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Meeting Report

Development and evolution of the eye: Fondation des Treilles, September, 2001

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In the idyllic setting of the Fondation des Treilles in the Provence region of Southern France, 15 scientists met to discuss their most recent experimentation examining mechanisms of eye development. The meeting was organized by Walter Gehring and Veronica van Heyningen and proved to be a watershed of complementary information. A huge range of interesting aspects of eye development and evolution were discussed at the meeting – to the extent that it would be impossible to summarize everything. For this reason, we have chosen to succinctly compile the major findings on the highlighted areas of vertebrate lens induction and *Drosophila* eye development.

1. Genetic pathways regulating vertebrate lens induction

Though the optic cup ablation experiments of Spemann (Spemann, 1901) were immediately controversial (Mencl, 1903), we have understood since that inductive signals can modify the fate of an adjacent tissue. Understanding the molecular mechanism by which the lens is induced has been of great interest as it represents a relatively simple system in which the process may be understood comprehensively.

One of the first molecular mediators of lens induction to be identified was the homeodomain and paired box transcription factor Pax6. This factor has a central role in lens development. Indicating that Pax6 is sufficient for lens development are misexpression experiments performed in the frog *Xenopus laevis* that result in the formation of ectopic lenses with all the characteristics of the endogenous structure (Altmann et al., 1997). A number of experiments also show that Pax6 is necessary for lens development. Tissue recombinations in which *Small eye* (*Pax6*) homozygous mutant tissues were recombined in explant culture with those from wild-type mice showed that Pax6 is required in the presumptive lens ectoderm but not in the optic vesicle for lens development to proceed (Fujiwara et al., 1994). This is also consistent with the observation that in chimeric mice, $Pax6^{Sey\text{-}1Neu/Sey\text{-}1Neu}$ cells are excluded from the presumptive and developing lens from E9.5 (Collinson et al., 2000; Quinn et al., 1996). Recently, the requirement for Pax6 in lens development has been elegantly demonstrated by the Gruss group through the generation of a genetically modified mouse in which a functional *Pax6* gene is eliminated only from the surface ectoderm using a lox-cre based system (Ashery-Padan et al., 2000). The conditional inactivation of *Pax6* from E8.75 in the presumptive lens ectoderm results in the lack of lens development (including thickening of the lens placode), absence of expression of established lens induction markers and interestingly, the lack of serious consequences for induction and differentiation of retinal neurons. Thus, besides indicating a role for Pax6 in lens induction, this suggests that the proposed reciprocal inductive signals (from presumptive lens ectoderm to presumptive retina (Nguyen and Arnheiter, 2000)) are not dependent on Pax6 or are delivered before the conditional inactivation of *Pax6* was complete.

In beginning to define the genetic pathways for lens induction, we can first look to an older experiment in which two phases of *Pax6* expression were defined (Grindley et al., 1995). An examination of the distribution of *Pax6* transcripts in the $Pax6^{Sey\text{-}1Neu/Sey\text{-}1Neu}$ mouse (a point mutant in which *Pax6* transcription is retained) has shown that the focused domain of *Pax6* expression in the lens placode (designated $Pax6^{placode}$; Fig. 1A) is dependent upon an earlier phase in the head surface ectoderm (designated $Pax6^{pre\text{-}placode}$; Fig. 1A). This describes the first two elements of a genetic pathway defining lens induction.

