Chapter 4
Lens Differentiation from Embryonic Stem (ES) and Induced Pluripotent Stem (iPS) Cells

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Abstract The formation of lens progenitor cells and differentiated lens tissue in cell culture conditions presents a number of experimental challenges, even though lens lineage formation and lens fiber cell differentiation are among the best characterized model systems at both genetic and molecular levels. Lens differentiation from ES cells in vitro appears to be a feasible goal. This chapter describes the significance of using ES and iPS cells for better understanding of embryonic lens development and formation of congenital cataracts. A discussion of how iPS cells can help studies of age-related cataract is also included. The chapter summarizes the current data on lentoid body formation from human and primate ES cells, and the molecular basis of directed differentiation of human ES cells into lens progenitor cells and lentoid bodies. Finally, current gaps in lens research and future directions to address these problems are discussed.

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Introduction

The central premise of embryonic stem (ES) cell biology is an unlimited potential of ES cells to form every cell type of the whole organism [33, 42, 91, 120]. The potential is fulfilled during ontogenesis. The major question is if it is possible to differentiate ES cells into all transient (embryonic germ cell layers and common cell progenitors) and terminally differentiated cell types in vitro. A large body of work using mostly human and mouse ES cells conducted during the last decade has shown that it is a generally feasible goal with major implications for our understanding of embryonic development; modeling of human disease and treatment of a wide range of diseases that require cell-based therapeutics [50].

The human eye is an excellent organ for in vitro studies of its organogenesis, modeling of human eye diseases through the generation of disease-specific-induced pluripotent stem (iPS) cells via nuclear reprogramming [121], and for cell replacement and paracrine rescue therapies [66]. To harness the power of ES- and iPS-cell-based ideas of treating human eye diseases, the essential first step is to develop procedures to form ocular cells and tissues using in vitro conditions. The main challenge for this research originates from our limited knowledge of cell fate specification processes that occur normally in a three-dimensional (3-D) context in developing embryos and what specific cell culture conditions may favor simultaneous formation of multiple cell types that might both positively and negatively influence the development of the desired cell type. While the cells can achieve the desired cell type, their terminal differentiation into a status comparable with tissues generated during ontogenesis often requires additional conditions that have to be determined empirically.

The formation of lens progenitor cells and differentiated lens in cell culture conditions presents a number of experimental challenges, even though lens lineage formation and lens fiber cell differentiation are among the best characterized model systems at both genetic and molecular levels [16, 18, 20, 23, 35, 59, 69, 82]. It has been shown that cultured *lens epithelial cells* can be differentiated into primitive lens-like structures termed "lentoid bodies." Lentoid bodies are 3-D structures that resemble the lens as they are both transparent and refract light. They can be generated in vitro either from primary, spontaneously transformed or viral oncogene-transformed lens epithelial cells [8, 45, 46, 51, 75, 76, 87, 112, 119]. Lentoid body formation can also be found in vivo in vertebrate embryos as a result of spontaneous or genetically engineered mutations in genes that operate in the pathways that control lens formation [56, 61, 101]. Finally, it is possible to transdifferentiate lentoids from retinal pigmented epithelium (RPE) cells [68, 72]. The formation of lentoid bodies in different experimental settings shows that the basic program to establish the 3-D structure of the lens is functional independently on the local environment such as in the absence of optic cup/retinal tissue [106]. Thus, lens differentiation from ES cells in vitro appears to be a feasible goal. This chapter first describes the significance of using ES and iPS cells for better

understanding of embryonic lens development and formation of congenital cataracts. A discussion of how iPS cells can help studies of age-related cataract is also included in "New Model Systems Based on ES and iPS Cell Differentiation to Understand Lens Development and Disease," section of this chapter. "Differentiation of ES Cells into Lens" summarizes the current data on lentoid body formation from human and primate ES cells, and the molecular basis of directed differentiation of human ES cells into lens progenitor cells and lentoid bodies. Finally, "Conclusions and Future Directions" provides a summary of current gaps in lens research and future directions to address these problems.

New Model Systems Based on ES and iPS Cell Differentiation to Understand Lens Development and Disease

Use of ES and iPS cells differentiated into lens cells offers a wide range of experimental approaches to better understand embryonic lens formation and lens fiber cell differentiation. Similarly, cataract-specific iPS cells offer a new array of approaches to evaluate various aspects of human lens homeostasis and identification of novel relationships between cellular processes and their impact on lens transparency.

