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Analysis of mouse eye development with chimeras and mosaics

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ABSTRACT Analysis of experimental mouse chimeras (chimaeras) and mosaics provides a means of investigating patterning and differentiation within the developing mammalian eye. Chimeric and mosaic mice carry two or more genetically distinct cell populations and extend the repertoire of analytical tools available to the geneticist. Here we review the impact these techniques have had on our understanding of eye organogenesis. Chimeras and mosaics are routinely used to investigate cell lineages, patterns of growth and gene function, and provide a means to clear analytical hurdles that otherwise limit standard genetic approaches. In particular, chimeras are used to investigate the roles of genes in tissues that do not develop in conventional mutant or knock-out mice, to test whether genes act cell autonomously or non-autonomously in different tissues and to dissect tissue-tissue interactions in less tractable, complex systems. Chimeras, in which cells of different genetic composition are mixed at a fine-scale cellular level, may provide qualitatively different data from mosaic mice with conditional knockouts. The uses of chimeras, Cre-*loxP* mosaics and *in vitro* tissue recombination for study of ocular organogenesis are compared. Wider use of mosaics and chimeras should provide further insights into eye development.

KEY WORDS: *eye, chimera, chimaera, mosaic, clonal analysis, chimeric rescue, cell autonomous gene action*

Introduction

The mammalian eye is a complex developmental system, with contributions from tissues of disparate embryological origins, dependent on several embryological inductive events requiring tissue-tissue interactions, and complicated by rapid morphogenesis. Many developmental genes are expressed in more than one ocular tissue, and some of the genes expressed in the eye have fundamental roles elsewhere that lead to early developmental failure in mutants and make conventional gene knockouts uninformative for analysis of later stages of eye development. Hence, an understanding of eye organogenesis requires rigorous genetic analysis, posing developmental questions about cell lineages, tissue morphogenesis, and autonomy of gene action. Here, we review the roles that mouse chimeras and mosaics have played in dissecting these developmental processes.

Production and analysis of mouse chimeras and mosaics

Chimeras

A chimera is a composite organism with two or more genetically distinct populations of cells that are derived from more than one zygote. This article considers the uses of experimentally produced primary chimeras, where the distinct cell populations are combined early in development (usually by embryo aggregation or blastocyst injection), so all tissues may be chimeric. The production of mouse aggregation chimeras was devised by Tarkowski (1961) and involves recovery of two genetically distinct 8-cell stage embryos, removal of their zonae pellucidae, aggregation, culture to the blastocyst stage and surgical transfer to the uteri of pseudopregnant recipients for further development. In addition chimeras can be produced using cultured embryonic stem (ES) cells combined with embryos by aggregation, co-culture techniques or (more frequently) blastocyst injection (Gardner, 1968).

Optimal use of mouse chimeras requires that the distinct cell populations differ for a combination of genetic markers that provide qualitative, quantitative and spatial information about the composition of different tissues. Ideally the genetic markers which are used to distinguish cells from the different aggregated embryos should be developmentally neutral (i.e. they do not themselves influence ocular development), should be expressed cell-autonomously (such that their presence or absence reliably identifies the origin of the cell) and be relatively easy to detect at the required

Abbreviations used in this paper: ES, embryonic stem; GPI, glucose phosphate isomerase; RPE, retinal pigment epithelium.

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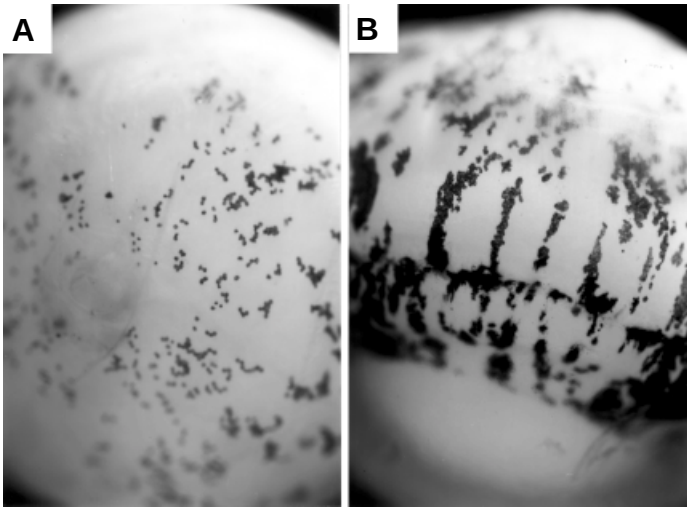

Fig. 1. **Patterns of proliferation and growth in the retinal pigment epithelium**. **(A)** *The back of an adult pigmented ↔ albino chimeric eye, showing small irregularly distributed patches in the retinal pigment epithelium (RPE).* **(B)** *Side-view of an adult pigmented ↔ albino eye showing stripes of pigment in the RPE (centre) near where it meets the iris (from West, 1999).*

level of analysis (in fresh tissue, fixed whole-mounts or histological sections). A common strategy is to combine embryos that differ for pigment markers, *Gpi1* variants (encoding the ubiquitous enzyme glucose phosphate isomerase, GPI1) and one or more transgenic markers. Pigment markers provide a simple means of identifying which individuals are chimeric and allow spatial analysis of cell distributions in the retinal pigment epithelium (RPE). GPI electrophoretic variants can be used for quantitative analysis of tissue composition (Chapman *et al.*, 1972; Gearhart and Mintz, 1972; West and Flockhart, 1994). Other molecular and biochemical genetic approaches have been used but GPI is widely favoured because the enzyme activity is ubiquitous, stable and simple to analyse. Although the contributions of the two cell populations to different chimeras varies widely, tissues within an individual chimera usually have a similar composition, so GPI analysis of several tissues usually gives a good guide to the overall composition of a chimera.

A variety of histological, histochemical and immunohistochemical markers have been used for spatial analysis of chimeras (Gardner, 1984; Kusakabe *et al.*, 1988; Ponder, 1987; West, 1984; Weinberg *et al.*, 1985; Yoshiki *et al.*, 1991) and DNA *in situ* hybridisation has also been used to detect species-specific satellite DNA sequences in *Mus caroli* ↔ *Mus musculus* chimeras (Rossant and Chapman, 1983; Siracusa *et al.*, 1983; Williams and Goldowitz, 1992). However, transgenic markers are now more commonly used. These include the highly reiterated *Tg(Hbb-b1)83Clo* transgene, which can be detected in histological sections by DNA *in situ* hybridisation (Keighren and West, 1993; Lo, 1986), and transgenes that express ubiquitously either *lacZ*, such as *Gt(ROSA)26Sor* (ROSA26) (Friedrich and Soriano, 1991), or green fluorescent protein (GFP) (Hadjantonakis *et al.*, 1998a; Pratt *et al.*, 2000).

