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Functional analyses of Equarin during chick lens development
（ニワトリレンズ発生時におけるEquarinの機能解析）

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Summary

The normal development of lens requires the elaborate control of cell proliferation, migration and differentiation. It is widely accepted that fibroblast growth factor (FGF) signaling is required for the differentiation of lens epithelial cells into crystallin-rich fibers, but this signaling is insufficient to induce full differentiation. To better understand lens development, investigatory and functional analyses of novel molecules are required. Here, we demonstrate that Equarin, which is a function-unknown secreted molecule, is expressed exclusively in the lens equator region during chick lens development. Equarin upregulated the expression of fiber markers, as demonstrated using in ovo electroporation. In a primary lens cell culture, Equarin promoted biochemical and morphological changes of lens epithelial-to-fiber differentiation. A loss-of-function analysis was performed using zinc-finger nucleases targeting Equarin gene. Lens cell differentiation was markedly inhibited while the endogenous Equarin was blocked, indicating that Equarin is essential for normal chick lens differentiation. Furthermore, a biochemical analysis showed that Equarin directly binds to FGFs and heparan sulfate proteoglycan, thereby upregulated the expression of phospho-ERK1/2 (ERK-P) proteins, downstream of the FGF signaling pathway, in vivo and in vitro. Conversely, absence of endogenous Equarin obviously diminished FGF-induced fiber differentiation. Therefore, our results suggest that Equarin is involved as an FGF modulator in chick lens differentiation.

During lens differentiation, epithelial cells undergo vertical elongation, with anterior and posterior tips of the elongating fiber cells sliding along the epithelium
and capsule, respectively, as these cell migrate inward. Lens differentiation involves dynamic regulation of cell-matrix and cell-cell adhesion, both of which are dependent on adhesion molecules. Equarin protein was found to be expressed in the extracellular region of lens elongating fiber cells and cell-cell borders. Equarin could promote lens epithelial cell adhesion through heparan sulfate proteoglycan. By biochemical analysis, we found Equarin directly bound syndecan 3 which showed similar expression pattern with Equarin. Consequently, Equarin is also involved in lens cell adhesion during lens development and differentiation.
List of Abbreviations

µg: microgram
ng: nanogram
ml: mililiter
µm: micrometer
pM: picomolar
nM: nanomolar
mM: milimolar
FCS: fetal calf serum
PFA: paraformaldehyde
DMEM: Dulbecco’s modified Eagle’s medium
DCDMLs: dissociated cell-derived monolayer cultures
HH: hamburger-Hamilton stage
ZFNs: zinc finger nucleases
FGF: fibroblast growth factors
FGFR: fibroblast growth factors receptor
CCDC80 coiled-coil domain-containing protein 80
HSPG: heparan sulfate proteoglycan
ECM: extracellular matrix
SDC: syndecan
IGF: insulin-like growth factor
PDGF: platelet-derived growth factor
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1. Introduction

1-1. Literature review

1-1-1. Overview of embryonic lens development

The normal development of an organism requires the elaborate control of cell proliferation, migration and differentiation. The eye is considered ideal for studying the embryonic development of an organ. In particular, owing to its simple structure and ease of manipulation, the ocular lens has been focused on by developmental biologists and has been considered for many decades to be an excellent tissue in which to uncover general mechanisms underlying embryonic developmental processes. Meanwhile, a detailed knowledge of the lens cellular features is central to an understanding of the pathology and therapeutics of the lens cataract.

Lens induction

The lens arises from a region of head ectoderm that lies adjacent to the region of neural plate from which the retina will form (Figure 1A). As the developmental proceeds, the region of presumptive lens ectoderm becomes closely associated with neutrally derived optic vesicle (Figure 1B). Just after the optic vesicle establishes close contact with the presumptive lens ectoderm, the first sign of lens formation appears. The presumptive lens ectoderm thickens to form a lens placode (Figure 1C). The tissue interactions that take place earlier in the embryo are important for lens induction (Lewis, 1904; Spemann, 1901). Over the years, the view that lens induction is a simple one-step model involving an interaction between presumptive lens ectoderm and optic vesicle has evolved into the recognition that lens induction is a multi-step process that involves a series of inductive interactions. Fisher and Grainger (2004) propose a current model for lens determination that includes five stages: competence, bias, specification, inhibition, and differentiation.
**Lens morphogenesis**

During the morphogenesis of the lens, the presumptive lens thickens to form the placode and then invaginates together with the optic vesicle to form lens pit and optic cup, respectively (Figure 1D and E). The lens pit deepens (Figure 1F) and it finally breaks away from the ectoderm to form the lens vesicle (Figure 1G). The optic cup completely surrounds the primary lens vesicle, except for the anterior side. Subsequently, the cells in the anterior part of the lens vesicle form a monolayer of epithelium covering the anterior surface, whereas cells in the posterior half of vesicle elongate and differentiate to form primary fibers (Figure 1H) (Lovicu and McAvoy, 2005; Reza and Yasuda, 2004; Robinson, 2006). In this way, lens acquires its distinctive polarity.

![Figure 1](image_url)

**Figure 1.** Schematic representation of vertebrate lens development. Gray represents retina lineage, green represents lens lineage and yellow indicates ectodermal tissue other than lens structure. Arrows show the direction of the successive stages of endogenous lens formation. OV, optic vesicle; SE, surface ectoderm; PLE, presumptive lens ectoderm; LP, lens placode; OC, optic cup; LV, lens vesicle; LF, lens fiber; LE, lens epithelium; NR, neural retina; RPE, retinal pigment epithelium (Reza and Yasuda, 2004).
Initially all of the cells of the lens vesicle are capable of proliferation, but the primary fiber cells quickly lose their ability to proliferate as fiber differentiation progresses. As development progresses, lens cell proliferation normally becomes largely restricted to a band of epithelial cells slightly anterior to the lens equator known as the germinative zone. The post-mitotic region of lens epithelial cells posterior to the germinative zone is known as the transitional zone, as these are the epithelial cells in transition to becoming secondary fiber cells. Within the transitional zone, the expression of many genes characteristic of fiber cell differentiation first becomes evident. Immediately posterior to the transitional zone (at the lens equator) cells line up into columns called meridional rows and begin elongating into secondary fiber cells (Figure 2; Harding et al., 1971; McAvoy, 1978a,b). In the avian lens, the epithelium thickens at the lens periphery, forming the annular pad. Mitosis at the border of the epithelium and the annular pad produces cells that will eventually differentiate into the fiber cells that comprise the bulk of the lens (Figure 3A and B).

The secondary fibers compose the layers, or stratum, of the lens. During their differentiation, fiber cells become progressively longer until their tips reach the sutures at the anterior and posterior poles of the lens. At the sutures, they contact fiber cells from opposite side of the lens (Figure 3B). Shortly after reaching the sutures, fiber cells detach from the lens capsule and are covered by the next cohort of differentiating cells. After fiber elongation is complete, fibers routinely eliminate their organelles, termed as fiber terminal differentiation. In this way the lens continues to grow throughout the life by progressively adding on layer upon layer of secondary fiber cells onto the lens nucleus composed of the primary fiber cells formed during embryonic development.
Figure 2. Diagram indicating the pattern of vertebrate lens cell behavior. The mature lens consists of an anterior epithelial layer composed of non-proliferating central lens epithelial cells (cuboidal cells with white cytoplasm) and a narrow band of proliferating cells known as the germinative zone (pink cells). Just posterior to the germinative zone is the transitional zone (blue cells) where many genes important for fiber cell differentiation are initially expressed. Just posterior to the lens equator (dotted line) transitional zone epithelial cells begin elongating forming secondary fiber cells (green cells). As secondary fiber cells progress through later stages of differentiation, they lose their intracellular organelles (represented by the shrinkage and loss of red nuclei). The lens nucleus (yellow) is composed of fiber cells that were present in the embryonic lens. The mature lens is bathed on the anterior surface by the aqueous humor and on the posterior surface by the vitreous humor. Adapted from Lovicu and McAvoy., 2005.
Figure 3. Chicken embryo lens sections along the equatorial (A) and midsagittal axes (B). (Bassnett, S. and Beebe, D. 2010)

1-1-2. The lens fiber differentiation

Following the differentiation and elongation of cells at the posterior of the lens vesicle into primary fiber cells during early embryogenesis, all further growth of the lens is due to proliferation of epithelial cells and their subsequent differentiation into secondary fiber cells at the equatorial region of the organ (reviewed in Piatigorsky, 1981; Wride, 1996). The transparency and refractive properties of the lens depend directly on cellular features that arise during fiber cell differentiation. Epithelial-to-fiber differentiation is characterized by distinct morphological and molecular changes: epithelial cells exit from the cell cycle, undergo extensive elongation, develop specialized cell junctions accumulate fiber-specific proteins including various crystallins, the intermediate filament protein CP49, and the plasma membrane protein MP26 (known as MP28 in the chick). Eventually, intracellular organelles are lost and synthesis of both DNA and protein ceases. Proliferation of epithelial cells and their differentiation into secondary fibers continue throughout the lifetime of the organism, albeit more
slowly postnatally than during embryogenesis (Harding et al., 1977).