Identification of a secreted signaling molecule involved

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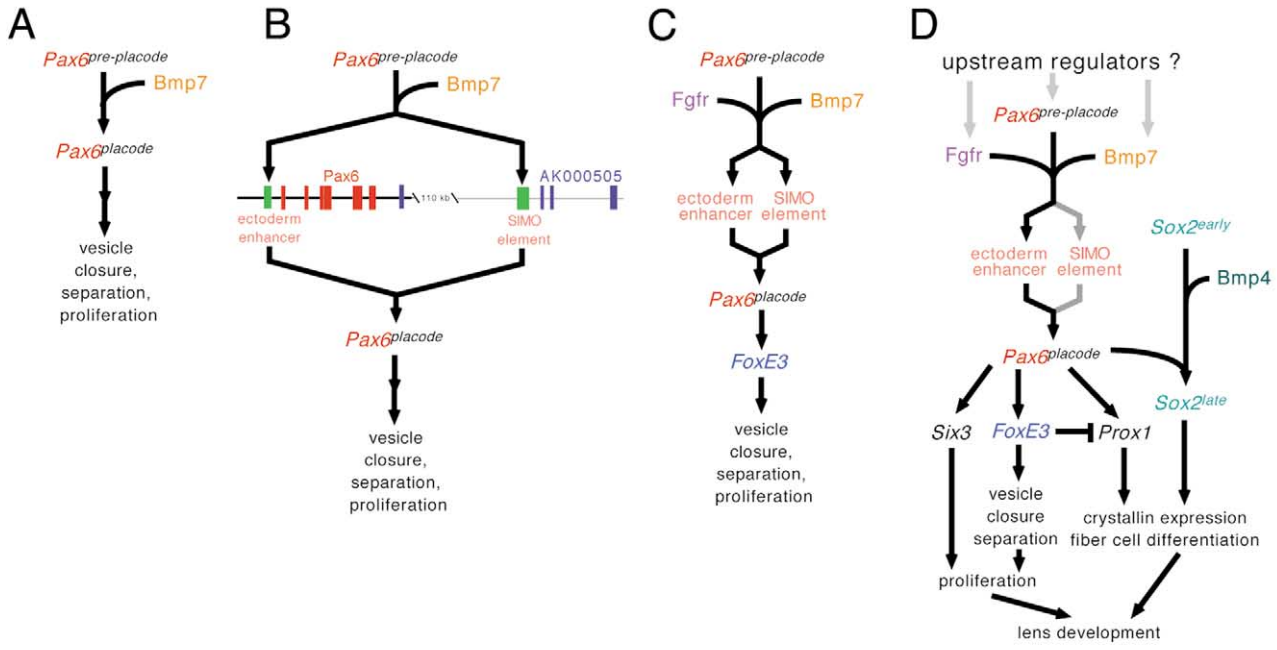

Fig. 1. The genetic pathways regulating lens induction in the mouse have been built up through a series of experiments that include assessment of gene expression in a selection of mutant mice as well as in tissue explants where signaling pathways have been modulated. In panel B, red and blue bars represent the exons of the *Pax6* and AK000505 genes, respectively, and the green bars, the approximate location of transcriptional control elements active in the lens lineage. See the text for details.

in lens induction grew out of the observation that Bone morphogenetic protein-7 (*Bmp7*) null mice have eye defects that range in severity from microophthalmia to anophthalmia (Luo et al., 1995; Dudley et al., 1995). Close examination of the early stages of lens development indicated that in severely affected embryos, the lens placode did not develop and that the placodal, but not pre-placodal phase of *Pax6* expression was lost (Wawersik et al., 1999). This suggested that in the genetic pathway regulating lens induction, Bmp7 signaling was required downstream of $Pax6^{pre\text{-}placode}$ but upstream of $Pax6^{placode}$ (Fig. 1A). Though *Bmp7* is expressed in the presumptive lens ectoderm and lens pit up to approximately embryonic day 11.0 (Wawersik et al., 1999) it is not currently understood which cells in the eye primordium respond to Bmp7.

The Lang, Maas and Gruss laboratories have previously identified and characterized a series of transcriptional control elements in the *Pax6* gene. A conserved region of 531 bp located approximately 3.5 kb upstream of the first promoter in *Pax6* showed activity in the lens lineage beginning with the presumptive lens ectoderm at E8.75. This suggested that this enhancer (designated the ectoderm enhancer) might mediate the expression of *Pax6* in its placodal phase (Fig. 1A). Recent analysis from the Lang laboratory (Dimanlig et al., 2001) has revealed a more complex situation. Through targeted deletion of the ectoderm enhancer, it was shown that while there were distinctive defects in lens induction and development, a small lens did form and *Pax6* expression remained albeit at a lower level. The simplest explanation was that the ectoderm enhancer was not the only control element mediating *Pax6* expression during the placodal phase.

