

The Chick as a Model for Retina Development and Regeneration

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“Our real teacher has been and still is the embryo, who is, incidentally, the only teacher who is always right.” Viktor Hamburger

THE CHICK EMBRYO AS A MODEL SYSTEM

Introduction

The ancient Egyptians and Greeks were history's first recorded embryologists, both of which used the chick as a model system to understand how human development occurred. In 343 BC (historians' best estimation), Aristotle studied the chick embryo as a means to discover secrets of the formation of life. In Book II of

Generation of Animals, Aristotle highlights the chick as the greatest model with which to study eye development (Aristotle, 343 BC), and later, in Book VI, he gives a gross anatomical description of the entire developmental process of the chick embryo (Aristotle, 343; 350 BC). Understandably, Aristotle takes note of one of the most noticeable and prominent features of the chick embryo – the eyes, telling the readers that the eyes are “swollen out to a great extent” and that “this condition of the eyes lasts on for a good while” (Aristotle, 343 BC). On the 10th day of development, he said, “the head is still larger than the rest of its body, and the eyes larger than the head, but still devoid of vision. The eyes, if removed about this time, are found to be larger than beans, and black; if the cuticle be peeled off there is a white and cold liquid inside, quite glittering in the sunlight” (Aristotle, 350 BC).

As vague as Aristotle's descriptions were, no observations of the chick eye surpassed those of the great teacher and philosopher until almost 2,000 years later. All of the gross anatomical features of eye development were first discovered in the chick, including two hallmarks of eye development; the choroid fissure which was first described by Marcello Malpighi in 1672, and much later, the evagination of the optic vesicle from the neural tube which was described by Christian Pander in 1817 (Adelmann, 1966). Descriptions of the embryonic retina were first recorded by Antoine Maitre-Jan in 1722, who said at the 9th day of development, it "is white and has the consistency of a coagulum" (Adelmann, 1966).

These and many other investigators using the chick as a model system paved the way for the late 19th and 20th century research to bring basic research to where it is today. Within this chapter, the reader will find a discussion of the advantages of the chick embryo as a model system for eye research, both in development and regeneration research. There is also a discussion of the techniques that have been used extensively with the chick embryo in the past, as well as new advances that will propel the use of the chick in eye research far into the future.

The Advantages of the Chick Embryo

Chick embryos are wonderful to work with in a variety of aspects. The following are general points that make chick embryos such a useful model system. (1) The eggs are a cheap and readily available source of material that is available year-round from a local or regional supplier. In the day and age of the transgenic mouse, it has become an issue to find systems that can be used, either as alternative vertebrate models or models to be used in conjunction with more expensive model systems, to define the functions of various genes. In comparison to the mouse, the chick is very inexpensive and has very little cost associated with housing. This has led to studies using the chick embryo as a high-throughput tool in which genes and reporter constructs driven by untranslated genomic sequences are introduced into the embryo as an initial determination of gene function, necessary cis-acting regions, etc. (Timmer *et al.*, 2001; Nakamura *et al.*, 2004; Uchikawa *et al.*, 2004). (2) Chicks undergo a series of successive and reproducible changes during development that have been well documented by several embryologists, most notably Malpighi, Lillie, Huxley, and Hamburger and Hamilton (Malpighi, 1672; 1675; Lillie, 1908; Huxley, 1934; Hamburger and Hamilton, 1951). This is a critical issue

primarily because investigators would like to be able to manipulate embryos at specific stages, so a time scale of approximately when embryos incubated at a specific temperature will become a particular stage is necessary. In addition, a large number of eggs can be incubated at one time in order to obtain embryos that are at the desired stage. (3) *In ovo* embryonic studies are more easily accomplished than *in vivo* mammalian embryonic studies. For instance, experiments in which dividing cells are labeled in the chick embryo do not have to deal with the label, tritiated thymidine or bromodeoxyuridine (BrdU), being diluted by the maternal vascular system. (4) For many tissues, including the eye, the tissue is easily accessible for various manipulations. Using some very cheap and readily available instruments, windows in the eggs can be opened, revealing the embryo and creating space for the insertion of instruments for surgical manipulations, etc. (5) Many experimental methods have been well established to study the chick eye, including retinal, lens and retinal pigmented epithelial (RPE) cultures, retinal wholemount *in situ* hybridization and immunohistology, *in ovo* electroporation, and expression of genes via retroviral infection (Belecky-Adams *et al.*, 1996; 1997; 1999; 2001; 2002; Weng *et al.*, 1998; Adler *et al.*, 2001; 2002; Sehgal *et al.*, 2006; Wilson *et al.*, 2007). There will be a discussion of some of these techniques later in the "Toolbox" section of this chapter. (6) The period over which the eye develops is relatively short and occurs entirely within the embryonic period of development. The short period over which the retina develops is a significant advantage when considering functional studies with genes of interest. In addition, it is also an advantage that the majority of differentiation within the retina occurs embryonically (Fujita and Horii, 1963; Prada *et al.*, 1991), hence investigators do not have the added stress that birth places on the developing systems to complicate analysis. (7) Chick embryonic eyes are enormous! (Fig. 8.1(A) and (B)). This can be a substantial advantage when considering techniques such as single cell or explant cultures, due to the availability of large amounts of tissue. (8) The chicken genome is available (Wallis *et al.*, 2004) and methods for making the chick embryo more accessible to genetic manipulations are being quickly developed. This may be of interest to investigators for a variety of reasons, including comparative analyses of various homologs or orthologs in other species, the study of gene organization and regulation, and the study of the evolution of genes, gene families, and signaling pathways. (9) The retina can regenerate during early development (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989; 1991; Spence *et al.*, 2004; 2007a,b) (Fig. 8.2). This is a substantial advantage if one wishes to study how the nervous

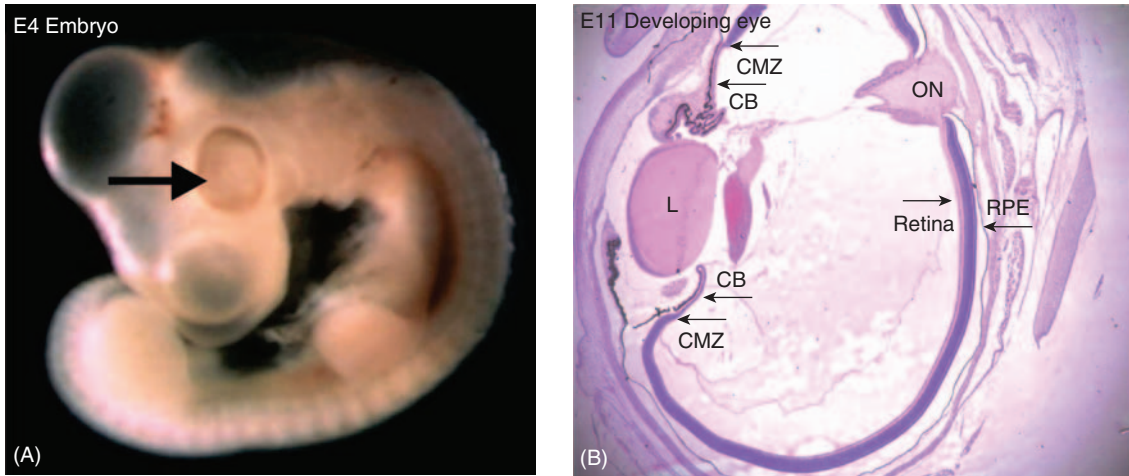


FIGURE 8.1 (A) A photograph of a chick embryo taken at E4 showing the location of the large developing eye. (B) A cross section of a developing eye at E11 showing the location of the retina, retinal pigmented epithelium (RPE), ciliary marginal zone (CMZ), ciliary body (CB), lens, (L), and the optic nerve (ON).