Mosaics

A genetic mosaic is similar to a chimera except that the genetically distinct cell populations arise from a single zygote. Females, heterozygous for X-linked genes, are X-inactivation mosaics since functional mosaicism arises after random X-chromosome inactivation occurs early in development (Lyon, 1961). Mouse X-inactivation mosaics can be generated easily by appropriate genetic crosses. Although no endogenous X-linked variants provide good cellular markers for spatial analysis in eyes, appropriate markers have been produced by mutagenesis and transgenesis experiments. The first useful X-linked cellular marker was Is(In7;X)1Ct (Cattanach's translocation), resulting from the insertion of an inverted piece of chromosome 7 into the X chromosome (Cattanach, 1961). The inserted length of chromosome 7 includes the wild-type *C* allele of the albino locus. Homozygous albino female mice (*c/c*) that are hemizygous for the Is(In7;X)1Ct insertion have variegated coat and eye pigment (Deol and Whitten, 1972; West, 1976a). A number of transgenic markers have now been incorporated into the

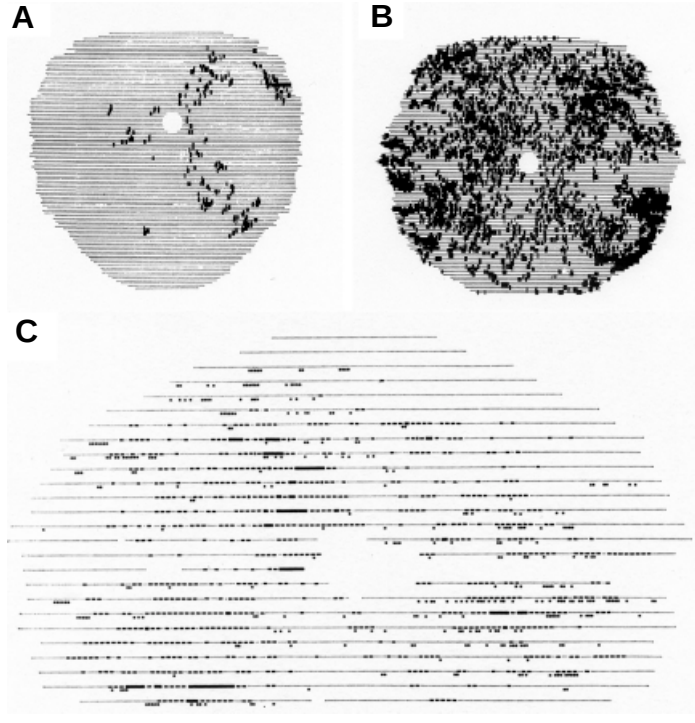

Fig. 2. **Two-dimensional reconstructions of serial sections of RPE in pigmented ↔ albino chimeras and the neural retina in *rd/rd↔+/+* chimeras. (A,B)** *Chimeric RPEs showing clusters of pigmented cells radiating from the optic nerve head, which probably represent descendent clones. Pigmented and albino cells appear less mixed at the periphery in (B) (also see Fig. 1B) (Reproduced with permission from Sanyal and Zeilmaker, 1977).* **(C)** *Two-dimensional reconstruction of the RPE and neural retina from a* rd/rd c/c ↔ +/+ C/C *chimera showing both the areas of degenerated neural retina and the pigmented RPE cells. Solid lines represent normal neural retina; dashed lines represent intermediate degeneration of neural retina; dotted lines represent completely degenerated neural retina. The small black squares below the section lines represent pigmented RPE cells. There is no concordance between the distribution of normal neural retina (solid lines) and pigmented (*C/C*) RPE cells. Areas of predominantly normal (+/+) and predominantly degenerated (*rd/rd*) neural retina appear to radiate from the central region near the optic nerve head. (Reproduced with permission from LaVail and Mullen, 1976).*

X-chromosome which allow analysis of X-inactivation mosaicism of unpigmented tissues. These include the *lacZ* transgenic strain H253 (Tan *et al.*, 1993; Tan and Breen, 1993) and GFP transgenics *Tg(CMV-GFP)1Nagy* (Hadjantonakis *et al.*, 1998b) and *Tg(CMV-GFP)1Jae* (Eggan *et al.*, 2000).

The pink-eyed unstable mutation (p^{un}) has a DNA duplication which mediates somatic reversion to wild-type, producing mosaicism with patches of pigmented $p^{+/un}$ cells in a largely unpigmented $p^{un/un}$ RPE (Brilliant *et al.*, 1991). Most other genetic mosaics used for studies of mouse development have been produced by transgenic methods. Exposure of mouse embryos to retroviruses results in transgenic mosaicism which provides a lineage marker in descendents of the infected cells (Soriano and Jaenisch, 1986; Turner et al., 1990). Mosaic *lacZ* expression has also been produced by engineering an inactive *lacZ* transgene with an internal duplication. This '*laacZ'* transgene undergoes intragenic homologous recombination at a low frequency to restore the active *lacZ* transgene and so generates *lacZ* mosaicism (Nicolas *et al.*, 1996). Conventional autosomal transgenes may also generate mosaic expression as an unintentional consequence of position effect variegation (Dobie *et al.*, 1997). In addition, conditional knockouts, produced using Cre-*loxP* technology induce mosaicism that may be generalised or restricted to one or a few tissues (Betz *et al.*, 1996; Rossant and Spence, 1998).

Analysis of organogenesis & cell mixing during eye development

Retinal pigment epithelium

The simple 2-dimensional distribution of pigmented and albino cells in a chimeric or mosaic retinal pigment epithelium (RPE) reflects the extent of cell mixing during development but retrospective estimation of the number of founder clones contributing to the RPE is more difficult. Spatial analysis of variegated patterns requires distinction of 'patches', 'coherent clones' and 'descendent clones', which are defined as follows. A *patch* is a group of cells of like genotype which are contiguous at the time of consideration, a *coherent clone* is a group of clonally related cells which have remained contiguous throughout development and a *descendent clone* is any group of clonally related cells irrespective of whether they remain contiguous (Nesbitt, 1974; West, 1976a, 1978a,b; West *et al.*, 1997). Each founder cell produces a descendent clone but these may fragment into smaller coherent clones or merge with neighbouring coherent clones of the same genotype, to form visible patches comprising several coherent clones.

The mean size of coherent clones can be estimated from the mean patch size by correcting for the effects of the proportions of the two cell populations, in the mosaic or chimeric RPE, on clone aggregation (Roach, 1968; West, 1975, 1976a). An early histological study of chimeras and X-inactivation mosaics suggested that between E12.5 and the adult, the number of coherent clones in the RPE increased about 3-fold and the mean number of cells per coherent clone increased by 4 to 4.5-fold (West, 1976a). Although such estimates of clone sizes and numbers may not be accurate in some situations (Schmidt *et al.*, 1986) they still provide a valid means of comparing different groups of mosaics or chimeras (West *et al.*, 1997; West, 1999).

In adult, pigmented ↔ albino chimeras, the retinal pigment epithelium (RPE) is usually obscured by pigment in the choroid but

