学　位　論　文

Doctor's Thesis

論文題名：**Studies on the role of a novel molecule (equarin) on eye formation**

（レンズにおける新しい分子の同定と眼の発生に関する研究）

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Contents

Summary

The lens plays an important role in eye development. To investigate the molecular mechanisms involved, we used signal sequence trap screens with a chicken lens cDNA library and identified a novel secreted molecule, equarin. Equarin encodes consensus repeat domains conserved in human SRPX and mouse *Urb*. In the embryonic eye, equarin transcript is detected exclusively in the lens from early stages and later persists in the lens equatorial region in a high-dorsal-to-low-ventral gradient. In vitro analysis of equarin protein indicated that after translation, it is modified, cleaved, and secreted to extracellular locations. Microinjection of equarin mRNA into Xenopus embryos induced abnormal eye development. These data suggest that equarin plays an important role during eye development.

List of Publications

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Abbreviation

Equarin-L: equarin-long

Equarin-S: equarin-short

SST: signal sequence trap

SRPX: sushi-repeat-containing protein, X chromosome

RGC: retinal ganglion cell

IPL: inner plexiform layer

SSG1: steroid-sensitive gene 1 protein

SRP: sushi-repeat protein

SCR: short consensus repeat

DCDML: dissociated cell-derived monolayer

MAO: morpholino antisence oligonucleotide

CP49/CP115: cytoskeletal protein 49/115

DIG: digoxigenin

DEPC: diethyl pyrocarbonate

SSC: saline sodium citrate

HISS: heat-inactivated sheep serum

NBT: *p*-nitroblue tetrazolium

BCIP: 5-bromo-4-chloro-3-indolyl phosphate

SDS-PAGE: sodium dodecyl sulfate –polyacrylamide gel electrophoresis

ECM: extracellular matrix

N-terminus: amino terminus

C-terminus: carboxyl terminus

1. Introduction and purpose

The development of the vertebrate eye depends on the coordinated interaction of three distinct tissues: the neuroepithelium, the overlying surface ectoderm, and the mesenchyme (Jean et al., 1998). The initial optic pit and subsequent optic vesicle are formed from an evagination of the forebrain into the anterior neural plate. When the optic vesicle makes contact with the overlying ectoderm, the ectoderm is induced to thicken into the lens placode. The lens placode then invaginates and pinches off to form the lens vesicle (Piatigorsky, 1981). Subsequently, cells on the proximal side of the lens vesicle elongate and differentiate into lens fiber cells. Once it has begun, the process of lens fiber cell differentiation is continuous throughout life (Jean et al., 1998). Simultaneously, the distal part of the optic vesicle invaginates to form the bilayered optic cup. The inner layer develops into the neural retina and the outer layer is destined to become the pigmented layer of the retina, the iris, and the ciliary body. The proximal part of the optic vesicle narrows to become the optic stalk and later the optic nerve. The mesenchyme invades the space between the lens vesicle and the overlying ectoderm, and forms the subepithelial cornea, the anterior chamber, and the vitreous body (Hyer et al., 1998; Jean et al.,

1998).

The development of the eye is controlled by a combination of intrinsic and extrinsic signals, and appears to require several different inductive interactions to form properly. For example, the lens is believed to be an important source of the signals mediating the inductive and regulative events during eye development (Beebe, 1986; Beebe and Coats, 2000; Thut et al., 2001). Removal of surface ectoderm at chick E1.5 (presumptive lens ectoderm, a rich source of FGFs) generated a rudimentary optic cup without lens at E4-7. No optic cup with well-defined neuroretinal and pigmented layers was formed, indicating that presumptive lens ectoderm provided positional cues organizing the bipotential optic vesicle into specific neural retina and pigmented epithelium domains (Hyer et al., 1998). The role of the lens in early eye development was further examined in transgenic mice carrying the cytotoxic diphtheria toxin A gene driven by hamster alpha A-crystallin promoter. Mice are microphthalmic, absent of lens and lack a pupil, aqueous and posterior chamber, vitreous humor, iris, and ciliary body and show extensive convolution of the neural retina. It suggests that a functional lens is critical for formation of the ciliary epithelium, iris, and the vitreous body, as well as for the growth, development, and maintenance of morphology of the retina, cornea, sclera, and optic nerve. (Breitman et al., 1989; Harrington et al., 1991). Mechanical removal of the lens or replacement of the lens at later stage of

E4 with a cellulose bead led to the formation a disorganized aggregate of mesenchymal cells beneath the corneal epithelium. No recognizable corneal endothelium, corneal stroma, iris stroma, or anterior chamber was found in these eyes. It provides evidence the formation of the corneal endothelium is regulated by signals from the lens (Beebe and Coats, 2000).