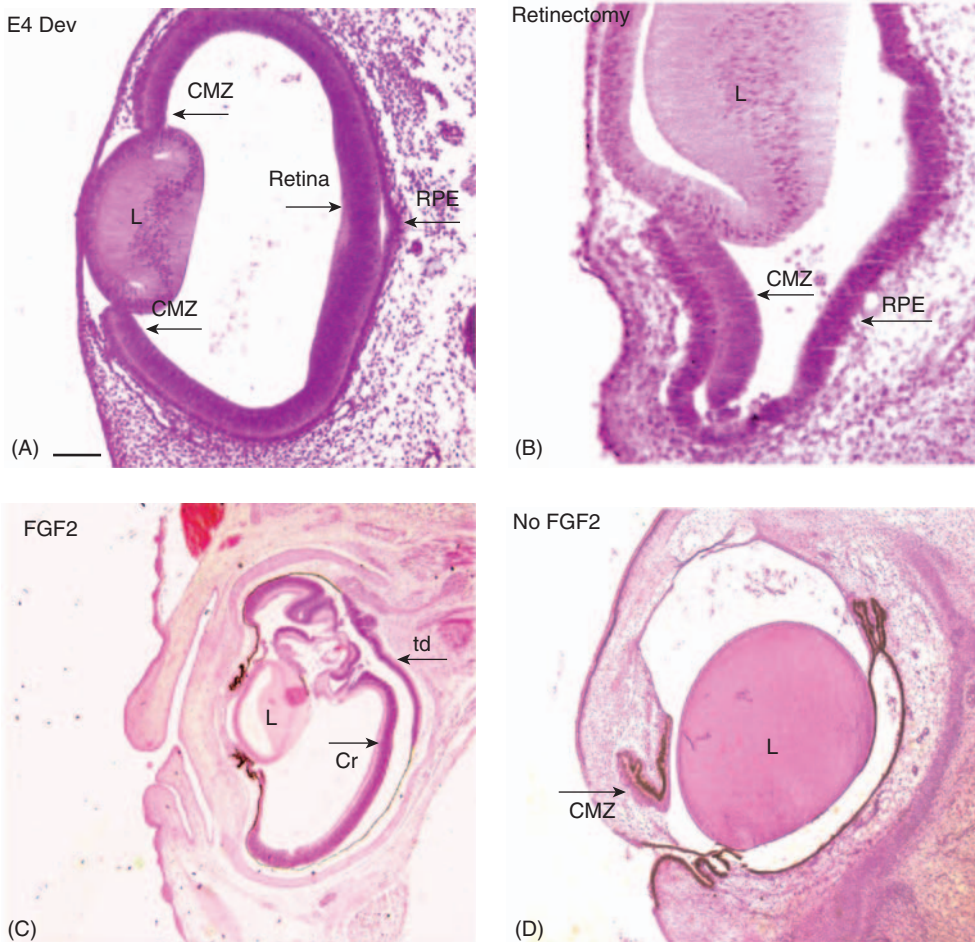


FIGURE 8.2 (A) A cross section of a developing eye at E4 showing the location of the ciliary marginal zone (CMZ), lens (L), retina, and retinal pigmented epithelium (RPE). (B) Cross section of the chick eye after retinectomy at E4. The CMZ is not removed and the RPE is thickened but not yet pigmented. (C) Cross section of retina regenerated in the presence of FGF2 at 7 days post-retinectomy (E11). Retina is regenerated from the retinal stem/progenitor cells present in the ciliary margin (cr) and transdifferentiation of the RPE (td). (D) Cross section of the chick eye 3 days post-retinectomy showing the lack of regeneration in the absence of FGF2.

system regenerates and/or compare early timepoints, when regeneration is possible with timepoints when it is not possible. Regeneration of the chick retina will be discussed in more detail in the second half of this chapter.

The Embryonic Chick Toolbox

Surgical Manipulations

The chick system has a long and venerable history using ablations, rotations, and auto-, allo- and xeno-transplantations. These surgical manipulations in the chick embryo have led to some of developmental biology's most important findings concerning induction of various tissues, fate mapping, patterning, axonal pathfinding, cell lineage, and differentiation. There have been several recent articles concerning the use of the chick in developmental biology that hit on many of these manipulations, so we will not repeat what was discussed in these articles (Stern, 2005). Rather, we will focus primarily on examples of procedures used in the visual system of the chick.

Naturally, the accessibility of the chick embryo has led to elegant analyses using chimeras of labeled chick cells transplanted back into host chickens or chimeras composed of quail and chick. Several of these studies have been directed at determining the fate maps of cells that give rise to the eye or parts of the eye (Hyer *et al.*, 1998; 2003). A large body of work using surgical manipulations has centered on the role of various tissues in patterning of the eye. For instance, removal of the lens ectoderm has shown the importance of the ectoderm in retinal differentiation and showed that the presence of the lens ectoderm is necessary for the morphological development of the optic cup (Fernandez-Garre *et al.*, 2002). Importantly, this same study has established that the lens *ectoderm* is necessary at a certain stage for development of the optic cup, however, the presence of the lens following its invagination into the optic cup appears not to be necessary for the survival and development of the optic cup (Fernandez-Garre *et al.*, 2002). To determine when dorso-ventral eye polarity is established, Araki and colleagues utilized rotations of optic cup explants, using the choroid fissure as a marker of polarity (Uemonsa *et al.*, 2002). Ablations and rotations and quail-chick chimeras of various portions of the optic vesicle have helped to determine when the naso-temporal development of the retina is specified (Dutting *et al.*, 1995a,b; Thanos *et al.*, 1996; Mueller *et al.*, 1998). Transplantation and rotation of the lens, has been used to show that the size and polarity of the lens can be changed *in vivo* (Coulombre and Coulombre, 1969). Using similar techniques, polarity of the chick tectum

and the role of the tectum in retinal differentiation and apoptosis has been defined (Cohen *et al.*, 1989; Ichijo *et al.*, 1990; Itasaki *et al.*, 1991; de Curtis *et al.*, 1993; Le Douarin, 1993; Nakamura *et al.*, 1994; Yamagata *et al.*, 1995; Cook *et al.*, 1998; Borsello *et al.*, 2002). Finally, there have also been several studies in which the interaction between the developing cornea and lens has been documented (Zinn, 1970; Lwigale *et al.*, 2007).