**The stages of fiber cell differentiation**

The lens grows by the steady addition of fiber cells at its polarity. All cells are retained within the lens. Consequently, fiber cell close to the surface of the lens are younger than the cells in the lens core. All lens fiber cells functions as optical elements and share an elongated prismatic shape. Although fiber cells are not always marker by gross morphological alterations, they can be discerned by the presence or absence of characteristic biochemical markers. Throughout the growth of fiber cells, the life of a fiber cell has been divided into four distinct stages (Bassnett, S. and Beebe, D. 2010) (Figure 4). *Fiber cell precursors* are produced by mitosis of cells in the periphery area of the epithelium. Fiber cells withdraw from the cell cycle and elongate from the columnar progenitor cells at the lens equator. *Elongating fiber cells* make distinct contact with the capsule and the epithelium at their basal and apical surfaces, respectively. When elongation is complete, fiber cells lose contact with the capsule and the epithelium. Distinct basal and apical adherens complexes can no longer be visualized by antibody staining. These *maturing fiber cells* appear to restructure their lateral membrane complexes, and there is a concomitant increase in the folding of their lateral membranes (Beebe et al., 2001; Kuszak et al., 1980; Willekens and Vrensen, 1982), presumably more firmly locking the fibers to their neighbors. At this point, fiber cell lateral membranes partially fuse, creating large pores between adjacent cells (Shestopalov and Bassnett, 2000). The final stage in the life of a fiber cell begins with the abrupt loss of intracellular, membrane-bound organelles. The removal of organelles is necessary because their retention would cause a significant diffraction of light and thereby comprise lens function. The resulting *mature fiber cell* is the fully differentiated cell type of the lens.
Figure 4. Stages in the differentiation of a fiber cells. (A) Mitotically active cell near the equatorial margin of the lens epithelium. (B) Elongating fiber cells. (C) Maturing fiber cells. (D) Mature fiber cells. (Bassnett, S. and Beebe, D. 2010)
Fibroblast growth factor signaling

How the two forms of lens cells differentiate in the lens has been a major focus of lens developmental biology. Studies over the last 20 years have mostly concentrated on identifying factor(s) that control fiber differentiation, whereas epithelial cells have been studied mostly in relation to their proliferative function. The ocular media are a rich source of growth factors, and the lens itself expresses members of the major growth factor families and a variety of growth factor receptors. Over 20 years of accumulated evidence from several different vertebrate species have suggested that fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs) play key roles in lens development. FGF signaling has been implicated in lens induction, lens cell proliferation and survival, lens fiber differentiation and lens regeneration (Le and Musil, 2001; Lovicu and Overbeek, 1998; Robinson et al., 1995, 1998; Robinson, 2006). Essentially, studies showed that FGF1 or FGF2 promoted morphological and molecular changes in lens epithelial explants that are characteristic of secondary fibre differentiation in vivo. To date, FGF is the only factor that is known to be capable of initiating epithelial-to-fiber differentiation.

It has been shown using this explant system that FGF induced different responses in lens epithelial cells with increased dosage; a low concentration of FGF induced cell proliferation, whereas higher doses were required to induce fiber cell differentiation (McAvoy and Chamberlain, 1989). This finding, together with the fact that more FGF is recoverable from vitreous than aqueous (Schulz et al., 1993), led to the proposal (Lovicu and McAvoy, 2005) that the distinct polarity of the lens in situ may be determined by an FGF gradient in the eye (Figure 5). This also fits well with the fact that vitreous humor (which bathes lens fiber cells in vivo), but not aqueous humor (bathes the lens epithelium), can induce fiber differentiation in rat lens explants (Lovicu et al., 1995).
Figure 5. Diagram indicating how ocular media govern the antero-posterior pattern of lens cell behavior in vivo. Epithelial cells and fiber cells are contained within a capsule of extracellular matrix (thick blue line). The germinative zone (red asterisks) is situated just above the equator. Cells that shift below the equator are exposed to the relatively high level of FGF in vitreous and initiate a signalling cascade that promotes fiber differentiation (Lovicu et al., 2011).
**The lens cell adhesion**

Adhesion molecules are known to be instructive for both development and differentiation. During lens differentiation, epithelial cells undergo vertical elongation, with anterior and posterior tips of the elongating fiber cells sliding along the epithelium and capsule, respectively, as these cell migrate inward. These cellular processes are highly coordinated through cell adhesive interactions, actin cytoskeletal reorganization and generation of contractile force (Bassnett et al., 1999; Kuszak et al., 2004; Piatigorsky, 1981; Taylor et al., 1996; Zelenka, 2004). The ability of a cell to recognize and interact with specific extracellular matrix (ECM) components is a fundamental requirement of cell migration and differentiation. Cells systematically create and dissolve cell-cell and cell-matrix adhesions, form connections between these adhesions and the cytoskeleton, and generate contractile force. Since errors in cell adhesion may lead to aberrant lens shape or misplacement of the lens sutures, precise regulation of each step is essential for the optical quality of the lens (Kuszak et al., 1994). Alterations in extracellular matrix composition that interfere with these interactions can lead to defects that alter tissue morphogenesis and the state of differentiation (Juliano and Haskill, 1993).

Examination of the in vivo expression patterns of extracellular matrix components, integrins, and adhesion proteins in the lens suggests that many of the proteins involved in fibroblast migration (Holly et al., 2000; Etienne-Manneville and Hall, 2002) will have similar roles in the lens. The lens is surrounded by an elaborated basement membrane composed of collagen IV, laminin, fibronectin (Cammarata et al., 1986) and a variety of proteoglycans, which are necessary for proper adhesion and migration. Integrins capable of binding these extracellular matrix proteins are expressed along fiber cell membranes (Menko and Philp, 1995; Menko et al., 1998; Menko and Walker, 2004). Focal adhesion proteins such as FAK, MLCK, caldesmon, and paxillin, are arranged at the basal tips of elongating
fiber cells in an unusual, two-dimensional array referred to as the basement membrane complex (Bassnett et al., 1999). In addition, many signaling proteins known to regulate aspects of fibroblast migration, such as Rho family, are also expressed in the lens. Thus, the expected cellular adhesion machinery and the signaling pathways that coordinate and regulate its operation are present in the lens.

1-2. Background and Specific aims

Background

The development of the eye is controlled by a combination of intrinsic and extrinsic signals. Lens plays an important role during eye development. Without a lens placode, the optic vesicle remains rudimentary, and no optic cup with well-defined neuroretinal and pigmented layers is formed (Hyer et al., 1998). Ablation of the developing lens using a toxin transgene inhibits the formation of the iris, the ciliary body, the vitreous body, and the retina (Breitman et al., 1989; Harrington et al., 1991). Therefore, lens is an important source of signals that influence the eye development and a variety of genes expressed by the lens has been identified. The identification of additional molecule(s), especially secreted ones that possibly mediated signals, will extend our knowledge of the molecular mechanisms of eye and lens development. In our search, we used signal sequence trap screens (Klein et al., 1996) to isolate molecule(s) from a chick E6 lens cDNA library. We found that a novel clone was expressed in the lens equatorial region and was therefore designated ‘Equarin’. Equarin was previously found to be involved in the retinal development (Mu et al., 2003). Since the lens equatorial region is the transitional zone from the anterior epithelial cells to posterior fiber cells, Equarin is predicted to play an important role in the cell differentiation during chick lens development.

Although FGF is essential for fiber cell differentiation, it is clearly not sufficient (O’Connor and McAvoy, 2007; Wang et al., 2010). FGF1- and FGF2-deficient mice exhibited no lens development
defects (Dono et al., 1998; Miller et al., 2000). Further evidence indicates that factors besides FGFs, including insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), (Wang et al., 2010), bone morphogenic proteins (BMPs) (Boswell et al., 2008a; Boswell et al., 2008b) and Wnt (Lyu and Joo, 2004), are required to coordinate with FGF for fiber differentiation. Therefore, the identity of novel molecules that play an important role in fiber differentiation and the precise mechanism of FGF function remain key questions in lens development.

Although important insights have emerged regarding the identity of genes controlling lens induction and differentiation, the signaling mechanism(s) regulating fiber cell adhesive interaction are presently far from clearly understood. Mouse CCDC80 (coiled-coil domain-containing protein 80; also known as DRO1 and URB), which show high homologue and structure similarity to chick Equarin, has been reported to be involved in cell adhesion and migration (Manabe et al., 2008). Meanwhile, immunocytochemical studies showed that Equarin protein was present in the extracellular region of transient Equarin expressing cells (Mu et al., 2003). Taken together, these data lead us to hypothesize the involvement of Equarin in chick lens cell adhesion during lens development.