Conversely, the presence of an additional lens in the developing eye causes abnormal development of the optic cup margin in vivo (Genis-Galvez, 1966). Organ culture recombination experiments showed that a chick lens is capable of inducing the expression of markers of the presumptive iris and ciliary body in the developing mouse neural retina. These studies provide molecular evidence that an evolutionarily conserved signal from the lens controls tissue specification in the developing optic cup (Thut et al., 2001).

Other studies in cave fish have shown that a transplanted surface fish lens can stimulate the growth and development of the cave fish optic cup, rescuing it from degeneration (Yamamoto and Jeffery, 2000). Our previously reported in vitro data also showed that the embryonic lens secretes chemorepellent(s) and influences the initial guidance of the earliest retinal ganglion cell axons growing toward the optic disc (Ohta et al., 1999).

These tissue interactions have long been recognized and have been characterized extensively, and a variety of genes expressed by the lens have

been identified (Furuta and Hogan, 1998; Kamachi et al., 1998; Hyer et al., 1998). However, the molecular mechanisms still need to be clarified. The identification of additional molecules, especially secreted molecules that possibly mediate signals as diffusible factors, will extend our knowledge of the molecular mechanisms of eye development. In this study, we used signal sequence trap (SST) screens (Klein et al., 1996) to isolate molecule(s) from a chick E6 lens cDNA library. We found five novel clones and analyzed one of them in detail. This transcript was expressed in the lens equatorial region and was therefore designated "equarin". Two alternatively spliced gene products, designated equarin-long (equarin-L) and equarin-short (equarin-S), were identified by screening a chick lens cDNA library. We analyzed their structures, expression patterns, proteins, and functions by microinjecting their transcripts into Xenopus embryos. We thus confirmed that equarin is involved in eye development.

2. Materials and Methods

2.1. Animals

White Leghorn chicken embryos obtained from a local supplier were incubated at 38 °C in a humidified incubator. Embryos were staged according to Hamburger and Hamilton (1951).

Xenopus embryos obtained by in vitro fertilization were dejellied in 2% cysteine (pH 8.0) and allowed to develop in 0.1 x MBS using standard methods. Embryos were staged according to Nieuwkoop and Faber (1967).

2.2 Screening by signal sequence trap

Signal sequence trap (SST) was performed according to Klein et al. (1996) to detect signal sequences on the basis of their ability to rescue the growth of a mutant yeast strain lacking invertase. Briefly, we used the pRK18 vector, which was purified after phagemid excision of λRK18 with M13 helper phage. E6 chick lens mRNA (5 μg) was converted to cDNA using standard protocols. After *Not*I/*Xho*I adaptor ligation, fragments between 500 and 1200 bp were cloned into the *Not*I site of pRK18. The cDNA pools were transformed into yeast strain SEY6210, and plasmids

were isolated from colonies that survived invertase selection. Positive clones were sequenced randomly and analyzed for any similarity to known genes.

2.3. Cloning of equarin

Clone 15 cDNA fragment (900 bp) was labeled with fluorescein-11-dUTP and used to screen 10^6 plaques from a chick E7 lens λgt10 cDNA library, using the ECL™ Random-Prime Labeling and Detection System (Amersham).

The structures of the cDNAs and the cognate amino acid sequences were analyzed using the following Internet sites: http://www.ncbi.nlm.nih.gov/BLAST/; NCBI conserved domain database and search, http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml; and signal sequences and O-glycosylation residues were predicted using http://www.cbs.dtu.dk/services/.