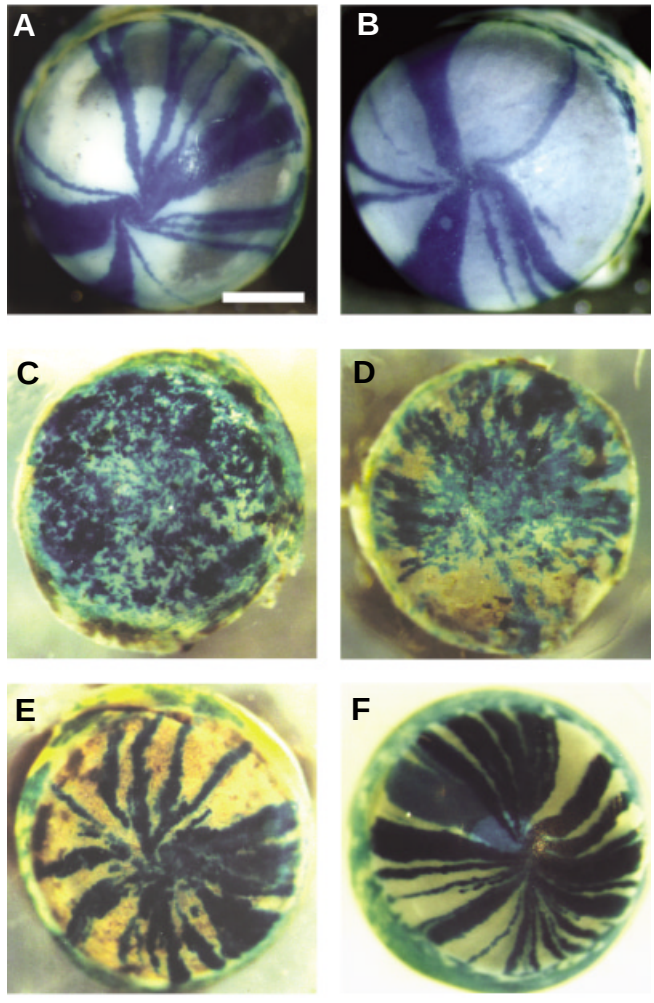

Fig. 3. Chimeric and mosaic analysis of growth and cell migration in the murine cornea. (A,B) *Radial stripes in the corneal epithelium of adult* lacZ$^+$ ↔ lacZ$^-$ *chimeric eyes stained with X-gal.* **(C-F)** *Development of radial stripes in the corneal epithelia of female X-inactivation mosaic mice, hemizygous for the H253, X-linked* lacZ *transgene. Stripes replace an initial pattern of randomly orientated patches. Eyes are from mice at 3 weeks (C), 7 weeks (D), 10 weeks (E) and 20 weeks (F). (Reproduced from Collinson* et al.*, 2002).*

in some chimeras the choroid is predominantly unpigmented, which allows the RPE to be visualised directly. In the proximal RPE (near the optic nerve head) the pigmented and albino cells are usually arranged as small patches, often clustered within broadly defined radial sectors, whereas in the distal RPE (towards the iris) there are larger patches, arranged as radial stripes (Figs. 1, 2A,B). The distribution of pigmented and albino cells in chimeric RPE was examined in more detail by 2-dimensional reconstructions from serial sections (Sanyal and Zeilmaker, 1977) (Fig. 2 A,B) and whole mount preparations (Schmidt *et al.*, 1986; Bodenstein and Sidman, 1987b). The broad radiating clusters probably represent descendent clones that were fragmented into smaller coherent clones proximally but remained less fragmented distally.

The formation of clearer stripes at the distal periphery (Figs. 1B, 2B) was explained by an elegant combination of computer modelling, mitotic analysis and analysis of chimeras and mosaics (Bodenstein, 1986; Bodenstein and Sidman, 1987a,b). Computer simulation

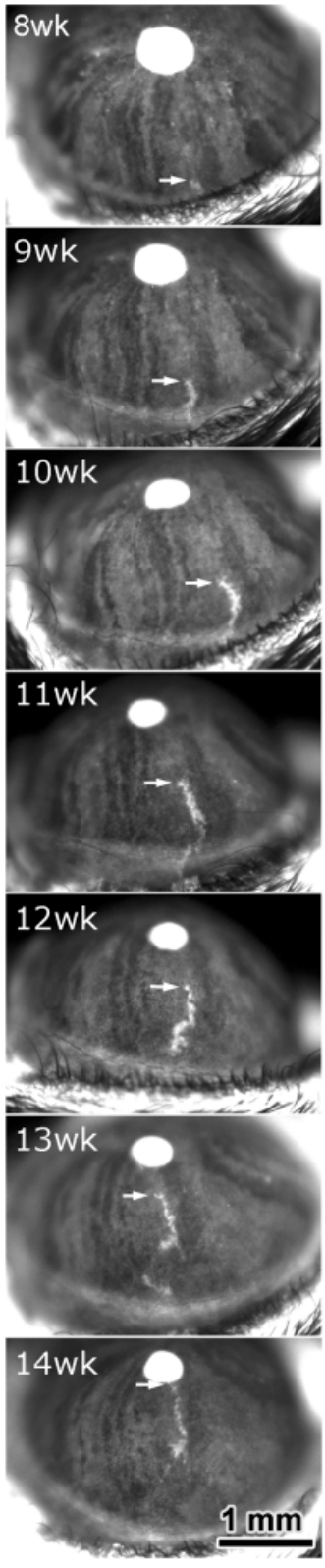

Fig. 4. Cell migration in the cornea. *Time lapse sequence of centripetal cell movement of a brightly fluorescent GFP-positive patch of corneal epithelial cells in a mosaic* Tg(GFPU)5Nagy *transgenic mouse between 8 and 14 weeks. The arrow indicates the leading edge of the patch of cells. (Reproduced with permission from Nagasaki and Zhao, 2003).*

predicted that extensive cell mixing would occur if dividing cells were scattered throughout the tissue during RPE growth. Conversely, if cell divisions were mostly confined to the edge of the growing tissue little cell mixing was predicted so radial stripes would form at the growing edge. Mitotic activity in the RPE was then shown to be widespread at E13 but between E13 and P7 it became progressively more restricted to the distal edge, implying that the peripheral (distal) stripes were formed during the later growth stage when dividing cells were confined to the edge thus reducing cell mixing. This reduction in cell mixing later in development is consistent with the earlier evidence, discussed above, for an increase in mean coherent clone size between E12.5 and the adult.

Corneal epithelium

Striking radial stripes have been reported in the corneal epithelium of adult chimeras and X-inactivation mosaics carrying *lacZ* transgenes (Collinson *et al.*, 2002) and mosaic GFP transgenic mice (Nagasaki and Zhao, 2003). The stripes in the corneal epithelium were much more marked than those seen in the periphery of the RPE spanning, in some instances, the full radius of the cornea (Fig. 3), and arose after birth. At three-weeks, *LacZ* mosaics had a pattern of randomly orientated patches rather than stripes. Stripes only emerged at the periphery at around 5 weeks and reached the centre by about 8 weeks (Fig. 3). The corneal epithelium is maintained throughout adult life by stem-like cells (limbal stem cells - LSCs) which reside around the edge of the cornea and produce progeny that migrate centripetally to replace cells lost during normal life. The radial stripes in mosaics and chimeras reflect this centripetal migration of corneal epithelial cells, as was confirmed by an elegant time-lapse study of movement of groups of GFP-positive cells in mosaic GFP transgenic mice (Nagasaki and Zhao, 2003) (Fig. 4). It is now clear that development of the corneal epithelium produces randomly orientated patches of cells in neonates which are subsequently replaced by cells derived from LSCs at the periphery of the cornea.

Neural retina

Organogenesis in the neural retina is complex and involves interactions of many cell types. The adult neural retina is arranged in concentric layers including six classes of neurons and Müller glia, all derived from pluripotent retina progenitor cells (Cepko *et al.*, 1996). Chimeras incorporating cells homozygous for a retinal degeneration mutation of the *Pde6b* gene (phosphodiesterase 6B, formerly *rd*) as a histological marker suggested that descendent clones in the neural retina are arranged as clusters of patches of mutant and wild-type cells radiating in sectors from near the optic nerve head to the ora serrata (Fig. 2C) (Mintz and Sanyal, 1970; Mintz, 1971; LaVail and Mullen, 1976). These original observations using retinal degeneration as a lineage marker are supported by more recent illustrations of similar distributions seen after *lacZ* staining of whole-mount chimeric retinas (compare Fig. 5A with Fig. 2C; also see Figs 2a-e in Reese *et al.*, 1999). One problem with using retinal degeneration as a morphological lineage marker in chimeras was that there were regions where degeneration was intermediate between wild-type and mutant (Fig. 2C). The use of modern cellular markers has shown that coherent clones span the full thickness of the neural retina as columns (see below), confirming the suggestion that the intermediate regions of retinal degeneration resulted from blur-

ring of the original boundaries between columns of mutant and wild-type cells after degeneration occurred (West, 1976b; Mullen, 1978).