Modeling of Embryonic Development

Although embryological studies on lens morphogenesis date to the beginning of the twentieth century, and have resulted in a comprehensive understanding of the origin of lens cell lineage, formation of the lens placode, formation of the lens vesicle, cell cycle exit regulation in the posterior compartment of the lens vesicle, lens fiber cell terminal differentiation, lens regeneration in specific amphibians, transdifferentiation of lens from other ocular and non-ocular tissues, and lens evolution in animal kingdom ([18–20, 30, 35–38, 48, 59, 63, 69, 82]), a number of important questions remain to be addressed, with three examples described below.

Based on studies in chicks and zebrafish, it has been proposed that lens progenitor cells originate from a common pool of pre-placodal cells [1, 38, 105]. Data to support this attractive model on mammalian lens development are still missing. A large body of data exists to support the role of FGF signaling at multiple stages of lens development [88]; however, little is known how the specificity of this signaling is established in the embryo in a 3-D space crowded with many signaling molecules, their agonists and antagonists [102]. The lens is also a unique tissue in terms of its terminal differentiation. To achieve transparency, lens fiber cells lose their subcellular organelles including the nuclei in a highly controlled process that ultimately preserves the lens fiber cells for the rest of the life [3, 4]. These questions

can be addressed through the use of ES cell differentiation as described in "Differentiation of ES Cells into Lens" and future experiments outlined in "Conclusions and Future Directions" of this chapter.

Congenital Cataracts

Congenital cataracts are typically caused by mutations in genes that control lens development and by mutations in genes encoding key lens structural proteins [36, 40, 98]. Although molecular mechanisms for many of these genes were established using mouse models, the power to produce lens cells from human patients that carry these mutations is unique. The advantage of this system is that one can prepare human lens cell extracts from genetically defined material and study protein–protein interactions of mutant crystallins and lens membrane proteins in their native environment [17]. Similarly, it is possible to derive lens cells from patients with mutations in DNA-binding transcription factors such as *FOXE3* [69], *HSF4* [11, 26, 100], *MAF* [17, 123], *PAX6* [43], and *PITX3* [7, 10, 96] to study molecular mechanisms of these mutations in their native biological environment. This approach should identify those specific genes with disrupted expression due to specific missense mutations and/or by their haploinsufficiency [17].

Age-Related Cataract

Age-related cataract is a disease of the ocular lens that is responsible for just under half of blindness worldwide, and is expected to increase as a result of extended life spans in industrialized, emerging-market, and underdeveloped countries [71]. Age-onset cataract develops between the age of 40–50 years as a result of the progressive breakdown of the lens microarchitecture [97]. Age-onset cataract is a complex disease involving both genetic and environmental factors that affect 42 % of the population between the ages of 52–64, and 91 % of the population for ages 75–85 [54, 103]. Genetic studies of age-related cataract point to both multiple genes and environmental factors influencing the phenotype [71, 97]. The Beaver Dam Eye Study suggests that mutations in a single gene/locus could be responsible for as much as 35 % of nuclear and up to 75 % of cortical cataract incidence [39, 47, 55]. Other studies using siblings and twins also demonstrate significant genetic influence on age-onset cataract [41, 97].

Age-related (or senile) cataract is defined as cataract occurring in people over the age of 50 in the absence of known mechanical, chemical, or radiation trauma. At the molecular level of age-related cataract, lens structural proteins, the crystallins, become oxidized and water-insoluble, and form high molecular weight aggregates. The continual accumulation of crystallin aggregates and other lens proteins causes opacification and loss of lens transparency. The current treatment

of senile cataract is surgery that replaces the opaque lens with an artificial intraocular lens. Although the surgery is routinely performed in the USA, numbering 1.5–2 million patients treated annually, it represents a major Medicare reimbursement category. It has been estimated that a 10-year delay in the onset of senile cataract could decrease the number of surgeries needed by almost one half, thus significantly decreasing vision care costs ([58]; www.nei.nih.gov/strategicplanning/np_lens.asp). Progress in human cataract research is hampered by the lack of genetically defined and abundant experimental materials as well as the absence of relevant animal models [6, 41]. The use of cataract-specific iPS cells offers a unique opportunity to develop well-defined human cell culture models to study cataract as a disease of lens protein homeostasis.

