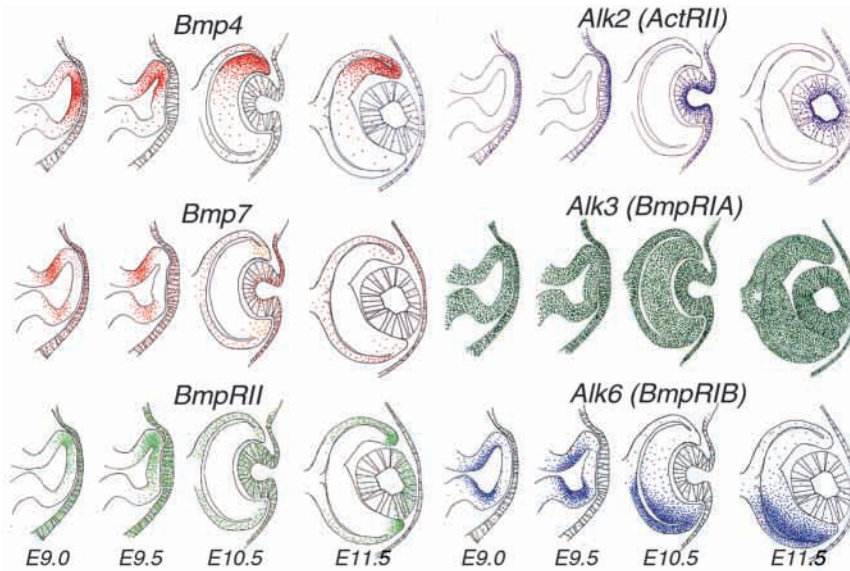
The anti-phospho-Smad1 antibody we have used in this analysis detects phosphorylated Smad1, Smad5 and Smad8 (Korchynskiy et al., 1999). In some circumstances this antibody crossreacts at a low level with phospho-Smad3 (that functions downstream of Tgf $\beta$  receptors) (Korchynskiy et al., 1999) potentially complicating interpretation of the current data. Importantly however, there is evidence that at physiological expression levels, Tgf $\beta$  receptors do not dimerize with Bmp receptors (Massagué, 1998) and so there is no expectation that dominant-negative receptors from each class would cross-react. Thus, with the current analysis and recent work in which the role of Tgf $\beta$  signaling in lens development was investigated (de Iongh et al., 2001) we can conclude that both Bmp and Tgf $\beta$  signaling are independently important in the development of the primary lens fiber cells. This suggestion is reinforced by the observation that primary fiber cell development can be inhibited in explant culture by noggin (Fig. 1), an inhibitor of BMP but not Tgf $\beta$  activity.

### Bmp signaling in the different phases of lens development

The published evidence indicates that both *Bmp7* and *Bmp4* are expressed in the eye primordium (see Fig. 7 for summary) and suggests that they have important roles in induction of the lens (Furuta and Hogan, 1998). *Bmp7*-null mice demonstrate that *Bmp7* regulates expression of the transcription factor *Pax6* in the lens placode (Wawersik et al., 1999). As *Pax6* is both necessary (Ashery-Padan et al., 2000) and sufficient (Altmann et al., 1997) for the earliest stages of lens development, this has argued that *Bmp7* has an important role in lens induction (Wawersik et al., 1999). By contrast, *Bmp4* does not regulate expression of *Pax6*, but is required for the upregulation of *Sox2* (Furuta and Hogan, 1998), a transcription factor that is involved in regulating the expression of crystallin genes sometimes in a complex with *Pax6* (Kamachi et al., 2001). As the ectoderm enhancer used to direct transgene expression in the *EE-1.0-K-*Alk6*<sup>DN</sup>* transgenic mice is expected to be expressed in the lens lineage from E8.75 (Williams et al., 1998), the question arises of why we do not see a defect in lens induction. There are several reasons. One explanation is that the level of *Bmp4* and *Bmp7* signaling activity in the induction phases of lens development is high, and that the transgene we describe does not provide a sufficiently high level of expression to inhibit Bmp signaling pathways. Another, more satisfying, explanation is based on the biochemistry of the Bmp receptor signaling system.