Approaches using either recombinant retrovirus-mediated transgenic mosaics, chimeras or X-inactivation mosaics showed that marked cell populations were arranged in stripes or columns spanning the entire thickness of the retina (Turner and Cepko, 1987; Turner *et al.*, 1990; Williams and Goldowitz, 1992; Reese *et al.*, 1995) (Fig. 5 B,C). (These columns are orientated perpendicularly to the original sectors of descendent clones.) Moreover, these studies showed that each coherent clone can produce all the differentiated cell types. Further analysis with X-inactivation mosaics and chimeras showed that, for four of the six retinal cell types (cone photoreceptors, amacrine cells, horizontal cells and ganglion cells), individual cells are also dispersed tangentially (Reese *et al.*, 1995; Reese and Tan, 1998; Fig. 5D). This tangential dispersal occurs over short distances characteristic of the cell type, which argues against passive displacement (Reese *et al.*, 1999).

The picture that emerged from studies with chimeras and mosaics is that during the early phases of optic cup formation the founder cells produce descendent clones that radiate outwards. As the layers of the neural retina stratify, further proliferation produces coherent clones, which form columns spanning the full depth of the retina, from the photoreceptors to the ganglion cell layer. Subsequently some cells disperse laterally.

Chimeric analysis of genetic control of eye development

Mutations in developmental genes often lead to an early interruption of organogenesis (e.g anophthalmia) or embryonic lethality precluding the analysis of later roles for the gene in specific organs. Chimeras composed of populations of wild-type cells and cells carrying a gene mutation of interest will commonly develop to appropriate embryonic stages such that mutant cells get the opportunity to contribute to tissues that do not form in the mutant mice. Mutant cells may be excluded from, or behave abnormally in, tissues where normal gene function is required. Hence by analysing the distribution and phenotype of mutant cells in chimeras the impact that a developmental gene has on cellular function can be determined.

Chimeras are the 'classic' tools for determining cell autonomy and non-autonomy of gene action in any particular tissue. The function of a developmental gene in a cell that expresses it is said to be cell autonomous if activity of the gene is required within that cell for it to fulfil its normal developmental programme. One would imagine, for example, that transcription factors and genes involved with signal transduction pathways will have a number of cell autonomous roles. A non-autonomous role for a developmental gene would be a situation where activity of the gene in one cell is required to direct normal development of another cell, for example a gene encoding a secreted molecule that directs the development of neighbouring cells or tissues in a paracrine manner. Developmental genes may have both cell autonomous and non-autonomous functions – a transcription factor may have non-autonomous roles through regulation of expression of secreted molecules. In chimeras comprising a mixture of wild-type and mutant cells, only the mutant cells show the mutant phenotypic effect if the gene acts cell autonomously (e.g. they may be absent, depleted in numbers or abnormally distributed). If the gene does not act cell autonomously the wild-type cells may rescue the mutant cells so that neither show an abnormal phenotype or, conversely, both mutant and wild-type cells may be affected.

The production of chimeric embryos containing wild-type cells intermixed with mutant cells puts the two cell populations in competition with each other. Their intimate mixing may in these cases allow fine-scale analysis of cell-cell and tissue-tissue interactions and may demonstrate subtle developmental defects in the mutants.

Analysis of cell autonomous versus non-autonomous requirement for developmental genes

The power of chimeric analysis to diagnose autonomy of gene action is maximised if:

1) the expression pattern of the gene of interest is known;

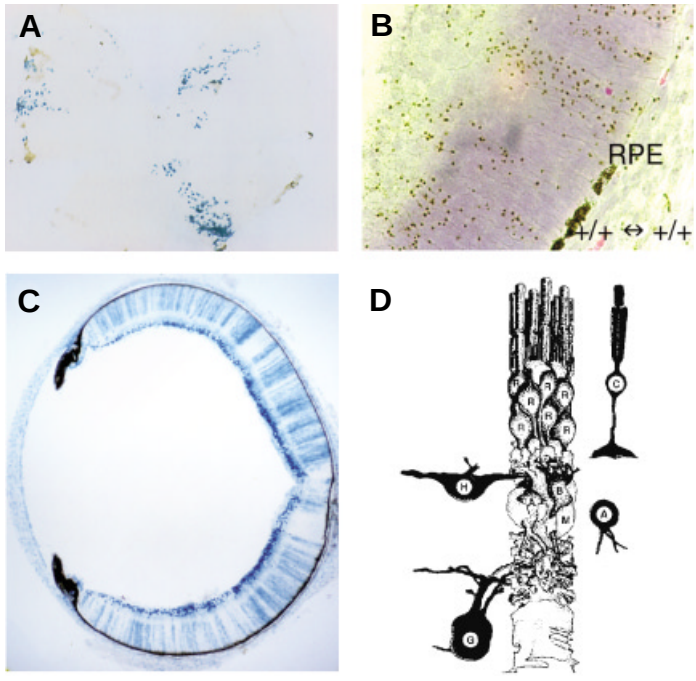

Fig. 5. Clonal growth in the neural retina. (A) *A neural retina flat-mount preparation, from a* lacZ^{+} ↔ lacZ^{-} *mouse chimera, stained for ß-galactosidase, showing radiating patches of* lacZ*-positive cells (blue). (Reproduced from Reese* et al.*, 1999, with permission).* **(B)** *A section from an E12.5 mouse chimera marked with the reiterated ß-globin transgene* Tg(Hbb-b1)83Clo. *Stripes of transgenic neuroblast cells, detected by DNA* in situ *hybridisation and visualised as small brown spots in the nucleus, span the full depth of the developing neural retina. Patches of pigmented cells are labelled in the RPE. (Reproduced from Collinson* et al.*, 2001)* **(C)** *A section from a newborn (P1) X-inactivation mosaic, mouse eye (female, hemizygous for the H253, X-linked* lacZ *transgene). The eye was stained for ß-galactosidase and shows stripes of* lacZ*-positive cells that span the neuroblast layer and tangential dispersion of the cells in the ganglion cell layer. In 3-dimensions the stripes appear as columns. (Colour version of figure from Reese* et al.*, 1999; reproduced with permission).* **(D)** *Diagrammatic representation of a clone of cells spanning the neural retina, showing coherent radial alignment of the rod cells (R), bipolar cells (B) and Müller glial cells (M), and the local tangential dispersion of individual cone cells (C), horizontal cells (H), amacrine cells (A) and ganglion cells (G). (Based on Reichenbach* et al.*, (1994), reproduced from Reese and Tan (1998) with permission).*