Differentiation of ES Cells into Lens

In this section, we will first summarize our knowledge about mammalian lens formation that is relevant to the design of experiments to differentiate lens cells from ES cells ("Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems"). We then provide examples of lentoid body formation in various ES culture systems ("Formation of Lentoid Bodies") and describe a procedure to produce highly enriched lens progenitor cells and "immature" lentoid bodies from human ES cells ("Lens Differentiation from Human ES Cells in Chemically-Defined Conditions"). Finally, we will discuss different strategies to improve the differentiation of human lentoid bodies ("3-D Cultures of Lentoid Bodies to Improve Their Differentiation Status").

Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems

Multiple signal transduction systems including BMP (bone receptor protein), FGF, Notch, TGF-β, and Wnt have been identified to control various stages of lens morphogenesis [18, 38, 59, 64, 102]. In addition, the origin of lens lineage from the pre-placodal region shown in chicken and zebrafish models suggests that early stages of the differentiation process require the formation of neuroectoderm and its subsequent "by-product," the pre-placodal ectoderm [105].

Neuroectoderm formation in cell cultures can be induced by a variety of growth factors, inhibitors of BMP signaling including noggin, follistatin, cerberus, chordin, ventropin, and gremlin [2, 92] as well as small drugs such as SB431542 [15]. It has been found recently that noggin is produced by a subpopulation of MyoD-positive cells in the epiblast; their immunologically mediated ablation interfered with lens and optic cup morphogenesis [31].

Loss-of-function studies of *BMP4* in mouse established a critical role of this growth factor for lens placode formation [27]. *BMP7* knockout mice also develop ocular abnormalities that were linked to the abnormal lens induction [65, 116]. In addition, studies of lens formation through conditional knockouts of two BMP receptor genes, *Acvr1* and *Bmpr1a*, further confirmed the essential roles of BMP signaling in lens induction, as reduced lens placode thickening and failure of lens invagination were observed [86]. In ex vivo explant assays using chicken embryonic tissues, BMPs have been shown to specify the formation of lens and olfactory placodes [80, 99]. BMP signaling not only plays a role in the formation of lens placode but also participates in lens fiber cell differentiation. BMP2, BMP4, and BMP7 have been shown to induce the expression of markers of fiber differentiation in primary chick lens cell cultures. In addition, expression of noggin, an inhibitor of BMP signaling, in the lenses of transgenic mice resulted in a postnatal block of epithelial-to-secondary fiber differentiation [9].

Numerous studies have shown multiple functions of the FGF (fibroblast growth factor) signaling pathway for the formation of the lens placode [105]. FGF signaling is well known as the key trigger for lens fiber cell differentiation [63]. The pioneering work conducted more than two decades ago showed that FGF2/bFGF is a potent inducer of lens fiber cell differentiation in vitro [14]. A recent study using conditional triple knockout mice with deletion of FGF receptors, *Fgfr1, Fgfr2*, and *Fgfr3,* provided evidence for the essential role of FGF signaling in lens fiber cell differentiation in vivo. The specific inactivation of these three FGF receptors at lens pit stage totally abrogated lens fiber cell differentiation, resulting in a hollow lens [126]. Transgenic mice expressing a dominant-negative *Fgfr1* in the presumptive lens ectoderm showed many early stage defects including reduced lens placode thickness and delayed lens placode invagination [25]. Studies on two genes, *Frs2* and *Ndst1*, also revealed that FGF signaling is critical for lens placode formation. *Frs2α* encodes a docking protein for linking FGFRs with a variety of intracellular signaling pathways. A mutation of this gene $Frs2\alpha^{2F/2F}$ led to the halt of the lens development at lens placode stage in severely affected mutant eyes [32]. *Ndst1* (*N*-acetylglucosamine *N*-deacetylase-*N*-sulfotransferase 1 enzyme) encodes an enzyme for biosynthesis of heparan sulfate proteoglycans, which is low affinity co-receptor of FGFRs. Inactivation of *Ndst1* in mouse resulted in invagination defects of the early lens [79]. The most recent study showed that inactivation of *Fgfr1* and *Fgfr2* at lens placode stage led to increased cell death and the formation of a thinner lens placode, suggesting that the primary role of autocrine or paracrine FGF signaling is to provide essential survival signals to lens placode cells [29].