2.4 Plasmid construction

For expression studies, equarin cDNAs were subcloned into the pEF/Myc-His vector (Invitrogen) to express a myc/His tag at the C-terminus. To produce eqaurin proteins with N-terminal myc tag, equarin

cDNAs without signal sequence were subcloned into EcoRI-XhoI site that located downstream of cDNA sequences encoding an N-cadherin signal sequence and six myc tags in the pCS2+-MT-Ncad vector (a kind gift from Dr. KG Johnson). Orientation and sequence integrity was verified by sequencing.

2.5 In situ hybridization

Digoxigenin-labeled anti-sense and sense probes were made from 500 bp and 900 bp templates of the 3' terminal non-coding regions of equarin-L and equarin-S in pBluescript SK (-) vector using T3 or T7 RNA polymerase, respectively. Probes for in situ hybridization on sections were alkali-treated to trim them to approximately 200 bp.

Embryos were dissected in cold diethyl pyrocarbonate (DEPC)-treated phosphate-buffered saline (PBS, pH 7.0) and fixed in 4% paraformaldehyde in PBS at 4 °C overnight.

In situ hybridization on sections was performed as described by Schaeren-Wiemers and Gerfin-Moser (1993). Briefly, after fixation, embryos were sunk in 30% sucrose in PBS and embedded in Tissue-Tek OCT compound (Sakura Fine Technical Co., Ltd., Tokyo, Japan). Sections were cut to 14–20 μm on a cryostat. Sections were treated with 10 μg/ml proteinase K. 1% Triton X-100, and then incubated in prehybridization

solution (50% formamide, 5 x saline sodium citrate [SSC], 5 x Denhardt's solution, 250 μg/ml yeast tRNA, 500 μg/ml herring sperm DNA) for 2 h at room temperature (RT). Hybridizations were performed with 0.4 μg/ml probe in prehybridization solution at 72 °C overnight. Specimens were washed in 0.2 x SSC at the hybridization temperature and blocked. Then alkaline phosphatase (AP)-conjugated anti-DIG Fab fragment (Roche) diluted 1:5000 in 1% heat-inactivated sheep serum (HISS) and buffer B1 (0.1 M Tris-HCl [pH 7.5], 0.15 M NaCl) was applied for immunochemical detection at 4 °C overnight. Samples were washed, and signals were detected using 337.5 μg/ml *p*-nitroblue tetrazolium (NBT) and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in NTMT solution (0.1 M Tris-HCl [pH 9.5], 0.1 M NaCl, 50 mM $MgCl_2$, 0.24 mg/ml levamisole) for 6–72 h at RT. Slides were dried overnight, dehydrated in a graded series of ethanol and xylene, and mounted. Sections were observed and photographed using a Nikon Eclipse E600 microscope equipped with a Hamamatsu color chilled 3CCD camera.

Whole-mount in situ hybridization was performed as described by Schaeren-Wiemers and Gerfin-Moser (1993). Briefly, after fixation, embryos were treated with a graded series of methanol in PBT (0.1% Tween 20 in PBS) and were stored at –20 °C. Embryos were rehydrated in a reversed graded methanol series in PBT, treated with 20 μg/ml proteinase K for 10–20 min, and refixed. Specimens were pretreated in

prehybridization solution for 1 h at 70 °C, and hybridized with 0.1 µg/ml probe in prehybridization solution at 70 °C overnight. Slides were washed and blocked, and reacted immunochemically with anti-DIG-AP Fab fragment (Roche) diluted 1:2000 in 20% HISS in TBST (137 mM NaCl, 2.68 mM KCl, 25 mM Tris-HCl [pH 7.5], 1% Tween-20) at 4 °C overnight. Samples were washed, and signals were detected in 337.5 µg/ml NBT and 175 µg/ml BCIP in a solution of 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl, 50 mM $MgCl_2$, 1% Tween-20, 0.48 mg/ml levamisole. Photographs were taken using a Nikon E990 digital camera.

The Xpax2 probe was kindly gifted by Dr. Perron, and whole-mount *in situ* hybridization for Xenopus embryos was performed according to Shain and Zuber (1996).