Bead Implantation

As investigators began overexpression/misexpression studies, the use of the chick as a model system was stymied for a short period of time because the cells of the chick were too small to inject DNA or mRNA (Stern, 2005). This led to the use of either grafts of transfected cells or insertion of inert beads to deliver factors to a given tissue. Acrylic, ethylene/vinyl acetate copolymer or agarose beads were used that had high affinity for many different molecules, and could slowly release the bound factors. Beads have been used to deliver a variety of growth factors to the developing eyefield and/or eye. One of the best known studies of this type is one in which the phenomenon of RPE transdifferentiation into retina was described by Park and Hollenberg (1989). Following removal of the retina, RPE treated with beads soaked in fibroblast growth factor (FGF) can generate a new retina (Park and Hollenberg, 1989; 1991; Spence *et al.*, 2004; 2007b). Beads have also been used to deliver growth factors in a number of studies to the developing forebrain and eye cup to affect eyefield and/or optic cup development (Ohkubo *et al.*, 2002). Further, beads can be used to deliver other substances to the developing eye, such as function-blocking antibodies or inhibitors of signaling pathways (Martinez-Morales *et al.*, 2005; Spence *et al.*, 2007a). Also explant cultures have been developed to allow optic vesicles to be exposed to growth factors (Trousse *et al.*, 2001).

Chemical Genetics

Chemical genetics is defined as the use of small molecules to affect biological events (Yeh *et al.*, 2003). This section includes examples of chemicals that have been used *in ovo* to specifically stimulate or inhibit various signaling pathways. The strength of the chick system here is that various reagents can be added dropwise to the egg, injected intravenously for systemic uptake, or injected into the optic cup at various stages. Further, multiple additions or injections over time can be easily done. While the list of chemicals that may be added is endless, we would like to consider molecules

that interfere with two signaling pathways: the sonic hedgehog (Shh) and FGF pathways.

Cyclopamine, a chemical originally identified as a teratogen, inhibits Shh signaling. Exposure of embryos to this chemical results in cyclopia stemming from the improper patterning in the ventral forebrain (Coventry *et al.*, 1998). Recent studies have compared the effects of cyclopamine to a cholesterol synthesis inhibitor *in ovo* to show that the mechanism of action within the forebrain was due to the direct antagonism of the Shh pathway rather than effects on cholesterol linkage of the Shh molecule (Incardona *et al.*, 1998). In a separate study, cyclopamine was injected directly into the developing eye cup to show the effects of decreased Shh signaling on axonal pathfinding within the retina (Kolpak *et al.*, 2005). On the other hand, SU5402 is a member of a family of FGF signaling inhibitors that bind specifically to the active sites of FGFR kinase domains (Mohammadi *et al.*, 1997). SU5402 has been used *in ovo* to block ganglion cell differentiation and lens fiber elongation (McCabe *et al.*, 1999; Huang *et al.*, 2003). Another more potent FGFR inhibitor PD173074 has been used to dissect the effects of FGF signaling during chick retina regeneration (Spence *et al.*, 2004; 2007a,b). These small molecules and many others have an enormous range of possibilities and combinations that can be tested.

Embryonic Cultures, Explants, Single Cell, Recombined Tissue

Culture systems are widely used to determine the effect of growth factors, toxins, inhibitors, and any other substance in different types of cells or tissues, when a certain amount of precision is required to ensure that all the cells are treated with a specific concentration of the given factor. Several types of chick culture systems have been used to tease out mechanisms of differentiation in the retina, namely eye cup cultures, explants, dispersed cell culture (low and high density), reaggregation of dispersed cells, and immortalized cell lines. We would like to consider three of the most common types of questions that have been addressed using chick retinal, RPE, lens, and corneal cultures and give a few examples of each from the literature. (1) How does one cell type or tissue affect the differentiation/development of another? In the first example of this type of study, Fuhrmann *et al.* (2000) showed that a signal from extraocular mesenchyme upregulated RPE markers and downregulated retinal markers in optic cup cultures. In a second example, the innervation of the developing cornea by the trigeminal nerve was shown to be dependent on the expression of semaphorin A in the adjacent lens

epithelium (Lwigale *et al.*, 2007). (2) How do cells from the same tissue influence one another during development? This question has been addressed using the various culture techniques listed above in a variety of permutations. For instance, low density cultures have been used to show that the stage at which retinal progenitor cells are cultured, determines the type of retinal cell they will form *in vitro*, demonstrating the importance of the *in vivo* environment in dictating cell fate (Adler *et al.*, 1989; Repka *et al.*, 1992a,b; Belecky-Adams *et al.*, 1996). Other studies have used heterochronic cultures to investigate the effects of earlier born cells on progenitor cell differentiation (Waid *et al.*, 1998), and the importance of cell-cell communication in retinal differentiation (Austin *et al.*, 1995). Finally, work from Layer and colleagues has explored the possibility of reconstituting the laminar formation and differentiation of the retina using reaggregation cultures of dispersed retinal cells (Rothermel *et al.*, 1997; 2006). (3) How does treatment of cells at various stages of development with growth factors affect development of retinal progenitors? There are an enormous range of growth factors that have been used in cultures of chick retina, including FGFs, BMPs, activins, CNTF, Shh, and NGF to mention only a few (Pittack *et al.*, 1991; Fuhrmann *et al.*, 1995; Matsuo *et al.*, 1997; Belecky-Adams *et al.*, 1999; Frade, 2000; Cirillo *et al.*, 2001; Le *et al.*, 2001; Zhang *et al.*, 2001; Belecky-Adams *et al.*, 2002; Nakagawa *et al.*, 2003; Kolpak *et al.*, 2005; Sehgal *et al.*, 2006).

DNA Transfer

In this section, we will consider several techniques that enable the investigator to introduce DNA, in the form of expression vectors, RNA interfering molecules (including morpholinos, siRNA, dsRNA, and shRNA) and reporter constructs to test cis-acting sequences in non-coding regions of the genome. Several techniques will be included in this section, including retroviral transfer, electroporation, and transfection using lipid-based reagents.

The chicken-specific replication competent retrovirus (RCAS) has been used for misexpression of genes in the chick since the late 1980s, and was the first technical revolution that allowed the introduction of exogenous genes into chick cells *in ovo* (Hughes *et al.*, 1984a,b; Morgan *et al.*, 1992; Riddle *et al.*, 1993). This retrovirus is derived from the SR-A strain of the Rous sarcoma (src) virus, and was made by deleting sequences that encode the src gene. Deletion of this portion of the viral genome allows insertions of genes of interest at this site (Hughes *et al.*, 1984a). It has become so commonly used in the chick

system that it has its own website (<http://www.retrovirus.info/RCAS>), run by one of the originators of the RCAS retrovirus, Stephen Hughes (Hughes *et al.*, 1984a,b). Since its arrival on the scene, there have been various modifications to the virus that allow it to be used in different ways. For instance, adaptor plasmids were made to aid in the insertion of exogenous genes into the RCAS retrovirus, mutations have been made in the genes that encode envelope proteins that allow investigators to target host range, other deletions have been made in viral genes to allow larger insertions, mutations to the long terminal repeat (LTR) enhancer allow the inserted gene to be expressed at different levels, and tetracycline inducible elements have been added to the RCAS A retrovirus so that expression of genes inserted into the retrovirus can be induced (Hughes *et al.*, 1987; Greenhouse *et al.*, 1988; Sato *et al.*, 2002). The RCAS system has been used by many to advance our understanding of the visual system, for example, the retrovirus has been used to study patterning (Nakamoto *et al.*, 1996; Schulte *et al.*, 1999; Sakuta *et al.*, 2001; Adler *et al.*, 2002; Kim *et al.*, 2006), mitosis (Crisanti *et al.*, 2001), axonal pathfinding (Kolpak *et al.*, 2005), differentiation (Blancher *et al.*, 1996; Jiang *et al.*, 1998; Ogino *et al.*, 1998; Yan *et al.*, 2000a; Li *et al.*, 2001; Liu *et al.*, 2001; Yan *et al.*, 2001; Esteve *et al.*, 2003; Canger *et al.*, 2004; Cho *et al.*, 2006; Moreira *et al.*, 2006), survival (Pimentel *et al.*, 2000), and regeneration (Spence *et al.*, 2004; Spence *et al.*, 2007a,b; Haynes *et al.*, 2007).