Recent genetic experiments, lens-specific inactivation of *Jag1* [60], *Notch2* [93] and *RBP-J* [90] have established role of Notch signaling in primary lens fiber cell differentiation.

Both canonical Wnt signaling, via β-catenin, and planar cell polarity (PCP/Wnt) non-canonical Wnt signaling play a range of roles in lens morphogenesis [64, 67]. Wnt/PCP signaling is required for organization of lens fiber cell cytoskeleton and lens 3-D architecture.

In summary, studies of lens development suggest that active BMP and FGF signaling are required for lens cell formation. FGF signaling is sufficient to induce lens fiber cell differentiation in in vitro cultures, and modulation of this process via Notch, Wnt/β-catenin and Wnt/PCP signaling pathways could provide additional tools to recapitulate lens ontogenesis from ES cell cultures.

Formation of Lentoid Bodies

Three earlier procedures identified lentoid body formation in primate and murine ES cells cultures. These methods were limited to a production of a small percentage of lentoid bodies along with a number of other cells types such as retinal pigmented epithelium (RPE) [44, 77, 107]. The protocols used in these earlier studies employed mouse feeder cells, and differentiation was induced by co-culture with mouse PA6 stromal cells ("SDIA, or cultures"). External FGF2 was added to some cultures [77]. The yield of lentoid bodies was between 200 and 300 colonies/10-cm dish after 30 days in culture. Formation of lentoid bodies was also detected when both mouse and human ES cells were cultured on matrix components of the human amniotic membrane ("AMED system") together with many other cell types including dopaminergic neurons, motor neurons, and RPE cells [111]. These experiments provided the "proof-of-principle" of lens cell formation from mammalian ES cells; nevertheless, they are not suitable for the standardized production of enriched lens cells and lentoid bodies.

Lens Differentiation from Human ES Cells in Chemically Defined Conditions

Using the information on normal lens formation ("Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems"), we established a new experimental three-stage protocol with defined growth factors to generate large quantities of lens progenitor cells and lentoid bodies from human ES cells as shown in Fig. 4.1. Inhibition of BMP signaling by recombinant noggin triggered differentiation of ES cells towards neuroectoderm. Subsequent reactivation of BMP and activation of FGF signaling elicited robust formation of lens progenitor cells marked by the expression of *PAX6* and αA- and αB-crystallins (*CRYAA* and *CRYAB*). The formation of lentoid bodies required the presence of FGF2 and the total number of the lentoids increased in the presence of Wnt3a yielding approximately 1,000 lentoid bodies per a 30 mm well. Lentoid bodies expressed and accumulated lens-specific markers including αA-, αB-, β-, and γ-crystallins, filensin/BFSP1, BFSP2/CP49, and MIP/aquaporin 0 [122]. Nevertheless, morphological and scanning and transmission electron microscopic analysis of these lentoid bodies identified nucleated lens cells and only moderately elongated