It has been established that type 1 and type 2 Tgf $\beta$  family receptors can heterodimerize in various combinations (Massagué, 1998). Expression studies have shown that the type 1 Bmp receptors *Alk2* and *Alk3* are expressed in the early lens





**Fig. 7.** Summary of the expression patterns of Bmp family ligands and receptors in the developing eye. The different stages of eye development (labelled underneath) are represented by the different line drawings. Dorsal is upwards. The distribution and approximate intensity of labeling for the different ligands and receptors is indicated by the colored stippling. The expression patterns of *Bmp4*, *Bmp7*, *Alk3* and *Alk6* are derived from in situ hybridization experiments presented in a prior publication (Furuta and Hogan, 1998). The distribution of *Alk2* is based on antibody labeling (Yoshikawa et al., 2000) and our understanding of *Bmpr2* distribution on the current analysis.

(Furuta and Hogan, 1998; Yoshikawa et al., 2000) (Fig. 7). Furthermore, the current analysis shows that *Bmpr2* is expressed in the developing lens from E9.5 to E12.5 (Fig. 6 and see Fig. 7 for summary). Thus, there are a limited number of receptor heterodimers that might mediate development. It has also been shown that despite the option to heterodimerize, the different type 1/type 2 receptor combinations have very different abilities to signal in response to a particular ligand (Table 2). Significantly, *Bmp7* has a higher affinity for the *Alk2/Bmpr2* combination than for any other including *Bmpr2/Alk6*. This argues that in the *EE-1.0-K-Alk6<sup>DN</sup>* transgenic mice, the dominant-negative *Alk6* is unlikely to impede *Bmp7* signaling at the stage of lens induction. Additionally, as *Bmp7* does not appear to signal efficiently through *Alk3* (Macias-Silva, 1998), it is likely that signaling through *Bmpr2/Alk2* (Liu et al., 1995) is the major effector of *Bmp7* action in lens induction. It is more difficult to explain why dominant-negative *Alk6* does not apparently inhibit *Bmp4* signaling in lens induction as it can clearly pair with *Bmpr2* and efficiently transmit *Bmp4* signals (Table 2). It will be interesting to observe the outcome of lens lineage conditional *Alk2* and *Alk3* targeting experiments that might definitively identify the type 1 receptor(s) crucial for the induction phases of lens development.

The current analysis provides strong evidence that as well as the early inductive action of *Bmp7* and *Bmp4*, lens development involves a later phase of Bmp signaling that

stimulates development of the primary lens fibers. The transcriptional control elements used to drive expression in the *αA-Alk6<sup>DN</sup>* and *EE-1.0-K-Alk6<sup>DN</sup>* transgenic mice have been well characterized and the lens expression patterns completely understood (Kammandel et al., 1999; Williams et al., 1998) (Fig. 2B). We have also confirmed lens expression (Fig. 2F-H). The *Pax6* ectoderm enhancer and the  $\alpha$ A-crystallin promoter have expression patterns that overlap in the equatorial cells of the lens vesicle starting at E11.5. Interestingly, these are precisely the cells that based on immunoreactivity for anti-phospho-Smad and anti-*Bmpr2* antibodies have the signaling machinery for Bmp signaling and are responding to Bmps at E12.5. Thus, we can suggest (Fig. 8) that the observed suppression of primary fiber cell differentiation is a consequence of dominant-negative *Alk6*-mediated inhibition of Bmp signaling in the presumptive primary lens fiber cells at the equator of the E12.5 lens vesicle.

Although there has been little published concerning the developmental distinction between primary and secondary lens

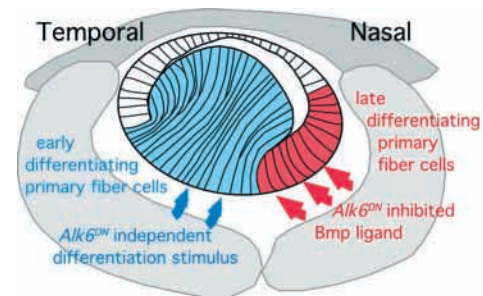
**Table 2. Ligand binding and signaling activity of type 1 and 2 Bmp receptors**

Ligand and assay type	Bmpr2 combinations		
	Alk2/Bmpr2	Alk3/Bmpr2	Alk6/Bmpr2
Bmp4 binding	None*	++ <sup>†</sup>	+++ <sup>†</sup>
Bmp4 signaling		+ <sup>†</sup>	++++ <sup>†</sup>
Bmp7 binding	++++ <sup>‡</sup>	+ <sup>‡</sup>	++ <sup>‡</sup>
Bmp7 signaling	+++++ <sup>‡</sup>	- <sup>‡</sup>	++ <sup>‡</sup>

\*ten Dijke et al., 1994.

<sup>†</sup>Nohno et al., 1995; Rosenzweig et al., 1995.

<sup>‡</sup>Liu et al., 1995; Macias-Silva et al., 1998.