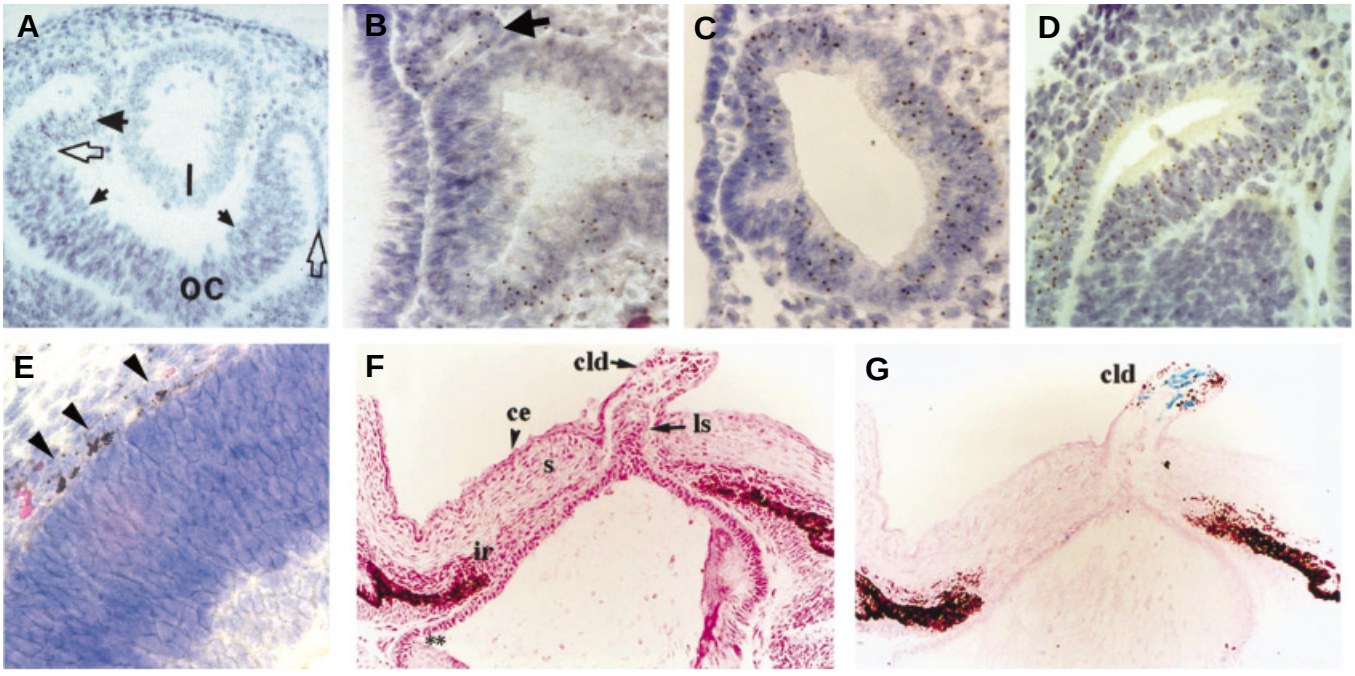

Fig. 6. Distribution and phenotype of mutant cells in chimeric eyes. (A) *The lens (l) of an E12.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera.* Pax6$^{-/-}$ *cells are identified by the presence of a brown spot in the nucleus after DNA-DNA* in situ *hybridisation to visualise the* Tg(Hbb-b1)83Clo (Tg) *marker transgene. The lens is entirely composed of* Pax6$^{+/+}$, Tg^{-} *cells. (From Quinn* et al., *1996).* **(B)** *'Exclusion' of an ectopic vesicle of* Pax6$^{-/-}$, Tg^{+} *cells (arrow) from the distal optic vesicle of an E9.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera.* **(C)** *Optic vesicle of an E9.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera with a higher percentage of* Pax6$^{-/-}$ *cells than in (B). The optic vesicle is segregated into regions of purely* Pax6$^{+/+}$ *or* Pax6$^{-/-}$ *cells. Only the* Pax6$^{+/+}$ *region of the distal optic vesicle has maintained contact with the head surface ectoderm. (B, C. From Collinson* et al., *2000).* **(D)** *Segregated regions composed purely of* Pax6$^{+/+}$ *or* Pax6$^{-/-}$ (Tg^{+}) *cells in the retina of an E12.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera. (From Quinn* et al., *1996).* **(E)** *Pigmented* Pax6$^{-/-}$ *cells (arrows) in the RPE of an E16.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera. (From Collinson* et al., *2003).* **(F,G)** *Persistent lens stalk in the cornea of an E18* AP-2$\alpha^{+/+}$ ↔ AP-2$\alpha^{-/-}$ *chimera (F). Blue X-gal staining (G) reveals the presence of* AP-2$\alpha^{-/-}$, lacZ^{+} *cells correlates with the lento-corneal dysgenesis (F, G. Reproduced with permission from West-Mays* et al., *1999). Key: l, lens; ls, lens stalk; cld, corneal lenticular defect; oc, optic cup; ce, corneal epithelium; s, corneal stroma.*

2) the gross contribution of mutant cells to the chimera can be assayed by, for example, GPI analysis and
3) the genotype of all cells in the chimera can be identified in a way that allows a quantitative analysis of their distribution.

Some earlier chimera experiments investigated the differentiation or degeneration of the lens and retina without prior knowledge of the genes underlying the mutations of interest, and without transgenic markers to identify the mutant cells.

Early studies of developmental genes with chimeras

Mutations in *Mip*, the major intrinsic protein of eye lens fibres, cause congenital cataracts. Chimeras were generated composed of cells from wild-type mice and cells from either the *Cat^{Fr}* (Cataract Fraser now known as *Mip$^{Cat\text{-}Fr}$*) or the *Lop* (cataract lens opacity, now *Mip$^{Cat\text{-}Lop}$*) mouse mutations (Muggleton-Harris *et al.*, 1987; Shiels *et al.*, 1991). A transposon-induced splicing error introduces a long repeat sequence replacing the normal Mip carboxy terminus in *Mip$^{Cat\text{-}Fr}$*, whereas a G to C transversion at position 151 of the encoded *Mip$^{Cat\text{-}Lop}$* mRNA inhibits targeting to the cell-membrane. Muggleton-Harris *et al.* (1987) produced adult *Mip$^{Cat\text{-}Fr}$*/*Mip$^{Cat\text{-}Fr}$* ↔ *+/+* chimeras and estimated the contribution of mutant cells to the lenses by GPI electrophoresis (as described earlier). Lenses composed entirely of mutant cells were cataractous but none of the chimeric lenses had congenital cataracts, even when mutant cells predominated. Similarly, the severely dysgenic lens fibre phenotype in LOP mice could be partly corrected in *Mip$^{Cat\text{-}Lop}$*/*Mip$^{Cat\text{-}Lop}$* ↔ *+/+* chimeras (Shiels *et al.*, 1991). These results suggested a non-autonomous effect whereby wild-type cells corrected the congenital cataractous phenotype usually associated with the mutants. This phenotypic rescue was incomplete because some lenses developed abnormalities after 2 months.

In addition Yoshiki *et al.* (1991) studied the role of the *eye lens obsolescence* gene *Elo*, (*Cryge*-crystallin gamma E (Cartier *et al.*, 1992)) and showed that a mixture of normal and *Elo/+* developing lens fibres (with abnormal nuclei) occur together in the posterior of the developing *Elo/+* ↔ *+/+* lens. The chimeric lenses were often morphologically similar to non-chimeric *Eld*/+ lenses, and it was concluded that there are autonomous roles for *Elo* during lens fibre differentiation and elongation, but that *Elo* probably did not affect lens epithelial proliferation. All the above results suggested that as expected for a crystalline structure such as the lens, a dysmorphic mutant lens fibre may be supported by surrounding wild-type cells, but equally it may disrupt the normal crystalline arrangement of the surrounding wild-type cells.

Other early experiments with mouse and rat chimeras revealed distinct modes of action for different retinal degeneration mutations (West, 1999). Patches of normal and degenerate photoreceptors (outer nuclear layer; ONL) were seen in *rd/rd* ↔ *+/+* and *Rds/+* ↔ *+/+* mouse chimeras and in *rdy/rdy* ↔ *+/+* rat chimeras. *Rds* is the mouse retinal degeneration slow gene, which affects both homozy-

gotes and heterozygotes, and *rd* is the mouse retinal degeneration gene (now known as *Pde6b*, phosphodiesterase 6B). The rat retinal dystrophy gene (*rdy*) causes a similar phenotype to mouse *rd* and *Rds*; however, chimera experiments showed that the mode of action differed significantly. No spatial relationships were seen between patches of pigmented and albino cells in the RPE and patches of degeneration in the ONL for the mouse *rd/rd↔+/+* and *Rds/+↔+/+* chimeras (LaVail and Mullen, 1976; Sanyal *et al.*, 1986; also see Fig. 2C). Hence the *rd* (*Pde6b)* and *Rds* genes act in the neural retina rather than the RPE. However, *rdy/rdy* ↔ +/+ rat chimeras showed that the ONL degenerated only in regions that were adjacent to patches of RPE comprising *rdy/rdy* cells (Mullen and LaVail, 1976) showing that the primary defect was in the overlying RPE and not in the neural retina itself. Thus the *rdy*-mutant RPE cells induces degeneration in the adjacent neural retina cells in a non-autonomous fashion.