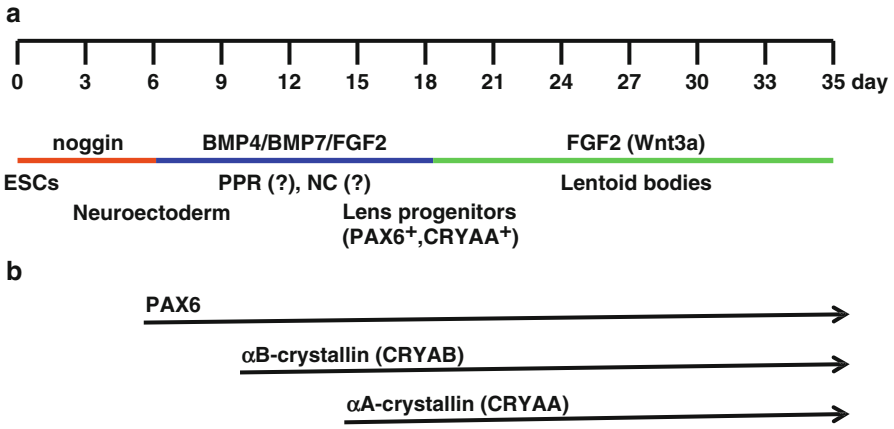

Fig. 4.1 Diagrammatic summary of a three-step procedure to differentiate human ES cells into lens progenitor-like cells and lentoid bodies. (**a**) Diagram of three steps: noggin treatment (days 0–6), BMP4/BMP7/FGF2 treatment (days 7–18), and differentiation in the presence of FGF2 (essential factor) and Wnt3a (modulatory factor) (days 22–35). Formation of putative cell populations including the neuroectoderm, pre-placodal region (PPR) and neural crest (NC) cells is indicated. (**b**) Sequential activation of PAX6, αB-crystallin (CRYAB) and αA-crystallin (CRYAA) indicates establishment of the lens progenitor-like cells around day 14 of the culture. At this time, the number of $PAX6^+$ and $CRYAA^+$ cells was 65 and 41 %, respectively [122]. Both αA- and αB-crystallins accumulate during the differentiation of lentoid bodies

lens fiber cells. These data indicated that while specific pathways of the lens fiber cell differentiation program such as synthesis and accumulation of both αA- and αB-crystallins were turned on in the "immature" lentoid bodies; however, activation of the denucleation pathway was not achieved. We conclude that this procedure can be immediately used to probe various aspects of human lens lineage cell formation focusing on the function of specific DNA-binding transcription factors, chromatin remodellers, and extracellular signaling; nevertheless, follow-up studies are necessary to address the culture conditions to achieve formation of "mature" lentoid bodies comprised of elongated enucleated lens fiber cells.

3-D Cultures of Lentoid Bodies to Improve Their Differentiation Status

A number of potential improvements of the differentiation procedure described above should be considered and empirically tested. In principle, the system can be improved through testing of different 3-D gels and extracellular matrix proteins that are found in the lens capsule, growth of lentoid bodies on lens capsule, specific activators and inhibitors of differentiation, chemical libraries, 3-D scaffolds to generate a gradient of growth factor(s), and any combination of these procedures. In addition, genetically engineered human and mouse ES cells that carry fluorescent

reporter genes, under the control of lens regulatory elements, can be used to aid in the analysis of the differentiation process.

There are at least three commercially available 3-D systems: ExtraCel hydrogel (Glycosan Biosystems), HyStem-C Cell Culture Scaffold kit (Sigma), and Cultrex 3-D Culture Matrix Extract (R&D Systems). Each system allows for the incorporation of variable amounts/ratios of laminin, collagen IV, entactin/nidogen, perlecan, fibronectin, collagen XVIII and sparc/osteonectin, extracellular matrix (ECM) proteins found in the lens [21, 117].

A number of drugs have been shown to promote cellular differentiation with some of the tested in lens cell cultures. These include specific inhibitors of DNA methylation such as 5-azacytidine and 5-deazacytidine [12, 49, 94], inhibitors of histone methyltransferases (cytarabine and decitabine [84]), inhibitors of histone deacetylases (valproic acid and sodium butyrate [22, 24, 34, 74, 78]), and inhibitors of cyclin-dependent kinases (olomoucine and roscovitine [70, 73, 89, 115]). Of particular interest are the rho-kinase (ROCK) inhibitors, Y27632 and PP-1, as the PP-1 drug has been successfully used to promote cell cycle withdrawal and commitment of lens cells to differentiate [113, 114].

Considering the specific roles of Notch and Wnt signaling pathways for lens fiber cell differentiation, and the role of Wnt signaling in the differentiation of lens epithelial cells described above ("Mammalian Lens Development and Lessons for a Rational Design of ES Cell-Based Differentiation Systems"), stimulation of ES cell differentiation may be considered. Recombinant Notch ligands, Jagged 1 and 2, can be added transiently during the thirds stage of the differentiation procedure. Concerning Wnt signaling, the situation is more complex as multiple Wnts and their receptors, the frizzled proteins, can regulate lens development both in the epithelial and fiber cell compartments. Nevertheless, inclusion of Wnt3a improved the quantitative parameters of the current procedure of lentoid body formation [122].

Ongoing experiments in the laboratory are aimed to improve differentiation of lentoid bodies using a combinatorial approach as outlined above. The procedure can be improved via genetically engineered ES cells [5] that carry fluorescent markers under the control of lens regulatory regions from genes known to control different stages of the lens lineage formation, cell cycle exit, and terminal differentiation. For this purpose, the EGFP, or enhanced green fluorescent protein marker can be inserted into a specific BAC clone with PAX6 (early marker), HSF4 (late marker), β-/γ-crystallins, DNase IIβ, MIP/aquaporin 0, paralemmin, and other genes expressed in terminally differentiated lens fiber cells as established for similar differentiation systems [83, 110].

iPS Cells and Cataract Research

For the first time in human lens research, we are about to establish a general strategy to model human lens development and diseases with an *unlimited* supply of lens cells that originate from *genetically and phenotypically defined* human source(s).