2.6 COS cell culture and transfection

COS-7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and 50 µg/ml gentamycin (all from Sigma). Cells were plated 12–16 h before transfection, at a density of 2 x 10^5 cells per well in six-well plates. Transfections were performed using Lipofectamine™ 2000 Reagent (Invitrogen), and 3 µg plasmid DNA was used for each well as specified by the manufacturer. After incubation with the DNA for 24 h, the transfection medium was

replaced with serum-free Opti-Medium (Invitrogen) supplemented with 50 μg/ml gentamycin. Cell cultures were incubated for another 48 h before the cells were collected for immunological analysis.

2.7 Western blotting

COS-7 cell-culture supernatants were collected 48 h after transfection and concentrated fourfold. Equal volumes of 2 x sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) sample buffer (20% glycerol, 250 mM Tris [pH 6.8], 4% SDS, 10% 2-mercaptoethanol) were mixed with the supernatants. Cells were washed twice with PBS, scraped from the culture dish, and collected into 1.5 ml plastic tubes. After brief centrifugation, cells were solubilized in 400 μl of 1 x SDS–PAGE sample buffer. Samples for SDS–PAGE, consisting of either 10 μl supernatant or 5 μl whole-cell lysate, were applied to gel lanes after boiling for 10 min. After electrophoresis, samples were transferred electrophoretically to PVDF membrane (Millipore) for 30 min at 250 mA. The resulting protein blots were blocked with 2% skim milk in PBS and incubated with anti-myc monoclonal antibody (9E10; DSHB) for 1 h at RT. Blots were washed, and the immune complexes remaining on the filter were visualized using a rabbit anti-mouse IgG horseradish peroxidase (HRP)-conjugated antibody (diluted 1:2000; Southern Biotechnology

Associates Inc.), followed by treatment with enhanced chemiluminescence (ECL) plus western blotting detection reagents (Amersam).

2.8 Immunohistochemistry

To observe the expression of equarin proteins in transiently transfected COS-7 cells, the cells were treated with trypsin and plated onto polyethyleneimine-coated cover slips in six-well plates at a density of 1 x 10^4 cells per well, one day after transfection. After incubation for 48 h, the cells on the cover slips were washed twice in PBS and either fixed in 4% paraformaldehyde in PBS for 10 min or remained unfixed. The cells were blocked in tyramide blocking buffer (NEN™ Life Science Products Inc., Boston) and incubated with an anti-His antibody conjugated with Alexa Fluor fluorescent dye (647; QIAGEN) and diluted (1:200) in tyramide blocking buffer, or anti-myc antibody (9E10; DSHB) for 1 h at RT. A fluorescein isothiocyanate (FITC)-conjugated secondary antibody (WAKO, Japan) was used to visualize the anti-myc primary antibody. The cells were extensively washed, and mounted in 4% n-propyl gallate glycerol in 90% glycerol/PBS.

Cryostat sections for immunohistochemistry were prepared as described above. Sections were washed with PBS for 5–10 min, then blocked and reacted immunohistochemically with anti-β-crystallin (DSHB)

and anti-Pax6 (DSHB) antibodies and anti-mouse IgG secondary antibodies conjugated with tetramethylrhodamine isothiocyanate (TRITC) or FITC, respectively.

2.9 Chick embryos in ovo electroporation

For in ovo electroporation, a BTM T-820 electroporation system (USA) was used. DNA solution contains 8.0 μg/μl pEF-equarin, 0.5 μg/μl of pEF-GFP-N1 and 1% fast green. In ovo electroporation was performed as described by Ogino and Yasuda (1998). White Leghorn chicken eggs were incubated at 38 °C until stages 10-11 of development or about embryonic day 1.5. A window of about 2-3 cm in diameter was opened at the top of the egg after removing 3-4 ml of albumin. Injection of Indian ink underneath the embryo facilitated visualization of it. PBS was poured over the embryo to obtain an appropriate resistance. When targeting at the lens, a tungsten anode was set on the left side of the ectoderm paralleling to the embryo. After DNA solution was being injected into the space near the right presumptive lens ectoderm with a sharp glass pipette (Drummond Scientific Company, USA), a sharp tungsten cathode was placed near the right ectoderm (Fig. 9a) and electric pulses (7-8V, 50ms) were applied three times. After sealing the egg-shells, embyos were allowed to develop in humidified incubators. DNA incorporation into chick lens by in ovo

electroporation was checked by GFP expression under fluorescent microscope on the following day. Only these embryos with strong GFP signals in lens vesicle were allowed for further 24 h incubation and were analyzed at E3.5.