Specific aims:

a) Since Equarin is expressed exclusively in the equatorial region of lens where is the transitional zone from the anterior epithelial cells to posterior fiber cells throughout the life, I explored the role of Equarin in lens fiber differentiation by both gain-of-function and loss-of-function assays.

b) Although our in vivo and in vitro studies clearly demonstrated that Equarin serves as a novel differentiation inducer, the mechanism of Equarin-mediated action remains unclear. Therefore, my second aim is to determine the mechanisms by which Equarin facilitates its effects on lens
cell differentiation.

c) Due to expression of Equarin protein in the extracellular region of cells and the involvement of mouse CCDC80 (homologue of chick Equarin) in cell adhesion, I examined the involvement of Equarin in cell adhesion during chick lens development and the molecular mechanism.
2. **Material and Methods**

2-1. **Embryos**

Fertilized White Leghorn chicken embryos that were obtained from a local supplier were incubated at 38°C in a humidified incubator. The embryos were staged as previously described by Hamburger and Hamilton (1951).

2-2. **Equarin protein purification and monoclonal antibody production**

To raise a monoclonal antibody against the Equarin protein, we purified an Equarin-Myc-His chimeric protein from the culture medium of Equarin-expressing HEK293 cells. The immunization was performed in accordance with previously described procedures (Ohta et al., 1996). The hybridomas were screened using an enzyme-linked immunosorbent assay and then by immunohistochemistry using both COS-7 cells that transiently expressed Equarin and lens sections from E10 chick embryos. Two hybridoma cell lines were established, which are referred to as clone 1 and clone 3.

2-3. **In vivo electroporation and analysis of phenotypes**

The Equarin-Myc-His cDNA fragment was subcloned into the pCAGGS vector (Niwa et al., 1991). The electroporation of the DNA constructs was performed as previously described (Figure 6). The pCAGGS-Equarin-Myc-His construct or empty vector was electroporated together with pCAGGS-GFP at a ratio of 9:1 into one side of the head ectoderm of Hamburger-Hamilton stage (HH) 10 embryos. The embryos were then cultured in ovo to the developmental stages of interest, and those embryos that displayed a GFP signal within the lens region were selected for further analysis. We confirmed that the development of the empty-vector-electroporated lenses was equivalent to the development of the contralateral un-electroporated lenses, and the Equarin-electroporated lenses were compared to the empty-vector-electroporated lenses.
**Figure 6.** Electroporation of head ectoderm of stage 10 embryos *in ovo*. The plasmids are electroporated into one side of the head ectoderm including the presumptive lens. Electroporation is carried out with five square pulses of 20 V for a duration of 50 ms and with 100 ms intervals using a pair of platinum electrodes with an interelectrode distance of 4 mm. Adapted from Uchikawa, 2008.

### 2-4. Cell cultures and treatments

Dissociated cell-derived monolayer cultures (DCDMLs) from lens epithelial cells were prepared from E10 chick lenses and plated at $1 \times 10^5$ cells/well on laminin-coated 96-well tissue culture plates, as previously described by Le and Musil (1998). The cells were cultured in Dulbecco’s modified Eagle’s medium that contained BOT (2.5 mg/ml bovine serum albumin [BSA] and 25 mg/ml ovotransferrin) and gentamicin in the presence or absence of additives at 37°C in a 5% CO$_2$ incubator. The cells were fed every 2 days with fresh medium. Recombinant bovine FGF2 was obtained from R&D Systems (Minneapolis, MN, USA), and recombinant FGF1 was obtained from Sigma-Aldrich (St Louis, MO, USA).

### 2-5. Construction of functional ZFNs

ZFNs targeting the Equarin gene were generated by one hybrid screening of a zinc-finger library
2-6. Transient transfection of lens cells

The DCDML cultures were transfected 1 day after plating in Opti-medium without antibiotics using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The pCAGGS empty vector, pCAGGS-Equarin-Myc-His, p3XFLAG-FGF2 or pCAGGS-ZFNs were used at a concentration of 0.2 μg DNA per well in a 96-well tissue culture plate. After incubation with the DNA for 6 hours, the transfection medium was supplemented with DMEM containing BOT. The cells were cultured for an additional 4 days prior to analysis.

2-7. In situ hybridization, immunohistochemistry and western blotting

To prepare samples for in situ RNA hybridization and immunohistochemistry, dissected embryos were fixed overnight in 4% paraformaldehyde (PFA) and embedded in Optimal Cutting Temperature compound (Sakura, Torrance, CA, USA) before being snap-frozen. The in situ hybridization was performed as previously described (Mu et al., 2003) using digoxigenin (DIG)-labeled antisense Equarin probes, which were produced from the corresponding DNA construct. Immunostaining was performed using specific primary antibodies, followed by incubation with fluorescent secondary antibodies and Hoechst staining to label the nuclei.

For the immunoblotting analysis, the DCDML cultures were solubilized in SDS-PAGE sample buffer and boiled for 10 minutes. An aliquot of 1 μg (β-crystallin), 3 μg (CP49) or the entire cell lysate (ERK) from a 96-well culture plate was analyzed using SDS-PAGE. Intact lenses from HH 20 embryos were dissected and immediately solubilized in lysis buffer; 20 μg of the total lens protein was analyzed using SDS-PAGE. After electrophoresis, the proteins were transferred to polyvinylidene fluoride
(PVDF) membranes (Millipore, Billerica, Massachusetts, USA). Immunoreactive bands were detected with enhanced chemiluminescence (ECL-Plus) using horseradish-peroxidase-linked anti-rabbit or anti-mouse IgG antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The following primary antibodies were used for immunostaining or immunoblotting: anti-Myc monoclonal antibody (9E10; the Developmental Studies Hybridoma Bank [DSHB], University of Iowa, Iowa City, IA, USA); anti-β-crystallin (DSHB); anti-Flag-M2 monoclonal antibody (Sigma-Aldrich, St Louis, MO, USA); rabbit anti-mouse CP49 polyclonal serum (#899, a generous gift from Paul FitzGerald, University of California), anti-p44/42 MAP kinase polyclonal rabbit antibody (Cell Signaling Technology, Beverly, MA, USA) and anti-β-tubulin (R&D Systems).

**2-8. Co-precipitation**

COS-7 cells were transfected with Myc-His-tagged Equarin and either Flag-tagged FGF1 or Flag-tagged FGF2. The conditioned media from both of the transfections were incubated together overnight at 4°C, and this was followed by incubation with ProBand Resin (Invitrogen, Carlsbad, CA, USA) on a rotator for 2 hours. Next, the beads were washed in IP buffer (containing 0.1% BSA, 150 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1.5 mM CaCl₂, 1.5 mM MgCl₂, 0.1% Triton X-100 and 0.1% CHAPS), and the protein-bound beads were analyzed using SDS-PAGE. The Myc-tagged and Flag-tagged proteins were detected using 9E10 (DSHB) and M2 (Sigma) antibodies, respectively.

**2-9. Binding of Equarin to heparin Sepharose**

We transfected pCAGGS-Equarin-Myc-His and p3XFLAG-CMV-FGF2-Flag into COS-7 cells. 500 μl of conditioned serum-free medium was incubated for 3 hours at 4°C with 30 μl of heparin-Sepharose CL-6B beads (GE Healthcare Bio-Sciences AB, Sweden). The bound materials were then eluted by boiling in SDS-PAGE sample buffer and detected by SDS-PAGE and western blotting. The
Myc-tagged Equarin protein was detected with an anti-Myc antibody (9E10; DSHB), and the Flag-tagged FGF2 was detected with an anti-Flag-M2 antibody (Sigma). Binding to heparin-Sepharose was performed either with or without the addition of 1.0 M NaCl.

2-10. Attachment assay

Purified Equarin protein and fibronectin that was derived from bovine plasma (Sigma) were used as the substrates. The negative control consisted of 1% BSA in DMEM. Nunc-Immuno™ 96-well plates with a MaxiSorp™ surface (Nunc) were coated with different substrates overnight at 4°C, rinsed with PBS and incubated with 1% BSA in DMEM medium for 1 hour at 37°C. Subsequently, 100 μl of the dissociated lens epithelial cell suspension (1×10^5/ml) was added to each well, and this was incubated at 37°C in a 5% CO2 incubator. After allowing the cells to attach for 1 hour, the wells were rinsed twice with DMEM, fixed for 20 minutes in 4% PFA, rinsed in PBS, and stained with 0.05% Toluidine Blue. Finally, the cells were solubilized in 1% SDS buffer and the absorbance was measured with UV-Visible Spectrophotometer 3000 (Pharmacia Biotech, Kenilworth, NJ) at 595 nm. Each assay point was derived from 3 separate wells. For the inhibition assay, the cells or wells were preincubated for 30 minutes with human IgG (10 μg/ml), RGD peptide (1 mM, Peptide Institute, Inc, Minoh-shi, Osaka, Japan) or heparin (100 μg/ml, Sigma).
3. Results

3-1. Part A: The role of Equarin in chick lens cell differentiation through FGF signaling

3-1-1. Expression of Equarin in the lens during early chick development

Localization of Equarin mRNA in the developing chick and mouse lens.