The RCAS retrovirus also has several drawbacks associated with it, including (1) the upper restriction on the size of insertions to the viral coding sequence is about 2Kb, so that it is unlikely that one could introduce more than one gene into the retrovirus, (2) it cannot be used to target post-mitotic cells, (3) there is an increase in the cost and time associated with making a retroviral stock, (4) it takes between 16 and 24h to get expression of the viral proteins *in ovo*, and (5) the investigator must use the substantially more expensive virus-free eggs.

Few technical advances have made the chick system more amenable to the types of studies performed today than electroporation. The basic idea behind electroporation is that an electrical pulse delivered by electrodes placed in the tissue disrupts the cell membranes, allowing DNA to enter cells. The negatively charged DNA will move toward the anode side of the electrode, resulting in transfection of tissue on the side of the anode. There have been a raft of articles discussing *in ovo* electroporation and the best parameters to use to enhance survival and increase transfection efficiency (Muramatsu *et al.*, 1997; Itasaki *et al.*, 1999; Nakamura *et al.*, 2000; Yasuda *et al.*, 2000; Yasugi *et al.*, 2000; Nakamura *et al.*, 2001;

Swartz *et al.*, 2001; Katahira *et al.*, 2003; Chen *et al.*, 2004; Krull, 2004; Nakamura *et al.*, 2004; Uchikawa *et al.*, 2004; Sato *et al.*, 2007). Several investigators have also determined how to introduce various types of interfering molecules into the developing chick using electroporation or viruses, making knock-down experiments feasible (Hu *et al.*, 2002; Katahira *et al.*, 2003; Kos *et al.*, 2003; Pekarik *et al.*, 2003; Chesnutt *et al.*, 2004; Rao *et al.*, 2004; Hernandez *et al.*, 2005; Canto-Soler and Adler, 2006; Harpavat and Cepko, 2006; Watanabe *et al.*, 2007). The use of electroporation has several advantages over the use of viruses to introduce DNA, such as there is no longer a need to clone sequences into the retroviral plasmid, no size restriction on insertions to the expression vector, no need to expend the effort and funds in making a viral stock with which to infect tissues, and no need to purchase the more expensive virus-free eggs. Further, because directionality of the transfection can be controlled somewhat by placement of the electrodes, the electroporation method has more precision over where DNA can be targeted. Introducing DNA via electroporation is not limited to dividing cells, as is the retrovirus, and the expression of plasmids introduced by electroporation is generally detectable within a few hours post-electroporation. One limitation that electroporation does have is that the DNA is not incorporated into the genome; hence its expression is lost over time. A recent advancement in this area is the stable incorporation of genes into the genome through the co-electroporation of a transposon-containing expression vector with a separate expression vector containing a transposase (Sato *et al.*, 2007). This combination led to the persistence of the electroporated green fluorescent protein (GFP) marker. This has also been combined with the tetracycline inducible elements, such that transgenes could be introduced fairly early in development, when accessibility of the embryo is at its highest, and turned on later in development by addition of tetracycline (Sato *et al.*, 2007). Until recently, electroporation had been used in very early embryos, primarily because later embryos turn inside such that the head is no longer visible and the embryo becomes covered with a dense vasculature. Two changes have been made recently to address introduction of genes into older embryos via electroporation. The first is the *ex ovo* electroporation of embryos grown in petri dishes and the second advancement is that of electroporation in hatchlings (Luo *et al.*, 2005; Yamaguchi *et al.*, 2007).

Last, there have been a variety of methods used to transfect cells with lipid-based technology (Iwakiri *et al.*, 2005; Muramatsu *et al.*, 1997; Yasugi *et al.*, 2000; Decastro *et al.*, 2006). The basis of this technique is the ability of liposomes loaded with DNA to fuse with cellular membranes and deliver their cargo to the cytosol.

This method has been used to generate chimeric chick embryos (Fraser *et al.*, 1993), and to transfect a number of tissues (Brazolot *et al.*, 1991; Demeneix *et al.*, 1994; Rosenblum *et al.*, 1995; Decastro *et al.*, 2006). While most early lipid delivery systems were not as efficient as electroporation in the developing embryo (Decastro *et al.*, 2006), enhanced Lipofectamine delivery through the addition of disulfide linked pegylated lipid has lead to a substantial increase in the transient transfection of a variety of tissues, including the neural tube and optic cup.

It is unlikely that these are the last of the advances for delivery of genes and other molecules into the developing and regenerating chick system (Kawakami *et al.*, 2008). One promising technology being developed is that of sonication (Ohta *et al.*, 2003; Fischer *et al.*, 2006). There have been some recent advances in "sonoporation" which make transfections *in vivo* more efficient and more likely to be used in the future (Gvili *et al.*, 2007; Saito *et al.*, 2007).

Disadvantages of the Chick Embryo

There are also some disadvantages of the chick system. Until a few years ago, the biggest disadvantage of the system was the inability to genetically modify

chickens (Stern, 2005). However, this difficulty has been overcome by several groups, and the practice of making transgenic chickens will soon become more standardized and catch up with the powerful techniques currently available for other models such mice, zebrafish, and *Xenopus tropicalis* (Mozdziak *et al.*, 2003; Koo *et al.*, 2004; 2006; Mozdziak and Petite, 2004; 2006; Kwon *et al.*, 2004; Chapman *et al.*, 2005). Another weakness that the chick model has been associated with is the lack of natural mutants and/or a long-term storage facility for such mutants. There are some mutants available, as has been reviewed recently, however, even some of those few are in danger of being lost (Delany, 2004). Coupled with this is the challenge of chemical mutagenesis in chickens. It is unlikely that the chick will ever be able to take advantage of mutagenesis screening that is common in models such as *Drosophila* and zebrafish.

