

Making Eyes – Lessons from Failed Miracles

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Preamble

Pick up any newspaper and there will be frequent reports detailing the identification of yet another human disease gene. What is the significance of such discoveries? How can we use the information to learn more about the mechanisms of disease and about possible paths to improved disease management and therapy? Our work on human eye malformations illustrates these principles.

The eye is the ultimate precision instrument. It needs to be “built” very precisely in order to fulfil its function. Charles Darwin described the eye as an ‘organ of extreme perfection’. When developing his theory of evolution, Darwin felt that the eye provided a strict test for the theory, which stipulates that an organ cannot pass through a stage where function is lost, as essential components disappear when selection is relaxed – the “use it or lose it” concept. Eyes exist in several fundamentally different designs in different animal groups; they are organised so differently that they must have evolved independently multiple times. And yet, as we shall see, a core-set of highly conserved interacting genes regulates eye development in all classes of creatures across evolution from flies to man, suggesting the re-use of common underlying components.

We have identified three major genes with key roles in eye development and function. All three work as DNA-binding transcription factors that regulate the expression of many other genes in eye, brain and some other sites. Subsequently we determined how different types of mutations cause abnormalities and were able to deduce some of the mechanisms through which such regulator genes control the complex processes of development. Each gene was found to fulfil multiple tasks in eye and brain development, and they interact with each other and additional eye and brain genes in different combinations, generating complex networks that ensure the tight regulation required for robust error-free development. Detailed analysis of how normal functions go wrong in humans and animals with known mutation-driven eye malformations has provided strong insight into the finely-tuned mechanisms of normal development and maintenance, as well as into the etiology of disease. Using animal models, we have also explored how the final outcome, the “phenotype”, can be influenced by environmental factors.

A brief introduction to eye development and organisation

The vertebrate eye starts to develop as an evagination of the brain, even before the neural tube is closed. The evagination balloons out and the curved surface of the neuronal precursor hemisphere touches the surface ectoderm, the outer layer of the body from which skin and also the lens and cornea will develop. The contact triggers an invagination of both the neural hemisphere and the surface ectoderm, so that a double-layered retina is formed and the lens pinches off, while the surface ectoderm reseals and eventually develops into the cornea. The double retina forms the outer pigmented retina and the inner layer becomes the soon-to-be-stratified neural retina. Photoreceptors develop on the outer curve of the neural retina, adjacent to the pigmented retina. The neural connections from the photoreceptors make connections with the visual cortex in the brain so that an exact map of retina is produced. It is not surprising that this complex developmental process occasionally goes wrong, often as a result of altered gene function.

Identification of disease genes.

Genetic eye disease is relatively frequent and often familial, partly because human societies look after people with visual impairment and they survive and reproduce relatively well. From the early 1980s efforts were made to identify genes responsible for inherited diseases. Genes were mapped using a variety of approaches to identify their chromosomal location and then candidate genes in the region were tested to assess whether they carried disease-causing mutations. One way to locate disease genes most easily then and even now, is by identifying cases where some demonstrable chromosomal rearrangement is associated with the disease of interest. The first developmental abnormality of the eye we studied was aniridia (absence of the iris) which had been previously associated with chromosomal deletions that caused two unrelated diseases because

the two disease genes were co-deleted by a single event, suggesting that the genes mapped close together. Although such deletions arise very rarely, they are highly recognisable because of the co-occurrence of two rare diseases. At that time, well before the Human Genome Project had properly begun, the search for candidate genes in a chromosomal region was a painstakingly slow process; but following extensive work internationally to map the deletions, a DNA-binding transcription factor gene called PAX6 was suggested as the candidate gene by colleagues in Texas. We set about helping them to prove that this was the gene. The process of identifying mutations in a gene was much harder in those days, but we succeeded, using both a mouse model for aniridia and collected human patient DNA.

More than a decade later, using similar chromosomal deletion approaches we identified two other transcription factors, each of which was shown to be mutated in a proportion of rare anophthalmia (no eye globe) and microphthalmia (small eye) cases. The two genes identified are SOX2 and OTX2.

The nature of the genes

All three genes are tissue-specific DNA-binding proteins, expressed in the developing eye and brain and some other tissues. Each one regulates the expression of multiple target genes important for development. The pattern of expression changes as development progresses. Each of these genes fulfils multiple roles during eye and brain development, in some other tissues too and also in adulthood.

Transcription factors (TFs) bind to chromosomal DNA at the required target sites. Access to the delicate DNA thread is modulated by chromatin conformation resulting from DNA interactions with support proteins such as histones. DNA transcription into RNA is facilitated by an open chromatin conformation. Switched off genes reside in regions of tightly-packed closed chromatin. TFs may work by turning target genes on or off. Frequently a single TF can function as an enhancer or repressor of gene expression under different circumstances. TFs work in concert with others of their kind, including some that are required for all gene expression (general transcription factors) while many, including our three eye genes, are tissue-specific regulators.