The expression of Equarin in the developing chick lens was first demonstrated by whole-mount in situ hybridization by Mu et al., 2003. Equarin mRNA was clearly detected to be expressed in the equatorial region in a high-dorsal-to-low-ventral gradient in the chick lens from the embryonic to postnatal stages.

In this study, we further studied Equarin mRNA expression in sections using in situ hybridization. Equarin mRNA was first detected at HH 13 in the lens placode (Figure 7A), which is defined as the thickened region of the ectoderm, and subsequently detected on the proximal side of the lens vesicle at HH 20 (Figure 7B). As lens development proceeded, Equarin was expressed in the lens equator region in a high-dorsal-to-low-ventral gradient (Figure 7C, D). The chicken lens presents an epithelial specialization known as the annular pad, which is composed of post-mitotic cells committed to and undergoing the initial stages of lens fiber formation. The annular pad cells of avian lenses are analogous to the transitional zone epithelial cells of mammalian lenses. Equarin was localized in both the annular pad (Figure 7C, D, between two arrowheads) and newly differentiated fiber cells (Figure 7C, D, arrows); this expression pattern seen in chicks was similar to that observed in mice (Figure 8A-C). Since the lens epithelial cells initiate differentiation at the equatorial region and many genes which are important for lens differentiation are expressed here, we hypothesized that Equarin may also play an important role in lens differentiation.
**Figure 7.** Spatial and temporal expression of Equarin in the developing chick lens. The mRNA (A-D) of Equarin was detected in coronal sections of chick eyes at HH 13 (A), HH 20 (E3) (B), and HH 36 (E10) (C, D). The orientation schematic is shown on A. Proximal is downward, and dorsal is toward the right. Note that Equarin was first detected in the lens placode at HH 13 and in the proximal side of lens vesicle at HH20. Gradually, Equarin was localized to the lens equatorial annular pad (C, D, between two arrowheads), newly differentiated fiber cells (C, D, arrows), and it displayed a high-dorsal-to-low-ventral gradient. Ip, lens placode; ov, optic vesicle; lv, lens vesicle; plf, primary lens fibers; (D), dorsal; (V), ventral. Scale bars: 100 μm.

**Figure 8.** Expression pattern of Equarin mRNA in the equator region in embryonic mouse lenses. Equarin mRNA expression was detected in the coronal sections of the mouse eye by in situ hybridization at E14 (A), E15 (B) and E16 (C) using a probe designed against mouse CCDC80/Equarin (Tremblay et al., 2009). The arrowheads indicate that the Equarin mRNA was expressed exclusively in the equator region of the mouse lens. Scale bar: 100 μm.
Distribution of Equarin protein in the developing chick lens.

To investigate the distribution of Equarin protein during lens development, we generated an anti-Equarin monoclonal antibody, which was screened by enzyme-linked immunosorbent assay and subsequently by immunohistochemistry using COS-7 cells that transiently expressed Equarin-Myc (Figure 9A). The Equarin protein that was detected, without fixation, by anti-Myc antibody staining was strongly expressed in the peripheral region of the cells. The staining pattern of the purified anti-Equarin monoclonal antibody (clone 3) was identical to that of the anti-Myc antibody (Figure 9A), indicating that the signal detected with the antibody corresponded to the Equarin protein.

During chick lens development, the Equarin protein distribution coincided with expression at the mRNA level, which was also detected in the lens placode at HH 13 (Figure 10A), in the proximal side of the lens vesicle at HH 20 (Figure 10B) and lastly in the equator region in a high-dorsal-to-low-ventral gradient that included the annular pad (Figure 10C, D, between the two arrowheads) and newly differentiated fiber cells (Figure 10C, D, arrows). Interestingly, Equarin protein was also expressed in the lens capsule (Figure 10C, D, open arrowheads). In addition to the distribution of Equarin protein in the lens, it was also distributed to the peripheral retina in a same manner. Conversely, there was no Equarin protein was detected in the central retina (Figure 11).
Figure 9. Specificity of the purified anti-Equarin monoclonal antibody. (A) COS-7 cells were transfected with an empty vector or pCAGGS-Equarin-Myc-His and were then processed without fixation for immunofluorescence, as indicated. Note that the immunostaining pattern that was obtained with the anti-Equarin monoclonal antibody (clone 3) is identical to the pattern that was obtained with the anti-Myc antibody. (B) No signal was observed when the first antibody was omitted from the incubation solution. Scale bar: 50 μm.

Figure 10. Distribution of Equarin protein in the developing chick lens. The protein distribution of Equarin was detected in coronal sections of chick eyes at HH 13 (A), HH 20 (E3) (B), and HH 36 (E10) (C, D). Proximal is downward, and dorsal is toward the right. Note that Equarin protein was detected in the lens placode at HH 13 (A), in the proximal side of the lens vesicle at HH 20 (B) and lastly in the equator region in a high-dorsal-to-low-ventral gradient that included the annular pad (C, D, between the two arrowheads) and newly differentiated fiber cells (C, D, arrows). Interestingly, Equarin protein was also expressed in the lens capsule (C, D, open arrowheads).
3-1-2. Overexpression of Equarin upregulates the expression of lens fiber-specific proteins in vivo

To address the role of Equarin during chick lens fiber development, we performed in ovo electroporation into the head ectoderm of HH 10 chick embryos. The pCAGGS-Equarin-Myc-His or empty vector was electroporated together with pCAGGS-GFP. After the embryos were cultured to HH 18, all of the empty-vector-electroporated embryos exhibited normal lens morphology and the same expression pattern of fiber proteins in the contralateral side and the electroporated side (Figure 12A-F).

In the Equarin-electroporated embryos, the Equarin protein was well distributed following electroporation. Equarin protein expression overlapped with that of the GFP signal, which represents the electroporation area, and it was also distributed in the remaining part of the lens (Figure 12L).
Importantly, Equarin was strongly trapped on the lens capsule (Figure 12L, arrowheads). The lens capsule is more than just structural support for the lens within the eye. It also provides necessary signals for proper lens cell proliferation, migration and differentiation (Blakely et al., 2000; Robinson, 2006). The binding of Equarin protein to the capsule suggested the involvement of Equarin in lens fiber differentiation.

As shown in Figure 12J, the Equarin-electroporated embryos showed lens structures that were phenotypically normal, suggesting that Equarin does not affect lens induction. To determine whether there was a change in the differentiation of the cells, we examined the expression of the fiber-specific marker β-crystallin, which is expressed in differentiated fiber cells of the developing chick lens (Reza and Yasuda, 2004). In 42% (15/35) of the embryos, although there was little primary fiber formation (Figure 12G) and weak β-crystallin expression (Figure 12H) in the contralateral lens, the Equarin-electroporated lens revealed the presence of the primary fiber that was going to fill the lens vesicle (Figure 12J) and exhibited strong β-crystallin staining (Figure 12K). Western blot analysis using lens lysates revealed that levels of β-crystallin were stronger in the Equarin-electroporated lens relative to the contralateral side (Figure 12M). These data suggest that Equarin upregulates β-crystallin expression during lens development.
**Figure 12.** Overexpression of Equarin promotes the expression of lens fiber proteins. HH 10 embryos were electroporated with either the empty vector (A-F) or pCAGGS-Equarin-Myc-His (G-L) together with pCAGGS-GFP and were incubated until HH18. Serial sections were made and stained with anti-Myc and anti-β-crystallin antibodies. Note that all of the embryos (n=6) that were electroporated with the empty vector (A-F) exhibited similar lens morphologies. In the Equarin-electroporated lenses (G-L), stronger β-crystallin expression was observed when compared with the contralateral lens (15/35). The arrowheads in panel L indicates the Equarin protein that was strongly trapped in the lens capsule. Scale bar: 100 μm. (M) Western blotting of lens lysates revealed the stronger expression of β-crystallin in the Equarin-electroporated lens relative to the level in the contralateral side (Student’s t-test, *P*<0.001; n=3). No statistical significance (N.S., n=3) was observed for the empty vector-electroporated embryos. β-tubulin was used as a loading control.