Enter the human geneticists to rescue the developmental biologists. At Fondation des Treilles, Veronica van Heyningen described a systematic identification of conserved *Pax6* gene intronic and flanking regions. Many conserved regions were described, but perhaps most interesting for the lens induction mechanism was the demonstration that the so-called SIMO element, situated about 135 kb 3′ to *Pax6* in the last intron of the adjacent gene (designated AK000505 and transcribed in the opposite direction) has activity in the lens lineage in reporter transgenic mice (Kleinjan et al., 2001). Since it appears that expression mediated by the SIMO element begins in the presumptive lens ectoderm at E9.5 or earlier, it probably represents the enhancer that permitted lens development to proceed in the *Pax6* ectoderm enhancer deletion mice (Dimanlig et al., 2001). With this, we can modify the genetic pathway describing lens induction to indicate that both the ectoderm enhancer and the SIMO element mediate *Pax6* expression during the placodal phase (Fig. 1B).

Foxe3 is a forkhead family transcription factor (first identified by the Jamrich laboratory and formerly known as *lens1* (Kenyon et al., 1999)) that is first expressed at E8.75 in a small patch of presumptive lens ectoderm. A simple analysis of *FoxE3* expression on the $Pax6^{Sey/Sey}$ background indicated that *FoxE3* expression was absent and therefore that *FoxE3* was positioned downstream of *Pax6*

in a genetic pathway defining lens development (Brownell et al., 2000). The *FoxE3* gene is mutated in the *dysgenetic lens* (*dyl*) mouse and in human anterior segment dysgenesis (Semina et al., 2001). The *dyl* mouse displays defects in lens vesicle closure and separation, as well as a reduction in the proliferation level of lens epithelial cells (Blixt et al., 2000; Brownell et al., 2000). The phenotypic resemblance of the *dyl* mouse with those in which the *Pax6* upstream ectoderm enhancer had been deleted (Dimanlig et al., 2001) prompted an examination of a possible genetic relationship. This revealed that *FoxE3* expression was undetectable in the enhancer null mice (Dimanlig et al., 2001) thus indicating that *FoxE3* is located downstream of the placodal phase of *Pax6* expression (Fig. 1B).

Inductive interactions are rarely a consequence of the action of one signaling pathway. This has been illustrated in different developmental processes and some themes have emerged. For example, it is frequently the case that Bmp and fibroblast growth factor (Fgf) signaling pathways cooperate in some way. Induction of the lens appears to be no exception with evidence that as well as Bmp7, Fgf receptor signaling is involved. Experimentation described at Fondation des Treilles (Faber et al., 2001) indicated that a lens lineage-specific transgene expressing a dominant-negative Fgf receptor would reduce the expression level of the two lens induction markers *Pax6* and *FoxE3* at E9.5. This was well before the appearance of morphological changes in the transgenic mice that included a lens pit of reduced size, a small lens and a Peters' anomaly-like persistent lens stalk. Furthermore, the dominant-negative Fgf receptor transgene displayed a genetic interaction with the *Bmp7* null allele. The genetic interaction manifested as an exacerbated lens phenotype and further reduced expression levels for the induction markers *Pax6* and *FoxE3*. Combined, this analysis indicated that Fgf receptor and Bmp7 signaling probably combine upstream of the placodal phase of *Pax6* expression in a genetic pathway defining lens induction (Fig. 1C).

Finally, based on analysis that has already been published, we can make further additions to the proposed lens induction pathway. The loss of *Six3* expression in the lens placode of conditional *Pax6* inactivation mice (Ashery-Padan et al., 2000) indicates that *Six3* is genetically downstream of the placodal phase of *Pax6* expression (Fig. 1D). The expansion and increased expression level of *Prox1* (a homeodomain transcription factor related to *Drosophila prospero*) in the lens epithelium of $FoxE3^{dyl/dyl}$ mice (Blixt et al., 2000; Brownell et al., 2000) also suggests that *FoxE3* normally inhibits the expression of *Prox1* (Fig. 1D). Bmp4 has been identified as a potential lens inducer (Furuta and Hogan, 1998). However, since it does not regulate *Pax6* expression at the induction phases of lens development (Furuta and Hogan, 1998), it likely functions in a pathway parallel to the one defined by the $Pax6^{placode}$ and $Pax6^{pre\text{-}placode}$ (Fig. 1D). Bmp4 does appear to regulate the expression of Sox2, a transcription factor that has been implicated in the regulation of crystallin genes (Kamachi et al., 1995). In particular, recent analysis has indicated that *Pax6* and *Sox2* form a complex that can regulate δ-crystallin gene expression in the chick (Kamachi et al., 2001). Combined, these data might imply that the main function of Bmp4 is to regulate early differentiation in the lens lineage. Perhaps the least well understood aspect of lens induction genetic pathways are the elements that function upstream. Presumably, identification of these genes will be a focus of future work.