**Fig. 8.** Model for the asymmetrical distribution of primary fiber cell differentiation stimuli in the developing eye. In this model, we propose that there are two distinct stimuli that direct primary fiber cell differentiation. In the ventronasal quadrant of the developing eye, we suggest, based on the phenotype observed in the *αA-Alk6<sup>DN</sup>* and *EE-1.0-K-Alk6<sup>DN</sup>* transgenic mice, that there is a Bmp ligand that can be inhibited by *Alk6<sup>DN</sup>*. We suggest that there must be other primary fiber cell differentiation stimuli that are not inhibited by *Alk6<sup>DN</sup>*.

fiber cells, it has been shown that there is a clear morphological difference (Shestopalov and Bassnett, 2000). Primary fiber cells, the first cells to elongate after the closure of the lens vesicle, appear to have a different cellular morphology and organization than secondary fiber cells in both the human and chick. Primary lens fiber cells are heterogenous in size and shape, while secondary fiber cells appear to be uniformly smooth. The current analysis suggests that Bmp signaling may be one determinant of the distinctions between primary and secondary lens fiber cells.

### Bmp ligands in the developing eye may be asymmetrically distributed

The data we describe shows that the lens normally goes through a stage where development is anatomically asymmetrical. Specifically, we show that primary lens fiber cells begin to elongate first in the ventrotemporal quadrant of the lens at E12.5, but by E13.5, the nasal side cells have also elongated and the obvious asymmetry is lost. To our knowledge, this is the first time that this aspect of mouse lens development has been recognized.

In the wild-type lens vesicle, Bmpr2 and phospho-Smad immunoreactivity are present in the entire equator and this suggests that all these cells are responding to Bmp signals. This raises the question of why, in the  $\alpha A$ -*Alk6*<sup>DN</sup> and *EE-1.0-K-Alk6*<sup>DN</sup> transgenic mice, where lens vesicle stage expression is not expected to be asymmetrical, the primary fiber cell differentiation defect is. The most obvious explanation is that there may be a Bmp ligand that is important for primary fiber cell differentiation that is restricted in its distribution to the ventronasal quadrant of the eye. A preliminary analysis of the expression patterns of many Bmp family members in the primordial eye shows that many are expressed and that the patterns are complex (S. C. F. and R. A. L., unpublished). Given the possibility that Bmps can act as both homodimers and heterodimers and have distinct signaling activity as a consequence (Suzuki et al., 1997), coupled with the existence of soluble modulators of their activity (Cho and Blitz, 1998), it is not unreasonable to suggest that Bmp responses within the eye may be finely restricted spatially.

Thus, we propose a model in which the normal lens vesicle contains two subpopulations of primary lens fiber cell progenitors that respond to distinct differentiation stimuli. We propose that in the ventrotemporal and dorsal lens vesicle, there is an early differentiating population (Fig. 8, blue) in which fiber cell elongation is unaffected by dominant-negative *Alk6*. This could mean that this domain of lens vesicle cells differentiates in response to non-Bmp ligands, or that if a Bmp ligand is involved (as the uniform distribution of phospho-Smad might suggest), it does not bind efficiently to *Alk6*-containing receptor heterodimers. We also suggest that there is a group of lens vesicle cells located on the nasal side that differentiates later (Fig. 8, red) in response to a Bmp ligand that can be inhibited by dominant-negative *Alk6*. This model explains both the normal asymmetry in primary fiber cell differentiation and the asymmetrical phenotype of the  $\alpha A$ -*Alk6*<sup>DN</sup> and *EE-1.0-K-Alk6*<sup>DN</sup> transgenic mice.

Other analyses of eye development have identified mutant phenotypes and gene expression patterns that reflect asymmetries. Gene targeting of the retinoid receptors *Rxra* and *Rxrg* has shown that this pathway is important for development

of the ventral eye (Kastner et al., 1994). In particular, compromised function of these receptors resulted in a ventral rotation of the entire lens suggesting that retinoid signaling is important for development of ventral eye structures. It may be interesting to determine whether retinoid signaling and Bmp-mediated primary fiber cell differentiation are developmentally related. Another example of interest is the expression patterns of the forkhead family members *Foxg1* and *Foxd1*. These are expressed in the nasal and temporal retina, respectively (Dirksen and Jamrich, 1995; Hatini et al., 1994; Tao and Lai, 1992), and it is possible that this type of gene expression pattern might reflect spatially restricted fiber cell differentiation stimuli that are likely to have their origin in the developing retina.

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