CHICK RETINA REGENERATION

Introduction

As mentioned earlier, one of the great advantages of working with the embryonic chick eye is that the retina

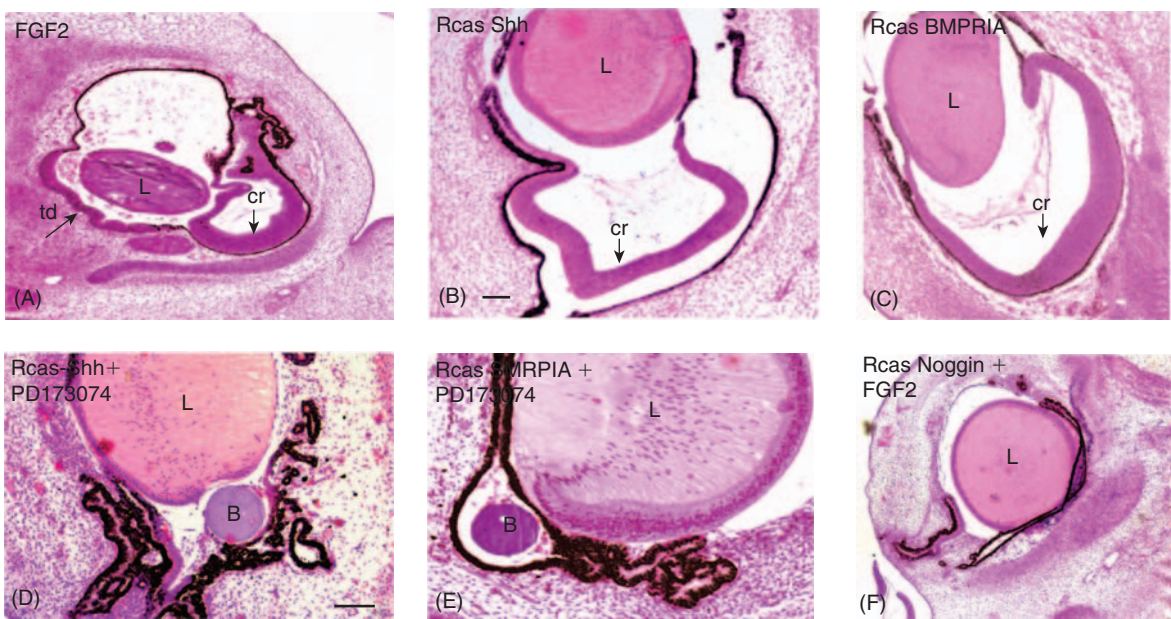


FIGURE 8.3 (A) A cross section from a regenerating eye 3 days post-retinectomy showing regeneration from both the retinal stem/progenitor cells (cr) and transdifferentiation of the RPE (td) in the presence of FGF2. (B and C) A cross section from a regenerating eye 3 days post-retinectomy showing regeneration from the retina stem/progenitor cells (cr) when the Shh (RCAS Shh) (B) or the BMP (RCAS BMPRIA) (C) pathway is constitutively activated. (D–F) A cross section from a regenerating eye at 3 days post-retinectomy showing the lack of regeneration in the presence of RCAS Shh and PD173074, an inhibitor of the FGF pathway (D), RCAS BMPRIA + PD173074 (E), and RCAS noggin, an inhibitor of the BMP pathway, and FGF2 (F).

can be repaired or replaced if damaged or removed. The accessibility to the embryo for microsurgery combined with the availability of molecular tools in the chick has made this a great system to study and dissect the early molecular events that take place during retina regeneration. The chick genome was also recently sequenced (Wallis *et al.*, 2004) and this provides a vast range of possibilities to study the early stages of retina regeneration, including the use of gene array technology to identify critical genes regulated during chick retina regeneration.

The embryonic chick can regenerate its retina via two modes. One requires the activation of stem/progenitor cells present in the ciliary margin, while the other involves the use of the classic process of transdifferentiation (Fig. 8.2). The phenomenon of retina regeneration in the embryonic chick has been observed since the early 1900s, however, it was not until Coulombre and Coulombre (1965) that the process of retina regeneration was described in more detail. Park and Hollenberg (1989, 1991) discovered that in order for any retina regeneration to take place a source of FGF had to be present. Recently, we have shown that other signaling pathways including the hedgehog (Hh) and bone morphogenetic protein (BMP) pathways regulate the process of retina regeneration (Spence *et al.*, 2004, 2007a, b; Haynes *et al.*, 2007). We will discuss the mechanisms by which each mode of retina regeneration is regulated.

Regeneration by Stem/progenitor Cell Activation

Regeneration from the stem/progenitor cells in the ciliary margin requires an induction process whereby the stem/progenitor cells are activated to proliferate and differentiate into the retinal cell types. The stem/progenitor cells in this region are used by the embryo to provide for the continuous growth for the retina, however, there is always a population of cells that remain undifferentiated and will not spontaneously respond to injury. However, after removal of the retina, the stem/progenitor cells can be activated with exogenous growth factors to proliferate and differentiate into each of the retinal cell types reforming a complete retina in about 1 week (Spence *et al.*, 2004; Fig. 8.2(C)). Activation of the retinal stem/progenitor cells is most robust if the retina is removed on embryonic day 4, although some activation does occur at later stages but at a reduced level.

Role of FGF/MAPK Signaling Pathway

As mentioned, FGF was the first exogenous growth factor to be identified as an inducer of retina regeneration

in the embryonic chick. Park and Hollenberg (1991) used FGF1 to induce chick retina regeneration from the stem/progenitor cells of the ciliary margin. More recently, we have used FGF2 (which was originally used by Park and Hollenberg (1989) to induce transdifferentiation in the chick retina) and studied its ability to activate the retinal stem/progenitor cells (Spence *et al.*, 2004, 2007a) (Fig. 8.2(C), 3(A)). FGF2 can activate several signaling pathways within the cell, but the activation of the mitogen-activated kinase (MAPK) signaling cascade by FGF2 is critical for retina regeneration since the addition of an inhibitor for this pathway in the presence of FGF2 results in a significant reduction in regeneration (Spence *et al.*, 2007a). The activation of MAPK by FGF2 induces proliferation of the retinal stem/progenitor cells and is required for cell survival (Spence *et al.*, 2007a).

Role of Shh Signaling Pathway

Other signaling pathways are also involved in the regulation of retina regeneration from the ciliary margin. One of these pathways is the Shh pathway. Like, FGF2, overexpression of Shh has been shown to induce retina regeneration from the stem/progenitor cells (Fig. 8.3(B)). However, induction of regeneration by either of these molecules is dependent on the other pathway being functional, since reduced regeneration from the ciliary margin occurs in eyes treated with either FGF2 and an inhibitor of the Shh pathway or a virus overexpressing Shh and an inhibitor of the FGF pathway (Fig. 8.3(D) and 8.4(A); Spence *et al.*, 2004).

Detailed studies have been done to dissect the role of FGF2 and Shh in retina regeneration. It has been found that Shh can induce regeneration from the ciliary margin by activating transcription of FGF ligands and FGF receptors thereby inducing proliferation through the FGF/MAPK pathway described above (Spence *et al.*, 2007a). A functional Shh pathway is also necessary because Shh works with FGF2 to promote cell survival and Shh alone is required for the maintenance of progenitor cell identity (Spence *et al.*, 2007a). In addition to stem/progenitor cell induction, overexpression of Shh has been shown to reduce the number of regenerating ganglion cells, demonstrating a role for Shh in retina differentiation (Spence *et al.*, 2004).