2.10 Preparing embryonic chick lens culture

2.10.1 Chick embryonic lens explants

Embryonic chick lens central epithelium explants were prepared as described by Piatigorsky et al. (1972). Briefly as shown in Fig. 10A. E6 chick lenses were excised and placed in a 4-well dish with the anterior epithelia down. The posterior capsule was then punctured and the fiber mass was removed though the tear, leaving the epithelia intact. A scalpel was used to cut out a square of approximately 0.5 mm^2 of central epithelium, which was subsequently cultured in basic culture medium (M199 medium with 0.1% bovine serum albumin [BSA]) for 12 hours for explants to attach to the bottom of the dishes. Then the explants were treated with equarin-containing medium and morpholino antisense oligonucleotide (MAO) of equarin (GENE TOOLS, LLC USA) according to its delivery protocol. For the equarin-containing culture 10%, 30% and 50% 1:10 concentrated equarin (prepared as described above) was added to the basic culture medium. Explants cultured in with and without 20% FCS

in basic culture medium used as positive and negative control respectively.

For MAO delivery, 1.8 µl of 0.5 mM Special Dilivery Morpholino/DNA stock solution was added in 62.7 µl distilled water and mixed by vortex with 1.8 µl of 200 µM EPEI Special Delivery Morpholino/DNA stock solution. After incubation for 20 minutes at room temperature, 0.6 ml of Opti-Med (GIBCO BRL) was added to the above mixture to make the complete delivery solution. The complete delivery solution (0.6 ml) was added in each well of the 4-well dishes. After 3 hour incubation, the media were changed to basic culture medium and the cultures were kept for further 6 days. A Xenopus Tsukushi morpholino antisense oligonucleotide was used as control.

2.10.2 Chick embryonic lens cell cultures

Dissociated cell-derived monolayer (DCDML) cultures were prepared from E10 chick lenses as described by Le and Musil (1998). Briefly, E10 lenses were excised, the attached ciliary body and vitreous body tissues were removed with fine tweezers in PBS under a dissecting microscope. Then the lenses were placed in TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na2PO4, 5 mM D-glucose, 0.025 M Tris Base, pH 7.4). They were dissociated at 37°C for 30 min with 0.08% trypsin (Sigma) in TD buffer. The digestion was performed on 20 lenses/6 ml tripsin in a 15 ml conical tube. The tubes were agitated at 15, 20, 25 min. At 25 min

agitation was continued until all the lens capsules were captured and the lens tissure was in small pieces; overagitation may disrupt cells. The cells were pelleted (15 min. 100g) and the supernatant removed. The pellet was resuspended in basic culture medium (M199 medium in 0.1% BSA) containing 20% FCS by pipetting. The cell suspension was filtered through three layers of lens paper (A.H. Thomas) to remove any cell clumps and capsule material. The filtrate which contains single cells was counted and the cells were plated at 1-2 x 10^5 cells/cm. Prior to cell plating, the substrate was treated overnight at 37 °C with 0.5 mg/ml poly-L-lysin in 0.15 M borate buffer (pH 8.4). After rinsed with distilled H2O, the substrate was coated with 0.03 mg/ml laminin in Earle's balanced salt solution (Sigma) for 4-5 h at 37 °C. One day after being plated, DCDML cultures were transfected with pEF-equarin plasmid DNA as described above. The pEF plasmid DNA was used as a control. After transfection the DCDML culture were supplied with basic culture medium and were analyzed after six days. The DCDML cultures transfected with empty vector were incubated with and without presence of 20% FCS as positive and negative controls, respectively.