2. *Drosophila* eye development

Pax6 also played a prominent role in the invertebrate studies described at the meeting. In contrast to the usual scenario of vertebrate redundancy and invertebrate efficiency, *Drosophila* has duplicated its *Pax6* gene; both *eyeless* (*ey*) and *twin of eyeless* (*toy*) have been shown to direct ectopic eye formation when misexpressed, although *toy* requires the function of the downstream *ey* gene to do this (Czerny et al., 1999; Halder et al., 1995). Several speakers discussed possible specialization of the functions of these two genes. Walter Gehring described likely null mutations of both genes. *ey* appears to be specifically required in the compound eye and not in the ocelli, additional light-sensing organs on top of the head. However, *toy* mutants lack almost the entire head, retaining only the proboscis. The expression patterns of the two proteins, described by Uwe Walldorf, fit this division of labor; Toy is expressed in a broader region of the eye disc than Ey, including areas that form head cuticle as well as differentiating photoreceptors just posterior to the morphogenetic furrow (MF).

The Ey and Toy proteins have diverged in the DNA binding specificity of their paired domains (Czerny et al., 1999); Serge Plaza reported that an enhancer of the downstream *sine oculis* (*so*) gene (Niimi et al., 1999) contains Ey-specific sites as well as sites bound by both Ey and Toy. His results suggest that the Ey-specific sites are more critical for expression in the eye anlagen, and the Toy/Ey sites for expression in the ocelli, consistent with the mutant phenotypes of the two genes. While the paired domain of Ey appears to be sufficient for its function in eye development (Punzo et al., 2001), the role of the homeodomain is less clear. Shoichiro Kurata noted that the homeodomain, but not the paired domain, is required to repress the antennal and leg determinant *Distal-less* (*Dll*) when *ey* is ectopically expressed, and probably to exclude *Dll* from the eye disc during normal development (Kurata et al., 2000). A tantalizing piece of data is the observation of Veronica van Heyningen's laboratory that multiple mutations in the Pax6 paired domain have been found in human patients with aniridia or other anterior segment defects (Hanson et al., 1999), while only one homeodomain missense mutation is known to cause a mild eye phenotype. Perhaps a different spectrum of human syndromes will turn out to be caused by mutations in the Pax6 homeodomain.

One focus of current research is to find other potential

transcriptional targets of these Pax6 proteins. Patrick Callaerts described two likely targets of Ey in the mushroom bodies of the *Drosophila* brain, genes encoding the cell adhesion molecules Fasciclin II and Neuroglian. A screen now being carried out in Walter Gehring's laboratory uses DNA microarrays to determine the differences in mRNA content between wildtype eye discs, wildtype leg discs, and leg discs ectopically expressing *ey*. One probable target found in this screen is *quail*, which encodes an actin-bundling protein (Mahajan-Miklos and Cooley, 1994) expressed just anterior to the MF. Interestingly, research in the Treisman laboratory has implicated Act up, a regulator of actin polymerization expressed in the same region, in the control of apical constriction in the MF and indirectly in limiting the spread of Hedgehog (Hh), the signal that triggers differentiation (Benlali et al., 2000). As this cell shape change does not occur in other discs exposed to Hh, it might be specifically regulated by Ey. A spectrum of genes similar to the Ey targets identified using microarrays have also been shown to be enriched in vertebrate photoreceptors by another genomics technique, SAGE, as Seth Blackshaw from the Cepko laboratory reported at the Fondation des Treilles. It will be very interesting to see how much overlap there is between the eye-specific genes and Pax6 targets of different species. Fly researchers will doubtless be searching for homologues of Necab, a calcium-binding protein shown by Peter Gruss to be an early target of Pax6 in the mouse retina.