Table 4.1 A representative list of distinct nuclear reprogramming procedures to generate human iPS cells

Starting cell type	Treatment	Abbreviation	References
Skin fibroblasts	[*Oct3/4,Sox2,Klf4,Myc*]-retroviruses	iPS	[108]
IMR90 cells, newborn foreskin fibroblasts	[*Oct4,Sox2,Nanog,Lin28*]-lentiviruses	iPS	[125]
Fetal, neonatal, and adult fibroblasts	[*Oct4,Sox2,Klf4,Myc*]-retroviruses + *hTERT* + *SV40LT*	iPS	[81]
Fibroblasts, liver cells	[*Oct4,Sox2,Myc,Klf4*]-adenoviruses	Adeno-iPS	[104]
Terminally differentiated amniotic fluid cells	[*Oct4,Sox2,Klf4,Myc*]-retroviruses	AF-iPS	[28]
Amnion-derived cells	[*Oct4,Sox2,Nanog*]-lentiviruses	hADC-iPS	[127]
Neural stem cells	[*Oct4*]-inducible lentivirus	NiPS	[53]
Peripheral blood mononuclear cells (PB-MNCs)	[*Oct4,Sox2,Klf4,Myc*]-retroviruses + *Htert* + *SV40LT*	BM-iPS	[57]
Umbilical cord matrix and amniotic membrane	[*Oct4,Sox2,Klf4,Myc*]-retroviruses, vitamin C, valproic acid		[13]
Human newborn fibroblasts (HNFs)	Proteins		[52]
Human foreskin fibroblasts	Episomal vector		[124]
Human embryonic fibroblasts (HEF)	*piggybac* transposon		[118]
Human peripheral circulating T cells	Sendai virus	TiPS	[95]

In addition, these materials can be shared between multiple laboratories to accelerate research. The pioneering work of S. Yamanaka at the Institute for Frontier Medical Sciences, Kyoto University, Japan, to establish the reprogramming procedure using skin fibroblasts provided proof-of-principle that the iPS cell can be established from somatic terminally differentiated cells, and these iPS cells behaved like authentic ES cells in a series of functional tests [108, 109]. A large follow-up effort in a number of laboratories worldwide resulted in expansion of the reprogramming procedures and cell types suitable for these manipulations. The majority of currently existing procedures are summarized in Table 4.1. It has been shown recently that iPS cells can be produced from a cataract patient using lens epithelial cells as the starting material [85]. Most importantly, these iPS cells were differentiated into lentoid bodies using the procedure described here (see Fig. 4.1) [85]. Nevertheless, whether iPS cells, generated through other reprogramming protocols and cell types, are capable of producing lentoid bodies similar to those generated from human ES cells, remains to be formally proven.

Conclusions and Future Directions

One of the most pressing objectives of medical research today is to develop novel approaches to model formation of human organs, tissues and diseases. Use of human ES and iPS cells differentiated into individual tissues provides the highest possible promise to achieve this objective as it is now possible to understand the contribution of genetic and environmental factors in various diseases including those related to aging such as age-onset cataract.

Thus, the present cell culture system can be used to modulate these common signaling pathways during lens formation [62] via siRNA technology and through the use of small drug molecules, inhibitors of FGF and BMP signaling (e.g., SB431542—an inhibitor of the Alk1 receptor, SU5402—an inhibitor of FGFR and U0126—an inhibitor of MEK) to study formation of lens lineage and formation of alternate cell fates that originate from the common pre-placodal region [105].

It is now possible to produce iPS cells from human patients that carry heterozygous mutations in regulatory genes such as *PAX6, FOXE3, MAF, HSF4, PITX3*, and others and to identify those genes that are not properly regulated during early stages of lens development. In contrast, studies of cataractogenesis using the system of ES/iPS cells seems be premature until procedures to generate enucleated lentoid bodies with distinct epithelium/fiber cell compartments are established. The long-term benefits of the research to model human cataract using iPS cells should stimulate our efforts to achieve this challenging goal.

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