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| β-crystallin   |                   |                     |
| β-tubulin      |                   |                     |
| Myc            |                   |                     |

| Empty vector   |                      |                     |
| Equarin        |                      |                     |
3-1-3. Equarin upregulates biochemical and morphological markers of lens fiber differentiation in primary lens cell cultures

To further assess the function of Equarin in lens fiber differentiation, we used an in vitro culture system. In the normal lens, the cells from the equatorial epithelial regions, but not the cells in the central epithelium, receive growth factors from the vitreous humor and initiate differentiation. Because Equarin is expressed exclusively at the lens equator region during chick lens development, we attempted to include these cell populations using primary embryonic chick lens dissociated cell-derived monolayer (DCDML) cultures, instead of central explant culture (Le and Musil, 2001).

Lentoids are a morphological hallmark of DCDML differentiation (Le and Musil, 1998; Menko et al., 1984), and β-crystallin has long been a specific marker for detecting lentoidogenesis in cultures of chicken lens epithelial cells. Thus, the differentiated state of the lens cells in vitro can be classified by an examination of the expression pattern of β-crystallin (Sawada et al., 1993). CP49 appears after 3 days of embryonic development in the chick, and the up-regulation of CP49 expression can serve as a fiber-specific marker for lens fiber development in all vertebrates (Ireland et al., 2000). During the culture of the DCDMLs, each well was plated with the same number of dissociated lens cells. We observed that the number and average size of the lentoids were notably increased in the Equarin-treated cells relative to the controls (Figure 13A). These multilayered clusters stained strongly for β-crystallin and CP49 (Figure 13A).

Low levels of β-crystallin and CP49 were detected in the DCDMLs that were maintained in control medium for 6 days. Although the same amount of protein was analyzed per lane, over the 6-day treatment period, the purified Equarin protein (20 nM) increased the expression of β-crystallin and CP49 in the DCDMLs to a similar extent as the known fiber-differentiation-promoting factors FGF1
and FGF2 (Figure 13B, C). The upregulation of both fiber markers required more than a 3-day exposure to Equarin. On the basis of both biochemical and morphological criteria, we concluded that Equarin promotes lens epithelial-to-fiber differentiation in DCDMLs.

Figure 13. Equarin upregulates the expression of epithelial-to-fiber differentiation markers in the primary culture of chick lens epithelial cells (DCDMLs). The DCDMLs were cultured in DMEM/BOT medium for 6 days in the absence (control), or presence of Equarin (20 nM), FGF1 (0.6 nM) or FGF2 (1 nM). (A) The DCDMLs that were cultured for 6 days with or without Equarin were imaged by phase-contrast microscopy to show the formation of the lentoids or were immunostained for β-crystallin or CP49. Scale bars: 50 μm. (B) An aliquot of 1 μg (β-crystallin) or (C) 3 μg (CP49) of SDS-solubilized whole cell lysate from a single well was analyzed using immunoblotting. The data are shown as the amount of β-crystallin or CP49 protein in the treated cells relative to the amount in the untreated controls within the same experiment. β-tubulin is shown as a loading control.
3-1-4. Generation of functional ZFNs targeting the Equarin gene

To gain insights into the function of specific genes during animal development, loss-of-function assays are widely used in most model animals. Recently, a new method for targeted mutagenesis using engineered zinc-finger nucleases (ZFNs) has been reported in Drosophila (Beumer et al. 2008), zebrafish (Doyon et al. 2008; Meng et al. 2008; Foley et al. 2009), plants (Shukla et al. 2009; Townsend et al. 2009) and human cultured cells (Urnov et al. 2005; Hockemeyer et al. 2009; Zou et al. 2009). ZFNs consist of a customized array of zinc fingers (ZFs) that bind to a specific DNA sequence and a nuclease domain of the restriction enzyme FokI (Figure 14A). When two ZFNs bind to their associated target sequences in the appropriate direction, their nuclease domains are able to dimerize and introduce a doublestranded break (DSB). The ZFN-induced DSB can then be repaired with high efficiency by either homology-directed repair or error-prone nonhomologous end joining (NHEJ) repair, independently of a DNA template for repair. Therefore, ZFNs induce a site-specific insertion or deletion at the site of the DSB after NHEJ repair (Figure 14B) (Doyon et al. 2008; Maeder et al. 2008; Meng et al. 2008).

To elucidate the role of endogenous Equarin in lens differentiation, we carried out loss-of-function experiments using ZFN technology in chick primary lens DCDML cultures to introduce targeted mutations of Equarin. We generated ZFNs targeting the Equarin gene (Figure 15A) in accordance with previously described procedures (Ochiai et al., 2010) and examined its efficiency and functionality. First, to determine whether the ZFNs caused site-specific mutations in Equarin, the DNA fragments amplified from Equarin-ZFNs-transfected primary lens DCDML cultures were digested with the
restriction enzyme BssSI, which has one recognition site in the target site for the Equarin ZFNs (Figure 15B). As non-homologous end joining (NHEJ) repair can lead to short deletions and insertions, we would expect the BssSI site to be lost in fragments amplified from Equarin-ZFN-transfected DCDML cultures. The fragments amplified from the empty-vector-transfected DCDML cultures showed complete digestion with BssSI. By contrast, a larger undigested band representing the original DNA fragment was observed in the DNA fragments from the ZFN-transfected DCDML cultures, indicating the absence of a BssSI site in the target sequence. These observations are consistent with the presence of a ZFN-directed mutagenic lesion at this site.

Next, to better characterize these ZFN-induced lesions and quantify the mutagenesis frequency, we cloned and sequenced PCR fragments from the Equarin-ZFN-transfected DCDML cultures (Figure 15C). Among the 61 clones examined, a variety of lesions comprising deletions (12 clones, 20%) or deletions and insertions (18 clones, 30%) were observed. In total, we identified lesions in 30 of 61 clones, indicating that the ZFN-induced mutagenic frequency was 49% in chick primary lens cultures. These results demonstrated that engineered ZFNs could introduce a double-stranded break (DSB) into the Equarin gene and the resulting error-prone NHEJ repair could introduce mutations into the Equarin gene in chicks.
Figure 14. (A) Each ZFN consists of two functional domains: A DNA-binding domain comprised of a chain of two-finger modules, each recognizing a unique hexamer (6 bp) sequence of DNA. Two-finger modules are stitched together to form a Zinc Finger Protein, each with specificity of ≥ 24 bp. A DNA-cleaving domain comprised of the nuclease domain of Fok I. When the DNA-binding and DNA-cleaving domains are fused together, a highly-specific pair of ‘genomic scissors’ are created. (B) A schematic representation to demonstrate potential outcomes of a double-strand break in DNA generated by a pair of ZFNs, DNA deletion or DNA insertion. Adapted from www.sangamo.com.
Figure 15. Generation of functional ZFNs that target chick Equarin gene. (A) A schematic representation of Equarin gene is shown. Exons are indicated by boxes. Gray and black boxes represent untranslated regions and coding regions, respectively. The ZFN-targeted sequence and a pair of ZFNs used in this study are shown above. (B) A schematic diagram of the Equarin genomic region used for the PCR-based analysis is shown on the left side of the panel. The target site for Equarin ZFNs contains one BssSI site. The PCR products extracted from the empty-vector-transfected DCDML cultures (C) or Equarin-ZFNs-transfected DCDML cultures (Z) were digested with BssSI to assay the presence or absence of the BssSI site. The control fragment showed complete digestion with BssSI. The larger
undigested fragment was caused by the loss of the BssSI site. (C) Sequence analysis of the Equarin-ZFNs-transfected DCDML cultures. Boxes indicate wild-type Equarin sequence. Color shaded boxes label the ZFN recognition elements. Red dashes or letters indicate the positions of deletions or insertions, respectively.

3-1-5. Endogenous Equarin is required for chick lens cell differentiation

To confirm the validity and specificity of the ZFNs targeting Equarin, we co-transfected COS-7 cells with the Equarin ZFNs and Equarin or Akhirin. Like Equarin, Akhirin is a function-unknown secreted molecule that was isolated from chick E6 lenses (Ahsan et al., 2005). Akhirin protein was expressed normally in the presence of the Equarin ZFNs. Conversely, Equarin protein expression was significantly decreased, whereas the ZFNs were transiently co-expressed (Figure 16A), indicating the specificity of the ZFNs for Equarin.

Subsequently, we examined the efficiency of Equarin ZFNs in chick lens primary DCDML cultures. Equal amounts of protein were loaded in each lane for blotting analysis. Like exogenously added purified Equarin protein, transiently expressed Equarin promoted epithelial-to-fiber differentiation. Importantly, the differentiation induced by transiently expressed Equarin was blocked by the targeted mutation of Equarin through the ZFNs. β-crystallin levels remained low in the DCDMLs after transient co-transfection with Equarin and the Equarin ZFNs indicating the validity of the ZFNs in lens primary DCDML cultures (Figure 16B).