In addition to transcriptional targets, proteins that interact directly with Pax6 aroused some interest at the meeting. Serge Plaza has investigated the block of eye development caused by misexpressing *Antennapedia* (*Antp*), a homeotic gene normally expressed in the second thoracic segment, in the eye disc. His results show that Antp acts not by repressing *ey* expression, but by binding to the Ey protein and blocking its function. The homeodomain of Antp is required to bind to Ey, but its own DNA-binding activity is not essential (Plaza et al., 2001). Although this interaction is unlikely to be important in normal eye development, since Antp is never expressed in the eye disc, it could affect Ey function in the CNS, and also hints at the possibility that other homeodomain proteins may alter Ey activity. Peter Gruss described another protein interacting with mouse Pax6, MAPL-1; interestingly, this protein also binds to microtubules and can retain cotransfected Pax6 in the cytoplasm. However, this mysterious protein also contains a nuclear localization sequence and a transcriptional activation domain, indicating the possibility of more complex interactions with Pax6.

Pax6 does not promote eye development in isolation, but by directing the expression of another set of transcription factors, the retinal determination genes (reviewed by Treisman, 1999). Maria Dominguez provided provocative evidence that several of these genes, *eyegone* (*eyg*), *eyes absent* (*eya*), and *so*, may function primarily in growth control. Activation of Notch at the dorsal–ventral boundary of the early eye disc is critical for the subsequent growth of this tissue (Cho and Choi, 1998; Dominguez and de Celis, 1998; Papayannopoulos et al., 1998). In an extensive search for genes acting downstream of Notch, Dominguez recovered *eyg*, a *Pax*-like gene with a partial paired domain (Jun et al., 1998) that like *ey* has a duplication partner, *twin of eyegone* (*toe*). Strong *eyg* mutants show a complete loss of the eye disc, and *eyg* overexpression can reverse the growth inhibition caused by blocking the Notch pathway. While *eyg* expression is strongest near the midline of the disc, *eya* and *so* are most highly expressed near the margins, and appear to be negatively regulated by Notch signaling. Combined with the strong overproliferation observed in *eya* or *so* mutant clones (Pignoni et al., 1997), these data suggest that *eya* and *so* may shape the growth of the eye disc by blocking proliferation at its edges. The role of Notch in promoting eye growth may be conserved in vertebrates, as Shoichiro Kurata showed that injecting an activated form of Notch into Xenopus led to an enlargement of the retina and duplication of the lens.

Although many eye specification genes are conserved across species, those specifically expressed in the vertebrate retina seem not to play a similar role in *Drosophila*. Rod McInnes described two fly homologues of the mouse *Chx10* homeobox gene, which is required in retinal progenitors and bipolar cells (Burmeister et al., 1996). The *Drosophila* genes appear to be expressed at the lateral margins of the eye disc, outside the eye field, and can actually block eye development when misexpressed within this field. The fly homologue of *Rx*, a homeoboxgene critical for retinal determination in mouse, *Xenopus* and medaka fish (Mathers et al., 1997; Winkler et al., 2000), as reported by Milan Jamrich and Felix Loosli, is never expressed in the eye disc at all (Eggert et al., 1998).

Finally, research reported from the Treisman laboratory gave some hints of the events downstream of the eye specification genes. Progression of a wave of photoreceptor differentiation across the eye disc is driven by the signaling protein Hedgehog (Hh) (Dominguez and Hafen, 1997; Heberlein et al., 1993; Ma et al., 1993). A mosaic screen for genes affecting photoreceptor differentiation has identified several novel regulators of Hh function. *sightless* appears to encode an enzyme that adds an essential fatty acid chain to the signaling domain of Hh (Chamoun et al., 2001; Lee and Treisman, 2001). Two components of the transcriptional mediator complex, TRAP240 and TRAP230, are encoded by the *blind spot* and *kohtalo* genes, and may assist in the regulation of some Hh target genes (Treisman, 2001). Orderly patterning of the disc requires a ubiquitin protein ligase encoded by the *hyperplastic discs* gene (Mansfield et al., 1994), which prevents ectopic Hh expression anterior to the MF.

The meeting proved very fruitful in identifying those aspects of eye development that have been conserved throughout evolution, as well as in impressing on us the variety of forms eyes can take and of mechanisms used to make them. We hope that the research stimulated by these

interactions will form the basis for another such meeting in the not too distant future.

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