Further analysis of mouse retinal degeneration focused on the mis-expression of a pig rhodopsin transgene; the transgenic expression both caused the *rd* phenotype and provided a convenient marker for affected cells detected by RNA *in situ* hybridisation (Huang *et al.*, 1993). This revealed patchy distributions of transgenic and wild-type cells in the neural retina but, unlike the retinal degeneration chimeras discussed above, the ONL did not show patchy distributions of degeneration. The chimeras had a uniform ONL of intermediate thickness implying uniform degeneration of both wild-type and transgenic photoreceptor cells. These observations suggested that the transgene acted non-autonomously, and that within the ONL cell interactions are crucial. The chimera experiments thus revealed an unknown complexity for generating retinal degeneration and uncovered a third mechanism that is distinct from the two identified by earlier chimera experiments with mutant genes.

Use of transgenic lineage markers to study developmental genes in chimeras

The cloning of genes underlying classical mutations and the availability of transgenic lineage markers have been crucial in extending the range of mouse chimeras in which conditions 1 and 3 above can be fulfilled, and have increased the power of chimeric analysis.

Lens development. The *Tg(Hbb-b1)83Clo* β-globin transgene (incorporated into the genome as approximately a thousand contiguous copies) was used by Liégeois *et al.* (1996) in examining the action of the recessive *aphakia* (*ak*) mutation. Homozygous *ak/ak* mice do not develop lenses and *ak* is almost certainly an allele of *Pitx3* (Semina *et al.*, 2000; Rieger *et al.*, 2001). The route for chimera production entailed the injection of wild-type ES cells carrying the *Tg(Hbb-b1)83Clo* β-globin transgene into *ak*/*ak* blastocysts. Chimeric animals manifested normal lenses that, however, were entirely derived from the Tg^{+} ES (wildtype) cells showing that *ak* gene function is autonomously required during lens development.

Similar results were obtained for analysis of the roles of the transcription factor Pax6 in the lens. *Pax6* is expressed strongly throughout eye development, in the undifferentiated optic vesicle and optic cup (in both the RPE and all neural retina precursors), in the facial epithelium, lens placode and developing lens and the epithelial layers of the iris and cornea (Walther and Gruss, 1991). Eye development fails at very early stages in the homozygous mutants (the interaction between the optic vesicle and facial epithelium, which leads to the induction of the lens placode, does not occur). Hence although the homozygous mutants do not develop lenses, it was not immediately clear whether this was due to an autonomous requirement for Pax6 in the developing lens lineage or a failure of lens induction by the optic vesicle. Quinn *et al.* (1996) showed that lenses normally developed in the eyes of $Pax6^{+/+} \leftrightarrow Pax6^{-/-}$ chimeras that contained less than ~50% mutant cells, but that no $Pax6^{-/-}$ cells were ever detected in those lenses by *in situ* hybridisation against the *Tg(Hbb-b1)83Clo* β-globin transgene (Fig. 6A). It could therefore be concluded that Pax6 is required in a cell autonomous manner for normal contribution to the lens.

A similar conclusion was drawn from $Mab21/1^{+/+} \leftrightarrow Mab21/1^{-/-}$ aggregation chimeras (Yamada *et al.*, 2003). *Mab21/1* is expressed in both the optic vesicle and the lens placode, and $Mab21/1^{-/-}$ mice are aphakic. Mutant cells did not contribute to the chimeric lens, showing an autonomous requirement for Mab21/1. The results are consistent with the observed down-regulation of *Mab21/1* in $Pax6^{-/-}$ mutants, and suggest a genetic network for lens formation in which *Mab21/1* expression is downstream of *Pax6*.

Retinal development. Presence/absence of a particular cell type in a chimeric tissue is only one of many possible 'end-points' of a cell autonomous role for a developmental gene. Mutant cells may be incorporated into a chimeric tissue, but be dysgenic (like the *Elo/+* lens fibre nuclei described above). Alternatively, if the gene controls parameters of proliferation, mutant cells may be over or under-represented in a chimeric tissue (in comparison to the global composition of the chimera). For example Robanus Maandag *et al.* (1994) found apparent selection against $Rb^{-/-}$ cells in the retina of $Rb^{+/+} \leftrightarrow Rb^{-/-}$ chimeras, by comparing the GPI1A:1B ratio of the retina with that of the other tissues in the chimera.

If a gene autonomously controls cell surface properties of the tissues in which it is expressed, then mutant cells may be present in normal numbers in those tissues in chimeras, but may fail to develop normally or may show patterns of segregation within or physical budding from those tissues. This is the case for $Pax6^{-/-}$ cells in the developing neural retina. During the interaction between the optic vesicle and the prospective epithelium in E9.5 $Pax6^{+/+} \leftrightarrow Pax6^{-/-}$ chimeras, the mutant cells are physically excluded and may form small ectopic vesicles abutting a primarily wild-type distal optic vesicle (Collinson *et al.*, 2000) (Fig. 6B). In chimeras that are >50% mutant cells, contribution of $Pax6^{-/-}$ cells to the convoluted chimeric neural retinal tissue continues to at least E16.5, but there is almost complete segregation of $Pax6^{+/+}$ and $Pax6^{-/-}$ cells into blocks of cells of a single genotype (Fig. 6 C,D) (Quinn *et al.,* 1996; Collinson *et al.*, 2000). Pax6 controls a number of cell surface and adhesion molecules (reviewed in Simpson and Price, 2002), and differential expression of subsets of these molecules in wild-type and mutant cells may explain their autonomous segregation in some chimeric tissues.

'Developmental delay' is another end-point of autonomous gene function that may be discerned in chimeras. $Pax6^{-/-}$ cells contribute to the developing retinal pigment epithelium of $Pax6^{+/+} \leftrightarrow Pax6^{-/-}$ chimeras, but in contrast to wild-type cells are not pigmented at E12.5 (Quinn *et al.*, 1996). However, by E16.5, some pigmented $Pax6^{-/-}$ cells are found in the chimeric RPE (Fig. 6E) (Collinson *et al.*, 2003), showing that *Pax6* is not absolutely

required for differentiation, but alternatively, controls the timing of the genetic cascade that leads to pigmentation.

Early embryonic lethality and developmental failure

Chimeric analysis of mouse mutants allows an understanding of gene function in tissues for which the mutation normally disrupts development at an early stage. For example, Pax6 is expressed in the cornea, but eye development fails before corneal induction in *Pax6*$^{-/-}$ mice. Collinson *et al.* (2003) used chimeras to investigate the roles of Pax6 in the developing cornea. *Pax6* is expressed at high levels in the corneal epithelium and perhaps, not surprisingly, mutant cells did not contribute to the corneal epithelium of *Pax6*$^{+/+}$ ↔ *Pax6*$^{-/-}$ chimeras. Less expected was a significant, though less dramatic, under-representation of *Pax6*$^{-/-}$ cells in the corneal stroma and endothelium of E16.5 chimeras, detected by comparing the proportion of *Tg(Hbb-b1)83Clo*$^{+}$ *Pax6*$^{-/-}$ cells in the different corneal layers with the global composition of the chimera determined by GPI in non-ocular tissues. The corneal stroma and endothelium express *Pax6* at very low levels (barely detectable by immunohistochemistry) transiently in the late-fetal and perinatal stages. The autonomous under-representation of *Pax6*$^{-/-}$ cells in these tissues was therefore good genetic evidence that the very weak detected expression may have functional significance during corneal development.