The role of PAX6, SOX2 and OTX2 in development and disease

These three transcription factors cause developmental malformations that affect the whole complex structure of the eye – the diseases associated with mutations at these three gene loci are panocular. Other TFs are associated with many different eye diseases, including retinal degenerations, glaucoma, corneal disease and cataracts.

Analysis of the expression pattern of each gene during different times in development – the spatiotemporal expression pattern – can be very informative about the nature of the phenotypes to be assessed. For these expression studies model organisms are generally used, and the mouse is highly favoured, because it is such an amenable, manipulable model. However, significant contributions are also made using zebrafish, as well as invertebrate models such as the fruitfly *Drosophila* and the nematode worm *Caenorhabditis elegans*. This is particularly true for PAX6 which is very highly conserved in terms of both function and amino acid sequence. Indeed, Pax6 mutations are known in all of the mentioned organisms and these mutations are associated most notably with the eye phenotype, or other sensory system abnormalities in the worm, which has no eyes. Careful examination of the mouse “Small eye” heterozygotes, and the neonatally lethal homozygotes, reveals that the heterozygote is a good model for human aniridia, while the homozygotes with no eyes have severe brain and olfactory system abnormalities, which lead to death within a short time of birth. Interestingly, heterozygous mice can be shown to have mild brain abnormalities as well as the aniridia-like eye phenotype.

Spurred on by knowledge of the expression pattern, and by the severe homozygous mouse phenotype, a selected group of long-term aniridia patients (all over 16 years old) was asked to participate in a research project to study their brain structure by MRI (magnetic resonance imaging), a non-invasive powerful analytical method for imaging brain structure. To everyone's surprise, a high proportion of the aniridia patients were found to have absent or hypoplastic anterior commissure, one of the key connections between the two hemispheres. Other abnormalities observed frequently include olfactory system deficits even to complete anosmia, but

this had been heralded by observations of considerably reduced olfactory bulb size in mice. Another surprise was the high frequency of cases with absence of the pineal, although no particular sleep pattern problems have been described. Finally one case with some hearing problems was seen by an audiologist, who found that each ear functions normally, but there is a problem with information transfer between the two hemispheres. Subsequently, a number of other adults and also some children were tested. Many were found to have similar auditory transfer deficits, though the children generally did not show absence of the anterior commissure, raising the possibility that the absence of the commissure is a progressive feature of aniridia.

What the details of the mutations tell us

Although classical aniridia, with absence of the iris, is generally caused by so-called null mutations where the most likely situation is that there is a reduction in protein levels because one copy of the gene does not produce protein, we do find a number of mutations which give rise to altered protein from the mutated copy. These cases, generally with a single amino acid change (missense mutation) often have a variant phenotype, sometimes milder and sometimes more severe than the classical case. There is a general trend to specific amino acid mutations to be associated with particular phenotypes. The most severe missense mutations are actually associated with microphthalmia which is indistinguishable superficially from phenotypes that are caused by mutations at the other two loci. SOX2 mutations are frequently found in the most severe cases with bilateral anophthalmia. All the mutations, which are mostly loss of function in one copy, seem to arise anew in the germ cells of one of the parents. Until very recently no vertical inheritance of SOX2 mutations had been observed. This suggests a highly penetrant dosage sensitivity for this gene. The anophthalmia is frequently associated with brain anomalies, developmental delay and seizures. Occasionally other associated abnormalities are also seen, such as tracheo-oesophageal joining – SOX2 is also expressed in the relevant epithelial cells, so this associated anomaly “makes sense”, although the variable occurrence is not understood. OTX2 null mutations also give rise to anophthalmia and microphthalmia which are not readily distinguished from the SOX2 phenotype. However, in this case we have seen a number of completely unaffected mutation carrier parents. There have also been differences in phenotype severity within a family. Anophthalmia and microphthalmia can be unilateral, particularly with SOX2 mutations. Recently a family with an inherited SOX2 missense change was reported with variable phenotypes some of which overlapped with PAX6-associated iris coloboma. We shall discuss the possible reasons for the phenotypic variability and overlaps observed with these genes.

Long-range regulation of gene expression

We were alerted to the existence of important distant regulatory elements outside the coding region of developmental transcription factor genes like PAX6 by several cases of classical aniridia where gene disruption arose by chromosomal breakpoints outside the gene. We showed that in the mouse Small eye model a stop codon mutation within the gene could only be corrected using a large genomic piece of DNA which included the intact PAX6 gene and extensive flanking sequence on either side of the gene. This led us to begin to explore the complex regulatory system of genes like PAX6 that fulfil multiple distinct roles in time and space, during development and even in adulthood. Once genomic sequences became available, it emerged very quickly that the regulatory functions are associated with highly conserved genomic elements upstream, downstream and within the introns of these genes. PAX6 has most of its regulatory region in the downstream region (relative to the direction of transcription). Surprisingly, all the downstream elements identified so far reside within the introns of a neighbouring gene called ELP4, which is apparently not affected by these elements, as it is ubiquitously expressed unlike PAX6 with its strict expression pattern. To assess the functional capacity of these conserved elements, we have used a system known as reporter transgenesis in mice and to some extent in zebrafish. We have shown for PAX6 that the predicted regulatory elements behave as enhancers, showing tissue-specific expression of the reporter gene in a pattern that is a sub-set of the total PAX6 pattern. Each regulatory element typically drives expression in more than one tissue and the pattern changes with developmental timing. Generally each PAX6-expressing tissue is regulated by several elements – for example the brain expression is controlled by a large number of enhancers which behave in a hierarchical manner in some instances. As a result of an ancestral genome duplication, zebrafish has two different copies of the pax6 gene, pax6a and pax6b. They have an