Role of BMP Signaling Pathway

In addition to FGF2 and Shh, BMP has also been shown to induce retina regeneration from the ciliary margin (Fig. 8.3(C)) (Haynes *et al.*, 2007). BMP can also activate the FGF/MAPK pathway by increasing the transcription of FGF receptors. The BMP pathway

and the FGF/MAPK pathway are both necessary for proliferation of retinal progenitor cells during the initial induction period of regeneration because if one pathway is blocked, proliferation and therefore regeneration does not occur (Fig. 8.3(E) and (F)). During this initial induction period of regeneration BMP activates the canonical BMP pathway (via SMADs). However, during the later stages of regeneration, BMP switches and activates a non-canonical BMP pathway (via TAK1) which leads to p38 activation and apoptosis. Inhibition of p38 is necessary to maintain BMP-induced regeneration otherwise the regenerated retina will undergo massive cell death. Even the addition of ectopic FGF2 does not prevent the high level of cell death because BMP decreases the transcription of FGF receptors at this stage. There is some evidence that BMP also regulates the differentiation of ganglion cells and the cells of the inner nuclear layer because these cells do not form in the absence of BMP (Haynes *et al.*, 2007).

While we are still deciphering how FGF2, Shh, and BMP pathways work together as well as in cooperation with other pathways yet to be studied, it is clear that functional FGF2, Shh, and BMP pathways are necessary for induction of regeneration from the stem/progenitor cells present in the anterior region of the eye. Further studies will help delineate whether the pathways work in concert or parallel to regulate proliferation, cell survival, and differentiation.

Regeneration by Transdifferentiation

An In Vivo Model

The second mode of regeneration that takes place in the chick retina is via the process of transdifferentiation. When a complete retinectomy is performed in embryonic day 4 chick eyes, and an exogenous source of FGF is introduced in the eye, the retinal pigmented epithelium (RPE) undergoes a reprogramming where the cells dedifferentiate, losing their pigment and become “embryonic-like.” These cells enter the cell cycle and build a neuroepithelium which will eventually differentiate to give rise to the newly regenerated retina. This process of transdifferentiation has been described histologically (Coulombre and Coulombre, 1965; Park and Hollenberg, 1989; Spence *et al.*, 2004) as well as with cell and molecular markers (Spence *et al.*, 2004; 2007b).

Other species can also regenerate their retina via transdifferentiation during early stages of their development (review in Lopashov and Stroeveva, 1964; Mitashov, 1996, 1997), however, studying this process *in vivo* can be challenging in animals such as mice or

even fish as these animal models are either not easily accessible or are too small to manipulate during early stages of their development. Some anurans such as newts have unsurpassed regeneration abilities and can regenerate their retina via transdifferentiation throughout their lifetime. A month and a half after retina removal, a complete functional retina is restored (Mitashov, 1996, 1997; Del Rio-Tsonis and Tsonis, 2003; Tsonis and Del Rio-Tsonis, 2004; Chapter 7). These virtues qualifies the newt as one of the best animal models to study transdifferentiation; however, the lack of molecular tools for newt studies has greatly limited the use of this model for dissecting the molecular regulation of transdifferentiation. The availability of molecular tools in the embryonic chick as well as the fast rate of retina regeneration (it only takes 7 days after retina removal to obtain a complete laminated retina with all the major retinal cell types present), qualifies this animal as the preferred model for the dissection of molecular mechanisms during RPE to retina transdifferentiation.

The embryonic chick eye only provides a good model to study early events of retina regeneration and transdifferentiation but will not address the restoration of vision since the transdifferentiated retina does eventually degenerate due to the lack of RPE, which fails to restore itself during the process of transdifferentiation (Coulombre and Coulombre, 1964; Park and Hollenberg, 1989). The lack of RPE in the transdifferentiated retina accounts for its reverse orientation when compared to the original retina or even to the one that regenerates via stem/progenitor cell activation (see Figure 8.2(C)).

A window of transdifferentiation

It is interesting to note that there is a small window during chick eye development where the RPE is competent to transdifferentiate (Coulombre and Coulombre, 1964; Park and Hollenberg, 1989). It is feasible to remove the retina as early as E3.5 and if a source FGF is added then, the RPE will transdifferentiate into retina. This competence is present until about E4.5. During this time, the RPE expresses microphthalmia (*Mitf*) and has stopped expressing Pax-6 (Spence *et al.*, 2007b). In the absence of neural retina (NR), RPE transdifferentiation in chick eyes has not been reported after E5 *in vivo* with any known treatment. However, RPE to retina transdifferentiation has been reported in developing eyes when Pax-6 is overexpressed in the RPE of chick eyes up to stage 35 (Azuma *et al.*, 2005), or in *in vitro* E5-6 (HH stages 28-29) explant cultures where activin/ TGF-beta/