Nottoli *et al.* (1998) and West-Mays *et al.* (1999) used chimeras to study the developmental roles of the transcription factor, AP-2α. Knockout *AP-2α*$^{-/-}$ mice show perinatal lethality, with dysgenesis of many organ systems. Severe craniofacial dysgenesis, including microphthalmia or anophthalmia were reported. Nottoli *et al.* (1998) and West-Mays *et al.* (1999) made chimeras by injection of *AP-2α*$^{-/-}$ ES cells into wild-type blastocysts. The chimeras showed ocular dysmorphologies including retinal and lens defects. A proportion of the chimeras showed eye defects in the absence of other craniofacial defects, dissociating abnormalities of the eye from secondary consequences manifested by the head abnormalities. The persistence of a lens-corneal bridge in many chimeras correlated with the presence of *AP-2α*$^{-/-}$ cells (expressing *lacZ*) in the lens stalk, suggesting an autonomous role for *AP-2α* in the morphogenesis of the lens vesicle (Fig. 6 F,G).

Taken a step further, it is possible to identify roles for important developmental genes during maintenance and ageing of adult tissues in chimeras, when conventional knockouts are embryonic lethal. This is of likely relevance to the study of oncogenes and tumour suppressors. Robanus Maandag *et al.* (1994) used chimeras to study the role of the *Rb* gene in adult ocular tissues. *Rb*$^{-/-}$ knockout embryos die at E13-E15 and this lethality is mediated via the placenta (Wu *et al.*, 2003). However, *Rb*$^{-/-}$ cells contributed to most adult chimeric tissues. There was failure of *Rb*$^{-/-}$ lens cell differentiation in the chimeras, and ectopic proliferation of retinoblasts, suggesting a failure of *Rb*$^{-/-}$ cells to respond to differentiation and growth-arrest signals. Liégeois *et al.* (1996) used their 'lens complementation assay' described above to investigate the role of Rb in the lens. Injection of *Rb*$^{-/-}$ ES cells into *ak/ak* blastocysts produced chimeric mice in which the lenses were entirely composed of *Rb*$^{-/-}$ cells. The lens fibres were disorganised with a large number of nuclei abnormally situated in the lens fibre region, some of which were mitotic.

Dissection of tissue-tissue interactions

Chimeras have been used to elucidate the functions of a developmental gene in tissue-tissue interactions in a situation in which both interacting tissues each express the gene of interest. Collinson *et al.* (2000) took advantage of the tendency of *Pax6*$^{+/+}$ and *Pax6*$^{-/-}$ cells to segregate in chimeric tissues to analyse the interface between the optic vesicle and the facial epithelium (prospective lens placode) in E9.5 mouse chimeras. Areas of the optic vesicle/prospective lens interface were identified where both tissues were wild-type, both mutant, or one tissue was mutant and the other wild-type. Lens placode formation and contact between the optic vesicle and the facial epithelium were scored. A strong correlation between the genotype of the facial epithelium and lens placode formation was found, showing that only *Pax6*$^{+/+}$ epithelium developed a placodal thickening and confirming the autonomous role of Pax6 in the process of lens induction. A second significant correlation was found, highlighting the role of *Pax6* in establishing and maintaining the adhesion at the interface between the optic vesicle and lens placode; *Pax6*$^{+/+}$ sectors of optic vesicle tended to maintain the contact whereas *Pax6*$^{-/-}$ sectors lost contact (Fig. 6C). This recapitulated the failure of the optic vesicle and facial epithelium to maintain contact in the non-chimeric *Pax6*$^{-/-}$ E9.5 embryo. Thus *Pax6* is required in both the optic vesicle and the facial epithelium to establish the full contribution of inductive and morphogenetic events that drive early eye development. Pax6 is required in the optic vesicle to maintain the adhesion during lens induction, and in the facial epithelium to autonomously control the genetic pathways that lead to lens differentiation.

Subtle developmental defects revealed by chimeric analysis

Collinson *et al.* (2001) showed that although *Pax6*$^{+/-}$ heterozygotes develop lenses, heterozygous cells are eliminated from the lens epithelium of *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ chimeras between E12.5 and E16.5, leading to the production of a wildtype lens, even in chimeras highly enriched (up to 80%) in *Pax6*$^{+/-}$ cells. Although lens development is delayed in non-chimeric *Pax6*$^{+/-}$ mice, there are no discernable defects, at the level of analysis of proliferation or apoptosis in heterozygous lenses, that might explain the complete 'disappearance' of *Pax6*$^{+/-}$ cells in chimeras.

A study of the localisation of *Pax6*$^{+/-}$ cells in the chimeric lens epithelia at E12.5, however, revealed that they were primarily distributed at the lateral edge of the lens epithelium, close to the lens equator. This suggests there may be some subtle adhesive or migratory defect of the *Pax6*$^{+/-}$ cells such that they are more likely than wild-type to be eliminated from the mid-lateral proliferative zone of the lens epithelium, and to move to the edge where cells leave the epithelium, become post-mitotic and differentiate.

Furthermore, it was noted that in adult *Pax6*$^{+/+}$ ↔ *Pax6*$^{+/-}$ chimeras, composed of up to 80% heterozygous cells but (as described above) with wild-type lenses, no other anterior segment defects were noted. The other eye tissues developed normally even though they were predominantly composed of *Pax6*$^{+/-}$ cells. The lens is a primary organiser for anterior segment development (Thut *et al.*, 2001; Strickler *et al.*, 2001), and the data from the chimeras suggested that if Pax6 dosage was restored in the lens, it could non-autonomously correct the mutant phenotype in surrounding tissues.

Chimeras, mosaics and tissue recombinations

The production of chimeras provides information about the *in vivo* development of complex organ structures. At a descriptive level, the analysis of the distribution of mutant cells in chimeras generates functional hypotheses about gene function that can be

tested further by molecular techniques. Chimeras are particularly useful for studying the roles of developmental genes in organs or tissues that do not form in conventional gene knockout mice, perhaps because of embryonic lethality, and they facilitate dissection of complex tissue-tissue interactions by allowing analysis of different roles for the same gene in interacting tissues. However, several other technologies exist that can be used to address these issues, and chimeras may not always be the most appropriate tool. Under what circumstances can similar information about ocular development be obtained by other, perhaps less labour intensive, techniques?

In vitro tissue recombinations can recapitulate many of the *in vivo* chimeric situations. If two tissues that express a developmental gene interact during normal morphogenesis, it may be possible in culture to study the interactions in a reconstituted system where one tissue is mutant and the other wild-type. *In vitro* experiments may produce data of equal or better quality, compared to chimeras, by achieving greater control of the genotype and arrangement of interacting tissues, but are only informative if culture systems are available that allow one to recapitulate a physiological situation. While culture systems exist for most ocular cell types, it is not yet possible to recombine a developing eye from its component tissues *in vitro*. Even if it were, there are still biologically important factors, such as the physical stresses of rapid three-dimensional craniofacial morphogenesis that are difficult if not impossible to reproduce in tissue culture. For example, Fujiwara *et al.* (1994) studied the roles of *Pax6* in the optic vesicle and lens placode by performing reciprocal recombinations of *Pax6*$^{-/-}$ facial epithelium with wild-type optic vesicle, and *vice versa*. They found that the genotype of the facial epithelium determined whether or not an invaginating lens placode would form, but that both *Pax6*$^{+/+}$ and *Pax6*$^{-/-}$ optic vesicles were capable of inducing lens development. This contrasted with the *Pax6*$^{+/+}$ ↔ *Pax6*$^{-/-}$ chimeric eyes in Collinson *et al.* (2000), which suggested that both optic vesicle and facial epithelium had to be *Pax6*$^{+/+}$ for lens morphogenesis to occur. Although Pax6 may not have a role in producing lens-inducing signals in the optic vesicle (Furuta and Hogan, 1998), it is required in the optic vesicle to maintain the adhesion with the facial epithelium, which may be necessary for mediation of (non-Pax6 dependent) lens-induction. This role is not apparent *in vitro* because the adhesion between the two tissues is maintained irrespective of the genotype of the optic vesicle.