DCDML culture cells have a limited potential to undergo fiber differentiation when cultured under control conditions without additional factors. We noticed that transient transfection with the ZFNs appeared to reduce the level of lens fiber cell autonomous differentiation. β-crystallin levels were markedly diminished when endogenous Equarin was blocked by ZFNs (Figure 16B). In all cases, the
ZFN-treated cells retained the same viability as the controls. This finding further supports the conclusion that Equarin is indispensable during lens differentiation.

Figure 16. Effect of ZFNs targeting Equarin on chick lens differentiation in DCDML culture. (A) Engineered ZFNs specifically knocked down Equarin. Co-transfection of Equarin ZFNs and Equarin or Akhirin was performed. The Equarin protein was not detected in the presence of the ZFNs, whereas the Akhirin protein was normally expressed. (B) The DCDML cultures were transfected with the pCAGGS empty vector or the pCAGGS-Equarin-Myc-His or pCAGGS-ZFNs plasmids one day after being plated. The cells lysates were prepared 4 days after the transfection and assessed for β-crystallin by western blotting. β-tubulin is shown as a loading control. While transiently expressed Equarin promoted fiber differentiation relative to empty-vector-transfected cells (*P<0.001; n=3), β-crystallin levels remained low after transient co-transfection with Equarin and the Equarin ZFNs (*P<0.001; n=3). Meanwhile, transient transfection with the ZFNs appeared to reduce the level of lens fiber cell autonomous differentiation relative to empty-vector-transfected cells (*P<0.001; n=3).
3-1-6. Equarin interacts with FGFs and heparan sulfate proteoglycan

Given the role of Equarin in epithelial-to-fiber differentiation, it is important to determine the mechanisms by which Equarin facilitates its effects on lens cell differentiation. FGF1 and FGF2 protein has been previously found to be distributed in the germinative and transitional zones of the lens and in the lens capsule (Lovicu and McAvoy, 1993). Importantly, we found that Equarin protein was also strongly expressed in the equatorior region and the lens capsule (Figure 10C, D, 12L). Therefore, we performed a co-precipitation assay to examine possible molecular interactions between Equarin and the FGFs. As shown in Figure 17A and B, both FGF1 and FGF2 were co-precipitated with Equarin, indicating a direct molecular interaction between Equarin and both FGF1 and FGF2.

The heparan sulfate proteoglycans, which act as co-receptor of FGFs to play a key role in its interaction with FGFR, are part of the core ultrastructure of basement membranes, such as lens capsule. FGF1 and FGF2 are secreted from various ocular sites into the capsule, where they bind to heparan sulfate proteoglycans (Schulz et al., 1997) and to specific FGF receptors (de Iongh et al., 1997). To investigate whether Equarin binds heparan sulfate proteoglycan(s), in vitro heparin-binding assays were carried out with heparin beads in either the presence or absence of 1.0 M NaCl; FGF2, which is known to strongly bind to heparin, was used as a control. The bound materials were eluted and analyzed with SDS-PAGE. As shown in Figure 17C, Equarin bound to heparin-Sepharose, and this binding was eliminated in the presence of high ionic strength; this result was also observed for FGF2. This data confirmed that Equarin interacts with heparan sulfate proteoglycan(s).
Equarin binds directly to FGF1, FGF2 and heparan sulfate proteoglycans. (A, B) Equarin binds to FGF1 and FGF2. Equarin-Myc-His- and FGF-Flag-containing conditioned media were incubated together, followed by incubation with ProBand Resin. The input amount of each protein was detected using immunoblotting with an anti-tag antibody. After immunoprecipitation, bound FGF1/2 was detected by immunoblotting with an anti-Flag antibody. Lane 1 indicates that in the absence of Equarin, FGF-Flag could not be detected. Conversely, Lane 2 indicated that FGF-Flag protein was visualized only in the presence of Equarin, suggesting that FGF was co-precipitated by Equarin. (C) Equarin binds to heparin-Sepharose. Lane 1 shows the starting material, lane 2 shows the bound material under physiological ionic strength, and lane 3 shows the bound material after the addition of 1.0 M NaCl.

Figure 17. Equarin binds directly to FGF1, FGF2 and heparan sulfate proteoglycans. (A, B) Equarin binds to FGF1 and FGF2. Equarin-Myc-His- and FGF-Flag-containing conditioned media were incubated together, followed by incubation with ProBand Resin. The input amount of each protein was detected using immunoblotting with an anti-tag antibody. After immunoprecipitation, bound FGF1/2 was detected by immunoblotting with an anti-Flag antibody. Lane 1 indicates that in the absence of Equarin, FGF-Flag could not be detected. Conversely, Lane 2 indicated that FGF-Flag protein was visualized only in the presence of Equarin, suggesting that FGF was co-precipitated by Equarin. (C) Equarin binds to heparin-Sepharose. Lane 1 shows the starting material, lane 2 shows the bound material under physiological ionic strength, and lane 3 shows the bound material after the addition of 1.0 M NaCl.
3-1-7. Equarin promotes lens fiber differentiation via the FGF-FGFR signaling pathway

It has frequently been stated that FGF is the only factor known to be capable of initiating the epithelial-to-fiber differentiation (Lovicu and McAvoy, 2005). Signaling via MAPK/ERK plays a central role and is required for the expression of several fiber-specific markers (Golestaneh et al., 2004; Le and Musil, 2001; Lovicu and McAvoy, 2001). We examined the effect of ectopic Equarin expression on the expression of phospho-ERK1/2 (ERK-P) proteins, which are the downstream effectors of the FGF-MAPK pathway. Using a phospho-specific antibody that was raised against ERK1/2, we detected endogenous ERK-P expression in the lens vesicle in the contralateral lens (Figure 18A). Interestingly, ERK-P expression was upregulated in the Equarin-electroporated embryos (Figure 18A). Western blot analysis of the lens lysates revealed a higher level of ERK-P expression in the Equarin-electroporated lens relative to the contralateral side (Figure 18B). Lens epithelial cells endogenously express low levels of FGF1 and FGF2 in vivo and in vitro (Lovicu et al., 1997; Schulz et al., 1993). Similarly, in DCDML culture, exogenously added Equarin also promoted the activation of ERK (Figure 18C). Moreover, this activation of ERK was reduced by the presence of the FGFR inhibitor PD173074, a widely used small molecule inhibitor of FGFR1-4 tyrosine kinase activity (Figure 18C). These results imply that Equarin upregulates ERK mainly via FGF-FGFR signaling.

It has been previously shown that FGF2 at a concentration of 55 pM only minimally stimulated lens fiber-specific marker expression (Le and Musil, 2001). We found that the minimum concentration of Equarin to induce fiber differentiation was 1 nM. Interestingly, minimal levels of Equarin and FGF together induced β-crystallin expression to nearly double the level observed with Equarin or FGF alone (Figure 18D). This finding further supports the hypothesis that Equarin is capable of upregulating FGF signaling.
Figure 18. Upregulation of FGF signaling by overexpression of Equarin. (A) ERK-P expression following Equarin electroporation. Serial sections were made for immunostaining. The samples with strong GFP and upregulation of β-crystallin were selected for staining with an anti-ERK-P antibody. The expression of ERK-P in the lens vesicle of the Equarin-electroporated side was upregulated. (B) Western blotting of the lens lysates (from the same samples as Figure 12) revealed that ERK-P was significantly induced in the Equarin-electroporated side relative to the contralateral side (Student’s t-test, *P<0.001; n=3). (C) Upregulation of fiber differentiation by Equarin required endogenous FGF
signaling. DCDMLs were incubated for 1 hour in either the absence or presence of PD173074 (PD) (added to cells 1 hour prior to the addition of other factors) with no additions (control), 20 nM Equarin or 1 nM FGF2. The cultures were lysed and assessed for ERK activation. ERK-P expression was also promoted by exogenous Equarin. However, it was diminished when PD173074 was added prior to the culture. (D) DCDMLs were cultured in a minimal concentration of Equarin (1 nM) and/or FGF2 (55 pM). The lysates were analyzed using immunoblotting with an anti-β-crystallin antibody. Minimal levels of Equarin and FGF together induced β-crystallin expression to nearly double the level observed with Equarin or FGF alone (*P < 0.001; n=3).