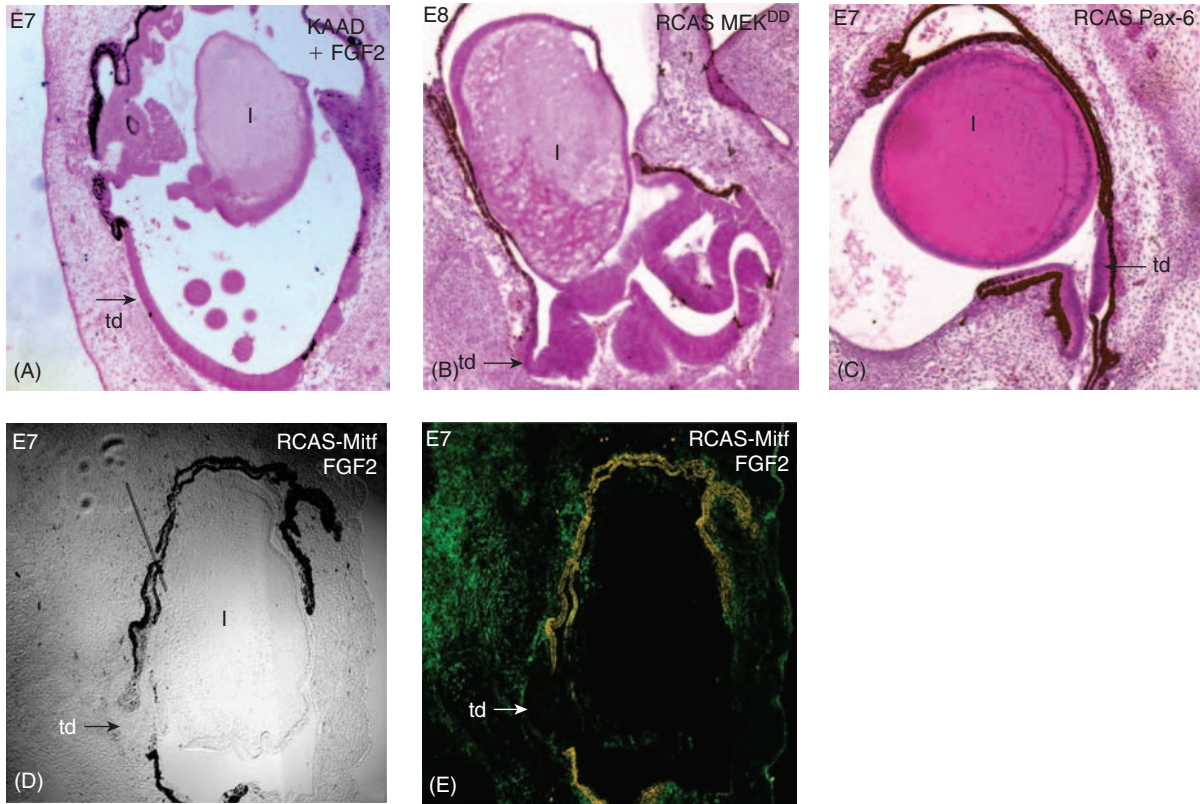


FIGURE 8.4 (A–C) A cross section of a regenerating eye at 3 days post-retinectomy (A, C) or 4 days post-retinectomy (B) showing transdifferentiation of RPE induced by KAAD, an inhibitor of the Shh pathway, and FGF2 (A), activation of MAPK pathway (RCAS MekDD (B), and overexpression of Pax-6 (RCAS Pax-6) (C). (D and E) Inhibition of transdifferentiation by overexpression of Mitf (RCAS Mitf) in the presence of FGF2 is shown by immunohistochemistry on a cross section of a regenerating eye at 3 days post-retinectomy using an antibody for Mitf (red) and an antibody for a protein from the viral coat (green). Yellow cells show the location of infected RPE (E). Transdifferentiation only occurs in area of the RPE that are not infected (td). DIC is shown in D.

nodal receptors are inhibited in the presence of FGF (Sakami *et al.*, 2008), or even in RPE explants of post-hatched chicks transfected with Optx2 (Toy *et al.*, 1998).

Dissecting the molecular pathway of RPE transdifferentiation

There are several molecular players involved in the process of transdifferentiation that have been unraveled by a disruption on their pathway or function during either retina development or regeneration. Two different groups of molecules have been identified in the saga of transdifferentiation. On one side, are the genes that protect the RPE phenotype and on the other, the ones that define the retina phenotype. Mitf (Mochii *et al.*, 1998a; b; Planque *et al.*, 1999; 2001; 2004; Bumsted and Barnstable, 2000; Nguyen and Arnheiter, 2000), Otx (Martinez-Morales *et al.*, 2001; 2003; 2004; Sakami *et al.*,

2005), Wnt13 (Fuhrmann *et al.*, 2000), BMPs (Muller *et al.*, 2007;), Shh (Zhang and Yang, 2001; Perron *et al.*, 2003; Spence *et al.*, 2004) and activin (Fuhrmann *et al.*, 2000; Sakami *et al.*, 2008) are associated with the induction and maintenance of the RPE, whereas Pax-6 (Belecky-Adams *et al.*, 1997; reviewed in Levine and Green, 2004; Chx10 (Rowan *et al.*, 2004; Horsford *et al.*, 2005), Msx-2 (Holme *et al.*, 2000), Optx2 (Toy *et al.*, 1998), Neuro D (reviewed in Yan *et al.*, 2005) and FGF/MAPK (Vogel-Höpker *et al.*, 2000; Galy *et al.*, 2002; and reviewed in Yang *et al.*, 2004) are associated with retina.

In chicks, Pax-6 overexpression in the RPE is sufficient for the induction of transdifferentiation during retina regeneration (Spence *et al.*, 2007b) (Fig. 8.4(C)) and even during development (Azuma *et al.*, 2005) while Mitf overexpression is sufficient to protect the RPE from transdifferentiating during FGF-induced retina regeneration (Spence *et al.*, 2007b) (Fig. 8.4(D) and (E)).

An In Vitro Model

The embryonic chick has been used for the study of transdifferentiation by several researchers using *in vitro* systems including isolated RPE cells or explants.

Transdifferentiation of RPE to NR

RPE explants

RPE cells have been cultured from chick embryos and tested for their ability to transdifferentiate into NR cells (Pittack *et al.*, 1991; Guillemot and Cepko, 1992). If the RPE is removed from the chick at E4.5-E5.5 (HH stages 24-28), dissociated and treated with FGF, the cultured RPE cells lose their pigment but do not express markers of neural cells (Pittack *et al.*, 1991). However, if the RPE cells are not dissociated, but instead left as an intact sheet of cells and treated with FGF, the RPE cells will lose their pigment and express markers indicative of retinal progenitor cells and even express markers of NR cells (Pittack *et al.*, 1991; Guillemot and Cepko, 1992). Recently, Sakami *et al.* (2008), had used this explant system to test the potential of activin to block FGF-induced RPE transdifferentiation using E4 explants, and have shown that when inhibiting the activin/TGF-beta/nodal pathway, E5 incompetent RPE can transdifferentiate. Interestingly, according to Zhou and Opas (1994), FGF does not act on the fully differentiated RPE, but only on those cells that have been stimulated to change their identity, probably via changes in their adhesive status. In addition, once FGF is able to direct RPE explants to transdifferentiate, the substratum where the cells are grown dictates their differentiation (Opas and Dziak, 1994). Transfecting RPE explants with key genes is another way to induce transdifferentiation effects such as the ones incurred by transfecting *Otx2* unto E7-E8 as well as post-hatched chick RPE explants (Toy *et al.*, 1998).

RPE isolated cultures

While the dissociated RPE cells did not express neuronal markers when cultured from chick embryos at E4.5-E5.5, they did begin the transdifferentiation process by losing their pigment if treated with FGF2. Additional studies of these cultured RPE cells revealed that an overexpression of *Mitf*, a transcription factor involved in defining RPE identity, inhibited FGF from triggering transdifferentiation of the RPE (Mochii *et al.*, 1998b). Furthermore, addition of *Msx-2*, a gene only expressed in NR, to the cultured RPE cells caused a decrease in *Mitf* and an increase in the neuronal marker, class III beta-tubulin (Holme *et al.*, 2000). Therefore, transdifferentiation of cultured RPE cells

from E4.5-E5.5 into neuronal cells requires the down-regulation of RPE genes, such as *Mitf*, and/or the upregulation of neuronal specific genes, such as *Msx-2*.

RPE cells cultured at a slightly later day in development, at E6, have also been used to study the ability of RPE to transdifferentiate into NR. Addition of FGF to cultured E6 RPE cells did result in an increase in cells expressing an early ganglion cell marker, RA4, but there was not a transdifferentiation to neuronal morphology. However, addition of *NeuroD* did result in a transdifferentiation of E6 RPE cells to photoreceptors (Yan and Wang, 1998; Yan and Wang, 2000a; b) while the addition of neurogenin 2 (*ngn2*) resulted in transdifferentiation of the E6 RPE cells to photoreceptors and retinal ganglion cells (Yan *et al.*, 2001). *Cath5* and *NSCL1* were also able to induce transdifferentiation of E6 RPE cells into retinal ganglion cells (Ma *et al.*, 2004; Xie *et al.*, 2004). Studying the induction potential of RPE cells *in vitro* will be beneficial in deciphering the molecules needed to induce transdifferentiation of the RPE *in vivo* at both E4 and at later stages.

Transdifferentiation of NR to RPE

NR from early chick embryos also possesses the plasticity to transdifferentiate into RPE *in vitro*. Studies performed by Opas *et al.* (2001) have shown that dissociated 6-day-old embryonic NR can transdifferentiate into RPE spontaneously. These pigmented transdifferentiating cells express RPE-specific protein, eRPEAG and lack of expression of the neural cell adhesion molecule, NCAM (Opas *et al.*, 2001).