Conditional gene inactivation using Cre-*loxP* or similar systems enables induction of mosaicism in defined tissues at specific stages in development. Once the conditional mutation is created there is (unlike chimeras) no further requirement for embryonic manipulation to produce the experimental animal. The Cre-*loxP* technology requires the site-directed insertion of two *loxP* recombinase target sites into the gene whose activity is to be eliminated. In order to conditionally disrupt the gene the Cre recombinase (from bacteriophage P1) is expressed from a tissue-specific promoter. Conditional mutations, thus far, have been used sparingly in the study of eye development. However, it is clear that the approach provides a different genetic perspective in understanding the developmental process.

Ashery-Padan *et al.* (2000) expressed Cre from elements of the *Pax6* promoter that drive expression in the lens, cornea and pancreas. The floxed *Pax6* allele was knocked out in the surface ectoderm of the head, leading to failure of lens development. The retina of these mice was convoluted at E14.5, with more than one optic nerve head in each eye, suggesting that several retinae were developing (Fig. 7 A,B). Molecular markers demonstrated that the patterning of these convoluted neuroretinae was normal. Quinn *et al.* (1996) produced superficially similar eyes in *Pax6*$^{+/+}$ ↔ *Pax6*$^{-/-}$ chimeras with a high proportion of mutant cells (Fig. 7C). Both the chimeras and the Cre-*loxP* lens knockouts demonstrated a requirement for Pax6 in the lens. In contrast they provided different information about the retina – the segregation of *Pax6*$^{+/+}$ and *Pax6*$^{-/-}$ cells in the chimeras suggested adhesive defects in the mutant neuroretina, which was not determined from the Cre-*loxP* retinae. However, the chimeras provided less information about the development of the wild-type retinal cells in absence of a lens, partly because of the complication of having both mutant and wild-type cells in the same tissue.

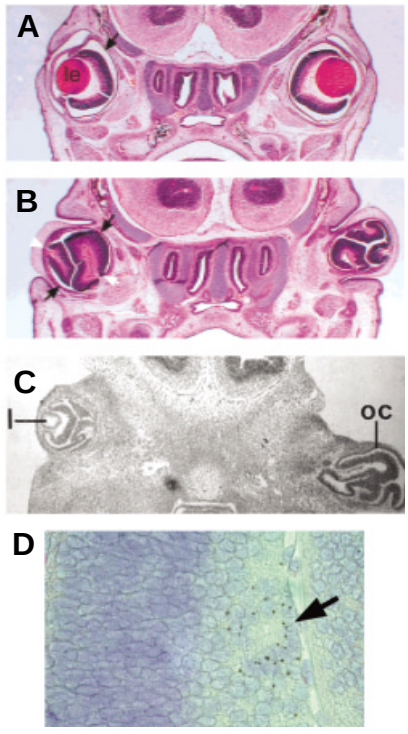

Fig. 7. Comparison of eye phenotypes produced in conditional knockouts and chimeras. *(A, B). Coronal section of head of E14.5 control embryo* **(A)** *contrasts with the absence of a lens and presence of multiple convoluted neuroretinae in the* Pax6 *lens-specific conditional knockout* **(B)**. *(A,B. reproduced with permission from Ashery-Padan* et al., *2000).* **(C)**. *Superficially similar section of an E12.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera. The right eye lacks a lens and has multiple convoluted neuroretinae. (Reproduced from Quinn* et al., *1996).* **(D)**. Pax6$^{-/-}$, Tg^{+} *cells in the prospective ganglion cell layer of an E16.5* Pax6$^{+/+}$ ↔ Pax6$^{-/-}$ *chimera (arrow). (Reproduced from Collinson* et al., *2003). Key: l, lens; le, lens; oc, optic cup.*

Marquardt *et al.* (2001) used the *Pax6* α-regulatory element to drive *Cre* expression in the distal neuroretina from E10.5. Retinal precursors normally express *Pax6* and are pluripotent, capable of differentiating into 6 neuronal cell types, and Müller glia, in the mature retina. *Pax6*$^{-/-}$ retinal precursors in the conditional knockouts expressed markers characteristic of amacrine cells only. In contrast, *Pax6*$^{-/-}$ cells in the retina of *Pax6*$^{+/+}$ ↔ *Pax6*$^{-/-}$ chimeras at E16.5 (Collinson *et al.*, 2003) were almost entirely confined to the prospective ganglion cell layer (Fig. 7D). This apparent discrepancy is possibly due to the different timing of Pax6 inactivation in

the chimeras and conditional knockouts. *Pax6* was inactivated at E10.5 by Marquardt *et al.* (2001), by which time the distal optic cup has been specified as neural retina; its subsequent differentiation reflects the fate of retinal precursors with or without Pax6. In the chimeras, the Pax6-negative retinal cells have never expressed *Pax6*, and the *Pax6* $^{-/-}$ cells in the chimeric ganglion layer may represent an earlier 'pre-retinal' stage of optic vesicle differentiation, expressing different cell surface markers from the amacrine cells in the conditional knockout.

In comparison to chimeras, Cre-*loxP* conditional knockouts are more reproducible and, for some purposes, may provide 'cleaner' experiments in that they can produce *in vivo* tissue combinations where all the cells of one tissue are mutant from particular stages of development, while surrounding tissues remain wild-type. Generally, the rate-limiting step for Cre-*loxP* investigations has been the availability of reliable promoters that drive *Cre* expression in the required temporal and tissue-specific patterns. In the eye, promoter dissection of important genes such as *Pax6* and crystallins allows the cloning of regulatory elements to drive *Cre* expression. Nevertheless, until promoter elements are cloned to cover all ocular cell types, the production of chimeras may remain the best way of getting a mixture of mutant cells into some ocular tissues.

Intimate mixing of wild-type and mutant cells *within a single chimeric tissue* provides information about cell-cell signalling and the autonomous cellular consequences of gene mutation *in vivo* that is not normally achieved in Cre-*loxP* conditional knock-outs. For example, in the *Pax6* $^{+/+}$ ↔ *Pax6* $^{-/-}$ chimeras produced by Quinn *et al.* (1996), lenses (derived entirely from wild-type cells) developed in chimeras that were less than ~50 % mutant cells, but not in those that were primarily composed of mutant cells. This suggested some sort of lens community effect or that a minimum essential 'cohort' of wild-type facial epithelium is required to maintain normal lens development. Mosaic patterns of gene inactivation within a single tissue *are* possible with Cre-*loxP*, e.g. by use of a weak promoter to drive *Cre* expression. A tamoxifen-inducible system for *Cre* expression has been demonstrated, which may also produce mosaic patterns of floxed gene inactivation in a single tissue (Hayashi and McMahon, 2002). Although we have yet to see widespread application of the tamoxifen-inducible system for this purpose, we believe it may combine the versatility and elegance of chimeric systems with the controllability and reproducibility of Cre-*loxP*.

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