3-1-8. Equarin is necessary for FGF-induced chick lens fiber differentiation

To further elucidate the role of Equarin in FGF signaling in chick lens differentiation, we examined whether FGF-induced differentiation was inhibited in the absence of endogenous Equarin. Co-transfection of FGF and Equarin ZFNs was performed in primary lens DCDML cultures (Figure 19). Like recombinant FGF2 protein, transiently expressed FGF2 also increased the expression of β-crystallin in the DCDMLs relative to empty-vector-transfected cells (Figure 19). Notably, lens epithelial differentiation was obviously reduced when FGF2 and Equarin ZFNs were co-expressed. Western blot analysis of the lens lysates showed a clear decrease in the level of β-crystallin in the cells co-expressing FGF2 and Equarin ZFNs relative to those expressing FGF2 alone (Figure 19). Therefore, the upregulation of fiber differentiation by FGF required the activity of Equarin that was endogenously produced by the lens cells themselves.
Figure 19. Equarin is required for the upregulation of fiber markers by FGF. DCDMLs were co-transfected with Equarin ZFNs and FGF2-Flag. Lysates were analyzed by immunoblotting with an anti-β-crystallin antibody. β-tubulin was used as a loading control. Transiently expressed FGF2 increased the expression of β-crystallin in the DCDMLs relative to empty-vector-transfected cells (*P<0.001; n=3). A clear decrease in the level of β-crystallin was shown in the cells co-expressing FGF2 and Equarin ZFNs relative to those expressing FGF2 alone (*P<0.001; n=3).

3-2. Part B: The role of Equarin in chick lens cell adhesion during lens differentiation

3-2-1. Expression of Equarin protein in chick lens cells

Lens cell differentiation involves regulation of cell-matrix and cell-cell interaction, both of which are dependent on adhesion molecules. A number of ECM components including laminin, collagens IV, fibronectin and proteoglycans have been localized in the lens (Cammarata et al., 1986). Many ECM molecules are secreted molecules that are immobilized outside of the cells. As shown in Figure 9A and Mu et al 2003, COS-7 cells transfected with Equarin cDNAs exhibited intense Equarin immunoreactivity around the extracellular region of the cells. In primary embryonic chick lens dissociated cell-derived monolayer (DCDML) cultures, lens cells form a cubodial packed epithelium
when they begin to differentiate (Menko et al., 1984). At this stage, Equarin protein was found localized in the extracellular region of the lens cells and cell-cell borders (Figure 20). This expression pattern of differentiating fiber cells suggests a unique role for Equarin in cell-cell or cell-matrix interactions of lens fiber cells.

![Figure 20. Localization of Equarin protein in chick lens cells. The Equarin protein of DCDMLs that were cultured for 3 days was visualized with an anti-Equarin antibody without fixation. Note that the Equarin protein was localized in the extracellular region of the lens epithelial cells. Scale bar: 50μm.](image)

3-2-2. Equarin promotes adhesion of the lens cells through heparan sulfate proteoglycan

It has been reported that mouse CCDC80 was able to promote cell adhesion and migration (Manabe et al., 2008). To investigate the role of Equarin in cell adhesion, we utilized an in vitro cell attachment assay that allows cells to attach for 1 hour in wells coated with different substrates. Similar to fibronectin, Equarin also serves as an adhesion substrate for lens epithelial cells (Figure 21A). We found that the attachment of lens epithelial cells to Equarin was concentration-dependent and that the maximum attachment was obtained at concentrations of 100 to 200 nM (Figure 21B and C). To confirm this adhesion ability of Equarin, we tested a large series of tumor cell lines. We found that although the ability to attach to Equarin or fibronectin differs in cell types, the HT1080, RD, C2C12, T98G, A549 cells adhered well to both Equarin and fibronectin (Figure 22A-E).
Next question is which receptor(s) and molecular mechanism were involved in Equarin induced cell adhesion. The integrin family of cell-surface protein is primary participants in cell adhesion to the extracellular matrix molecules and other cells (Hynes et al., 2002; Plow et al., 2000). We found that the RGD peptide, an inhibitor of integrin-ligand interactions, did not inhibit the attachment to Equarin, whereas it efficiently perturbed the attachment to fibronectin (Figure 21C). Another candidate cell-receptor family is the sulfated cell membrane proteoglycan family (Bernfield et al., 1992). The cell attachment to Equarin was almost completely inhibited by heparin when heparin was preincubated with Equarin-coated wells for 30 minutes (Figure 21C). Because the heparin incubation occurred prior to the addition of the cells, heparin did not bind to the cell surface; rather, it bound to Equarin and thereby blocked cell adhesion to Equarin. No significant heparin effect was observed regarding cell attachment to fibronectin (Figure 21C). The same inhibitory heparin effect was observed in tumor cell lines, such as HT1080 cells (Figure 22F). These results suggest that the heparan sulfate chain is critically involved in Equarin-mediated cell attachment.

3-2-3. Equarin interacts with SDC-3

Previous studies have demonstrated that syndecan receptors are reported to regulate FGF signaling and cell-matrix adhesion. In the chick lens, only syndecan-3 (SDC-3) displays persistent expression by lens fiber cells after the head ectoderm differentiates into lens placode (Gould et al., 1995). We demonstrated by co-precipitation that Equarin bound to chick SDC-3 (Figure 23A) but not syndecan-2 (SDC-2) (Figure 23B).
Figure 21. Equarin mediates lens epithelial cell adhesion through heparan sulfate proteoglycan. (A) Lens epithelial cell attachment to Equarin at different concentrations. Scale bar: 50μm. (B) Dose-dependent cell attachment of lens epithelial cells to Equarin or fibronectin. Blue: Equarin, red: fibronectin. The ordinate represents the OD values at 595 nm and the abscissa represents the concentration of the substrate in nM as indicated. (C) Inhibitory activity of human IgG (10 μg/ml), RGD peptide (1 mM) or heparin (100 μg/ml) on the attachment of lens epithelial cells to Equarin (50 nM) or fibronectin (10 nM). Note that the RGD peptide inhibited the attachment to fibronectin and heparin completely perturbed the attachment to Equarin. Blue: Equarin, red: fibronectin. The ordinates represent the percentage adhesion of the control. The data in B and C represent the mean±SEM of triplicate wells.
Figure 22. Cell attachment of human tumor cell lines in response to Equarin or fibronectin. Cell attachment of (A) HT1080 cells, (B) RD cells, (C) C2C12 cells, (D) A549 cells and (E) T98G cells to Equarin- (blue) or fibronectin- (red) coated wells at different concentrations. The ordinate represents OD values at 595 nm, and the abscissa represents the concentration of the substrate in nM as indicated. (F) HT1080 cell attachment to Equarin is mediated through heparan sulfate proteoglycan. This figure demonstrates the inhibition effect of human IgG (10 μg/ml), the RGD peptide (1 mM), function-blocking β1 integrin antibody (AIIB2) or heparin (100 μg/ml) on the HT1080 cell attachment to Equarin and fibronectin. Note that (AIIB2) and RGD peptide inhibited the attachment to fibronectin. Conversely, the attachment to Equarin was sensitive to heparin. The data represent the mean±SEM of triplicate wells.
Figure 23. Equarin directly binds to SDC-3 but not SDC-2. Equarin-Myc-His- and SDC-3-Flag- or SDC-2-Flag-containing conditioned media were incubated together, followed by the incubation with ProBand Resin. The input amount of each protein was detected using immunoblotting with an anti-tag antibody. After immunoprecipitation, bound SDC was detected by immunoblotting with an anti-Flag antibody. (A) Lane 1 indicates that in the absence of Equarin, SDC-3-Flag could not be detected. Conversely, Lane 2 indicated that SDC-3-Flag protein was visualized only in the presence of Equarin, suggesting that SDC-3 was co-precipitated by Equarin. (B) Lane 2 indicated that SDC-2-Flag protein was not co-precipitated by Equarin.
4. Discussion

Lenses depend for their function on having transparency and refractive properties, which directly relate to cellular features that arise during fiber cell differentiation. Therefore, a detailed knowledge of the differentiation process is central to an understanding of the fundamental optical properties of the lens. It also provides the basic information necessary to understand the etiology of certain types of cataract. The formation of lens fiber cells is one of the most extreme examples of cell differentiation in nature, culminating in a degree of specialization scarcely equaled by any other cell type. In this study, we demonstrated a novel secreted molecule, Equarin, plays an essential role during lens fiber cell differentiation and development.

4-1. Role of Equarin in chick lens epithelial-to-fiber differentiation

Recent studies have indicated that, although FGFs play important roles in lens epithelial-to-fiber differentiation, they are insufficient for the full differentiation of the lens fiber cells (O’Connor and McAvoy, 2007; Wang et al., 2010). This concept has come from three main lines of investigation. Firstly, FGF1- and FGF2-deficient mice exhibited no lens development defects (Dono et al., 1998; Miller et al., 2000). Secondly, further evidence indicates that factors other than FGF, including IGF, PDGF, EGF, (Wang et al., 2010), BMPs (Boswell et al., 2008a; Boswell et al., 2008b) and Wnt (Lyu and Joo, 2004), are required to cooperate with FGF to regulate fiber cell differentiation. Although a low dose of vitreal factors such as IGF, PDGF and EGF alone cannot induce fiber cell differentiation, when combined with a low dose of FGF, a fiber-differentiation response is elicited to varying degrees, depending on the specific growth factor combination (Wang et al., 2010). Finally, studies directed at interfering with signaling by the various other growth factor families that are expressed in the eye have identified pathways that also appear to be critical for regulating specific events in the complex process.
of lens fiber differentiation (Wang et al., 2010). Thus, to fully understand lens development, investigatory and functional analyses of novel molecules will be required.