overlapping but distinct expression pattern. *pax6a* is more widely expressed in brain, and *pax6b* has taken on the role of pancreas control; both are expressed in the eye. We were interested to find that evolutionary changes in the regulatory elements can be linked to the changes in expression pattern.

It is now considered that changes in gene regulation are a major mechanism for evolutionary change. Not surprisingly, it is also very likely that regulatory element variation is involved in many disease associated mutations. Some of these variants are likely to be implicated in the more subtle genetic predispositions to later onset common diseases. Genome-wide association studies place about half of all the recently identified disease-associated variants in regulatory regions. One of the continuing mysteries is how all the different regulatory elements work together to bring about the complex control of individual genes and their fine coordination with other genes.

Enhancer function and transcription factor networks

It is clear that enhancers fulfil their role under the direction of transcription factors that bind to them. There is plenty of room for a large number of transcription factors to bind to each predicted, sequence conserved, regulatory element. It is therefore not surprising that these enhancers are controlled by multiple, often interacting transcription factors. Thus it emerged that SOX2 and PAX6 interact at the protein level by co-binding to neighbouring sites in more than one target site, including in controlling the expression of a lens crystallin protein and also in an auto- and cross-regulatory loop modulating SOX2 expression (and also PAX6 expression – target element not yet clearly identified). The actual sequences binding SOX2 and PAX6 at the two known targets are very different probably because the exact affinity for the complex varies from tissue to tissue where the expression levels of the two transcription factors is probably critical and very finely tuned. This is just one example of interaction between two or more transcription factors working in the same developmental pathways. It is becoming clear that transcription factors participate within complex finely tuned networks which have nodes and some hierarchical characteristics. Network architecture, which is probably continuously evolving, is a very important determinant of developmental robustness. It is not surprising that developmental abnormalities are very often caused by dosage-altering transcription factor mutations.

In order to expand our knowledge of transcription factor networks, we have used bioinformatic approaches to predicting novel PAX6 (and SOX2) targets, using a few already defined binding site sequences in a method termed Hidden Markov Modelling. We are now in the process of validating the predicted targets using zebrafish as a model, since the predicted targets are by definition conserved between mammals and fish.

Phenotype modulation in health and disease

We have remarked on the significant phenotypic variation that can be associated with the same mutation between families, within families where we sometimes even see non-penetrance of disease in mutation carriers, and even within a single individual (eg unilaterality in eye disease, kidney disease, deafness etc). We wanted to explore the mechanisms that might underlie such variation. An important pointer was work in the fruitfly *Drosophila*, where it was shown in 1998 that cryptic mutations could be uncovered if the function of the chaperone system based around HSP90 (heat shock protein 90) was perturbed. HSP90 has multiple roles in facilitating protein folding for newly produced proteins, maintaining structure for metastable proteins and helping denatured proteins to refold (or chaperoning them to their destruction). We decided to see whether the same system is also at work in more complex vertebrates and used zebrafish for this purpose. We showed that we were able to modulate eye phenotype in two zebrafish mutants, both of which turned out to be caused by missense mutations. We were also able to uncover repeatedly rare microphthalmia and anophthalmia cases in one particular strain of zebrafish, initially at low frequency, but this was increased when we inbred selected predisposed parents.

To pursue the molecular mechanisms further, we set out to identify novel interacting proteins that associate with HSP90. Interactors which turned out to belong to a family of proteins now known to be involved in chromatin modification were identified. Pursuing the function of one of these further, we have very recently found that these proteins fulfil multiple roles, by showing a presence in the cytoplasm as well as the nucleus, which is expected for a chromatin modifier. It turns out from looking further at its interactors that our protein associates with intraflagellar transport proteins and

can now be identified by immunohistochemistry in primary cilia and in known ciliated tissues, such as the zebrafish lateral line structures. This is very exciting since ciliary abnormalities are implicated in many diseases with variable phenotypes, including obesity and diabetes, kidney anomalies and developmental heart defects. It is most exciting to be able to associate the ciliary functions of environmental sensing with chromatin modification which would lead to changes in the regulation of gene expression.

Epilogue

It has been, and continues to be, an exciting journey from the study of human malformations to begin to unravel some of the deeper mysteries of biology and gene regulation and hopefully also to open up some possible avenues for improvements in disease management and phenotype modulation.