In Vitro-In Vivo

RPE cells cultured *in vitro* have also been transplanted into the embryonic chick eyes and shown to integrate into the developing eye. Cells cultured from the developing RPE of an E5.5 chick embryo and grown until they develop the morphology of RPE cells will integrate into the developing RPE when transplanted into the embryonic chick at E11-E18 (Liang *et al.*, 2006). However, if, before transplantation, the cultured RPE cells are treated with an RCAS virus expressing *NeuroD*, which has been shown to be important for photoreceptor development (Yan and Wang, 1998), the infected RPE cells will begin to express *visinin*, an early marker for cone photoreceptors, and integrate into the outer nuclear layer of the retina indicative of transdifferentiation of the transplanted RPE cells into photoreceptor cells (Liang *et al.*, 2006). These transplanted cells continue the differentiation process expressing advanced photoreceptor markers such as opsin and extend axons into the inner nuclear layer

or ganglion cell layer. Although these transdifferentiated photoreceptor cells do integrate into the correct location and express the appropriate markers for photoreceptors, the photoreceptors are not all organized perpendicular to the RPE and some advanced markers are expressed in the cell body instead of the axon (Liang *et al.*, 2006). This is believed to occur because there is not an intimate association of the transplanted cells with the developing RPE that is needed for proper organizational cues. Despite the organizational problems that need to be solved, these studies involving the chick embryo provide hope that transplanted RPE cells can someday be directed to differentiate *in vivo* to replace lost or damaged photoreceptors.

Using the Embryonic Chick Eye to Probe for Retina Repair Potential of Mammalian Cells

Embryonic stem cells isolated from the mammalian blastocyst and retinal stem cells isolated from rodents and post-mortem humans have been cultured and directed to differentiate into ocular structures including lens (Oota, *et al.*, 2003; Takahashi, *et al.*, 2006), retina (Zhao *et al.*, 2002; Hirano *et al.*, 2003; Haruta, 2005; Banin, *et al.*, 2006; Lamba, *et al.*, 2006; Limb, *et al.*, 2006; Zhao, *et al.*, 2006, and Vugler, *et al.*, 2007) and RPE (Haruta, *et al.*, 2004; Klimanskaya *et al.*, 2004; Aoki, *et al.*, 2006; and Takahashi *et al.*, 2006). The embryonic chick has proven to be an excellent model to determine the ability of these stem cells to integrate and differentiate *in vivo* (Coles *et al.*, 2004; Aoki *et al.*, 2006).

Embryonic Stem Cells

Mouse embryonic stem cells incubated with basic FGF, cholera toxin, dexamethasone and Wnt2b resulted in these stem cells expressing retinal precursor markers and differentiating into eye-like structures resembling lens, RPE, and retina with a high frequency within 10–12 days *in vitro* (Hirano *et al.*, 2003 and Aoki *et al.*, 2006). When these eye-like structures were developed for 11 days *in vitro* and then transplanted into the developing chick eye, they most often migrated to the developing RPE layer and differentiated into mature RPE cells expressing the RPE marker, RPE65 (Aoki *et al.*, 2006). A few of these transplanted eye-like structures also expressed markers indicative of a ganglion cell lineage (Aoki *et al.*, 2006; 2007). Embryonic stem cell transplanted after only 6 days in culture also integrated into the retina of the chick and were induced to form lens tissue or express markers of a ganglion cell lineage. Based on these studies, we can speculate that human embryonic stem cells have the potential to

integrate into different tissues of the eye and differentiate into functional cells of the lens, retina, and RPE if manipulated correctly.

Adult Stem cells

Retinal stem cells isolated from the ciliary margin of post-mortem human eyes were also tested for their potential to differentiate *in vivo* using the embryonic chick eye. These retinal stem cells were able to proliferate and differentiate spontaneously into all retinal cell types when cultured *in vitro* although the addition of FGF, epidermal growth factor (EGF), and heparin increased the rate at which this occurred (Coles *et al.*, 2004). These retinal stem cells were able to respond to environmental cues in the developing chick eye and express markers of ganglion and horizontal cells when transplanted at the time these cells would normally be developing in the chick eye (Coles *et al.*, 2004). These studies show great promise for the future use of either embryonic or adult stem cells in the treatment of retina degenerative diseases. They also demonstrate the conservation between environmental cues in human and chicks making the chick a reliable model in which to study the potential of these cells.

The Post-hatch Chick and Its Potential Sources of Retina Repair

The Ciliary Margin

Although retinal stem/progenitor cells continue to proliferate for up to 3 weeks after hatching, they are unable to regenerate a complete retina even in the presence of exogenous growth factors (Fischer and Reh, 2000). The ciliary margin which houses the retinal stem/progenitor cells is composed of two distinct regions in the fully developed chicken eye. The more anterior structure is the ciliary body (CB) which is composed of two cellular layers, the pigmented epithelial layer (PE) and the non-pigmented epithelial layer (NPE). Posterior to the CB at the tip of the NR is the ciliary marginal zone (CMZ) (Fischer and Reh, 2003a). EGF, insulin, and IGF-1 increase proliferation and induce differentiation of the cells in the CMZ (Fischer and Reh, 2000; 2003a), whereas FGF2, insulin and EGF stimulate the cells of the NPE to proliferate and differentiate (Fischer and Reh, 2003a). While differentiation can be induced, it is limited in the post-hatch chick. Cells in the CMZ will differentiate into amacrine and bipolar cells and cells from the NPE differentiate to form amacrine and ganglion cells but other cell types including photoreceptors are not

formed by either group of cells. Regardless, retinal injury will not stimulate the cells of the CMZ to regenerate or repair the retina (Fischer and Reh, 2000).

Müller Glia

Müller Glia are another possible source of regeneration in the post-hatch chick. Injection of toxins that cause cell death in certain retinal neurons or the addition of FGF2 or insulin causes the Müller Glia to proliferate, lose their characteristic Müller Glia markers and begin to express markers indicative of retinal progenitors (Fischer and Reh, 2001; Fischer *et al.*, 2002; Fischer and Reh, 2003b). Many of the activated Müller Glia remain undifferentiated but a small percentage of them do differentiate into ganglion, amacrine, or bipolar cells (Fischer and Reh, 2001; Fisher *et al.*, 2002) under certain treatments. It has been shown that the Notch pathway is necessary for the dedifferentiation and proliferation of Müller Glia but if the Notch pathway remains active, it will inhibit the differentiation of the newly formed progenitors into neural cells (Hayes *et al.*, 2007). In addition, NeuroD has been shown to induce dedifferentiation of Müller Glia cultured from toxin-damaged retina and promote the differentiation of immature photoreceptors (Fischer *et al.*, 2004).

CONCLUSION

The chick provides an excellent system to explore cell and molecular events during retina development and regeneration, including cell fate determination, stem and progenitor cell biology, cell differentiation, cell division, cell death, cell signaling, axon path finding, retinotectal projections and neural circuitry to name a few. It is an inexpensive, molecularly friendly system with many tools currently available.

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