Here, we showed that a novel secreted molecule, Equarin, is expressed in the lens equator region in chicks and mice; this region is the transitional zone from the anterior lens epithelial cells to the posterior fiber cells. This expression pattern prompted us to examine the involvement of Equarin in lens development. Using both in ovo and in vitro analyses, we demonstrate that Equarin has the potential to promote epithelial-to-fiber differentiation. To better elucidate the functions of specific genes during animal development, loss-of-function assays are frequently used. Recently, a new method for targeted mutagenesis using engineered ZFNs has been reported in several model organisms, including drosophila (Beumer et al., 2008), zebrafish (Foley et al., 2009), sea urchin (Ochiai et al., 2010), plants (Shukla et al., 2009) and human cultured cells (Hockemeyer et al., 2009). In this study, we tested targeted gene inactivation using ZFNs in chick lens epithelial cell cultures. Our findings suggest that ZFN-induced genomic mutation in chick cells is feasible and quite robust; nearly half of screened clones carried lesions at the target sites. Our results provide a foundation for performing focused gene modification in vertebrate genomes and will allow the development of techniques for more subtle genomic manipulation. Regarding the function of Equarin, our results using ZFN technology provide convincing evidence that Equarin serves as a bona fide differentiation factor during chick lens development. This is first report demonstrating the molecular function of Equarin in chick lens formation; our findings will open up a new avenue for the investigation of lens differentiation.

Given the distribution of the Equarin protein at the peripheral retina in a dorsal-high-to-low-ventral gradient (Figure 11), one hypothesis is that Equarin affects retinal polarity. This possibility is supported by the fact that microinjection of Equarin mRNA into Xenopus embryos causes ventral eye
malformation (Mu et al., 2003).

4-2. Role of Equarin in the FGF signaling pathway

A fundamental question concerns the mechanism used by Equarin to promote lens fiber differentiation. We have shown that the Equarin protein is strongly distributed to the posterior lens capsule. FGF1 and FGF2 have previously been reported to be secreted from various ocular sites into the capsule where they bind to heparan sulfate proteoglycans (Schulz et al., 1997) and specific FGF receptors (de Iongh et al., 1997). In this study, we demonstrated the direct binding of Equarin to FGF and showed that Equarin exerted its function by modulating FGF signaling. These conclusions are based on the following observations: (1) the overexpression of Equarin upregulated the expression of ERK-P, the downstream effector of FGF-MAPK, both in vivo and in vitro; (2) the activation of ERK was diminished by the presence of the FGFR inhibitor PD173074; and (3) a minimal level of Equarin and FGF upregulated the fiber differentiation. Furthermore, we showed that loss of endogenous Equarin by ZFNs diminished the upregulation of fiber markers by FGF. Thus, all these observations support the conclusion that Equarin serves as a novel extracellular regulator of FGF signaling during lens development. Nevertheless, other molecules may also be necessary for upregulation of differentiation by FGF during chick lens development because only a partial reduction of FGF function was induced by Equarin ZFNs.

We showed that Equarin bound to heparan sulfate proteoglycan. It has been established that heparin or heparan sulfate also bind to FGF and FGFR and that heparan sulfate is essential for FGF signaling at the extracellular level, raising the possibility that Equarin interacts with heparan sulfate to enhance complex formation between FGF and FGFR. We propose a model in which Equarin, cell-surface heparan sulfate proteoglycans, and FGF form a ternary complex on the cell surface; Equarin associates
with heparan sulfate proteoglycans to serve as a reservoir of FGF at or near the lens cells; this increases
the local FGF concentration in close proximity to the FGFR, which results in the upregulation of the
FGF-FGFR signaling pathway (Figure 24). In this manner, Equarin can function as an organizing
center for the FGF signaling complex on the cell surface.

We cannot exclude the possibility that other signaling pathways may also be involved in Equarin
function. This possibility is supported by the following results: (1) the ERK pathway also mediates the
EGF, IGF, VEGF and integrin receptor signaling pathways; and (2) PD173074 could not completely
inhibit the activation of ERK that was induced by Equarin. These observations imply that Equarin may
also participate in other signaling pathways during lens development.

Further investigation will be necessary to identify the binding domains that mediate the interactions
between Equarin and FGF and heparan sulfate proteoglycans. It will also be interesting to investigate
the significance of the gradient pattern of Equarin expression.

Figure 24. A model describing the role of Equarin in chick lens differentiation.
4-3. Role of Equarin in lens cell adhesion

How the cell differentiation processes are regulated involves the particular array of matrix components and adhesion proteins that are expressed through their signaling pathways (Lauffenburger and Horwitz, 1996; Roskelley et al., 1995). Within an individual embryonic lens all stages of cell differentiation are represented. The anterior epithelial cells proliferate and divide. In the lens equatorior zone, the cells begin to form complex intercellular connections as they elongate and differentiate. The differentiating fiber cells slides along the lens apical and basal surface, respectively. The lens differentiation process involves a progression from cell-substrate to cell-cell interaction. Recently, mouse CCDC80, which is homologue of Equarin, have been reported to be involved in the assembly of the extracellular matrix and the mediation of cell adhesion (Manabe et al., 2008). Meanwhile, Equarin protein is present on the extracellular region and cell-cell border of lens cells. To test the ability of Equarin to mediate cell adhesion, we utilized an in vitro attachment assay. We found Equarin can serve as a cell adhesion protein in a dose-dependent manner. This result suggests that Equarin is capable of mediating cell-cell adhesion to promote lens fiber differentiation.

We further explored the molecular mechanisms of Equarin-mediated cell adhesion. We found that lens epithelial cells attach to Equarin by engaging to cell-surface heparan sulfate proteoglycans. This conclusion is based on the demonstration that the attachment of lens epithelium cells to Equarin was completely inhibited by heparin and was not perturbed by the RGD peptide. By a biochemical assay in Figure 17, we have proved that Equarin binds to heparan sulfate proteoglycan. Therefore, Equarin mediates and triggers cell adhesion through heparan sulfate proteoglycan (Figure 25). Moreover, integrins and heparan sulfate proteoglycans are the primary ECM adhesion receptors that coordinate in signaling events and determine the signaling outcomes (Iba et al., 2000; Kim et al., 2011). Regarding
the specific mechanism that is involved, we need to confirm whether integrin is responsible for the transduction of the Equarin-mediated cell adhesion response.

Several receptors that contain heparan sulfate chains are expressed on the cell surface, including the syndecan family of cell-surface proteoglycans, the phosphatidylinositol-linked glypican, and the part-time proteoglycans betaglycan and CD44E (Bernfield et al., 1999; Carey, 1997). Previous studies have demonstrated that syndecan receptors have been reported to be involved in the regulation of FGF signaling and cell-matrix adhesion (Carey, 1997; Iba et al., 2000; Rapraeger, 2001). Their heparan sulfate chains bind to a large number of molecules, including extracellular matrix components such as fibronectin, collagen, and heparin-binding growth factors such as the FGFs. In the chick lens, only SDC-3 persists to express in the lens fiber cells from lens placode stage (Gould et al., 1995). We demonstrate that Equarin binds to SDC-3, but not SDC-2. Therefore, these data suggest SDC-3 may represent a potentially important receptor for Equarin to regulate cell adhesion.

Although we have demonstrate that Equarin is capable of mediating cell adhesion of lens cells, it is essential to examine the in vivo effect of this cell-cell interaction on actin cytoskeleton, fiber cell elongation and differentiation.

Figure 25. A model describing the role of Equarin in chick lens adhesion.
5. Conclusion

Equarin is expressed exclusively in the equatorior region of chick lens and serves as a novel differentiation inducer through FGF signaling and cell adhesion.

![Diagram of chick lens](image)

**Figure 26.** Equarin serves as a novel differentiation inducer through FGF signaling and cell adhesion. Equarin is specifically expressed in the lens equator region. Equarin binds to FGF, thereby upregulates FGF signaling and promotes lens differentiation. During lens differentiation, adhesion activity is necessary for differentiating fiber cells. Equarin protein is also localized in the extracellular region of differentiating fiber cells and promotes lens cell adhesion activity. Therefore, Equarin is involved in chick lens differentiation by two aspects: upregulating FGF signaling and mediating cell adhesion.
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