2.11 In vitro transcription and embryo microinjections

Capped synthetic RNAs were synthesized from linearized equarin-L-

CS2+ and equarin-S-CS2+ plasmids using an SP6 mMESSAGE mMACHINE™ kit (Ambion). Capped mRNAs were injected unilaterally into the dorsal animal blastomere of Xenopus embryos at the 4- or 8-cell stage. Tadpoles injected with varying amounts of transcript were grown to stage 41 or 46. The embryos were embedded in paraffin at stage 41, sectioned to 4 μm, and stained with hematoxylin and eosin.

3. Results:

3.1. Identification of a novel molecule, equarin, from chick embryonic lens by SST

We used SST screening of a chick E6 lens cDNA library to isolate molecules possibly involved in eye formation. From a total of 2 x 10^6 yeast cells, 177 plasmid DNAs were recovered from the selected colonies, and the cDNA inserts were sequenced. Of these, 84 clones were identified as potential orthologs of known genes, 18 clones represented novel sequences, and 75 clones were false positives (Table 1). Computer analysis to identify signal peptides confirmed that five novel cDNAs encoded genuine signal sequences (clones 15, 1, 153, 103, and 179). In situ hybridization revealed that all five novel clones were expressed in the lens (data not shown). We were particularly interested in clone 15 that was expressed in the equatorial region of the lens, and named it "equarin".

Two forms of equarin were isolated from a λgt10 cDNA library from chick E7 lens. Equarin-L (deposited in DDBJ under accession no. AB086824) has a 4.2-kb cDNA with open reading frame of 958 amino acid residue, and equarin-S (accession no. AB086823) has a 3.9-kb cDNA with open reading frame of 601 amino acid residues. Equarin-S is identical to

the N-terminal part of equarin-L except for nine amino acid residues at its C-terminal end. Therefore, equarin-L consists of 592 amino acid residues that are identical to those of equarin-S and an extra 366 amino acid residues in the C-terminal region (Fig. 1a). Both cDNAs have identical nucleotide sequences in the 5' non-coding and coding regions including the same translation start site, suggesting that equarin-L and equarin-S are alternatively spliced forms encoded by the same gene.

Equarin-L has relatively high homology to mouse (65.6%; Aoki et al., 2002) and human (66.4%; GenBank accession no. AF506819) Urb (upregulated in BRGS-3-deficient mice). Part of equarin-L protein also shares a comparatively high homology (52.7%) with another protein, rat steroid-sensitive gene 1 protein (SSG1; Marcantonio et al., 2001). The functions of Urb and SSG1 have not yet been determined.

A signal peptide cleavage site occurs between residues 20 and 21 in both equarin amino acid sequences (Fig. 1a, b). This was confirmed by the fact that equarin proteins were secreted into the cell supernatant when equarin cDNAs were transfected into COS-7 cells (see below). The homology search showed that three separate regions (residues 140–281 in both of equarin-L and equarin-S, residues 623–768 and 778–921 in equarin-L) display similarity to a domain in the human sushi-repeat-containing protein encoded on the X chromosome (SRPX) (Meindl et al., 1995; Dry et al., 1995). As well as SRPX, the rat homolog (drs) of SRPX

(Pan et al., 1996), and human (GenBank accession no. NM-014467) and mouse (GenBank accession no. AB028050) sushi-repeat proteins (SRP) also showed the same similarity. Further alignment of the above three regions of the equarin proteins and the corresponding domains in SRPX, the rat drs gene product, and human and mouse SRP indicated that 11 separate residues are conserved between all the domains, which is about 140 amino acid residues each (Fig. 2a). Therefore, we designated the three regions in the equarin-L amino acid sequences consensus repeat domains (Fig. 1a, 2b).

A Thr-rich region and a Lys-rich region were also found in both equarin-L and equarin-S amino acid sequences (Fig. 1a, 2b). Three potential N-linked glycosylation sites were also identified in equarin-S and five in equarin-L, together with several dibasic protease-susceptible sites (Fig. 1a).

3.2. Embryonic expression of equarin

To analyze equarin expression during chicken embryonic eye development, embryos were examined by in situ hybridization with DIG-labeled antisense RNA probes. In the eye, equarin mRNAs are expressed exclusively in the lens until postnatal day 2. Equarin-S is first detected in the lens placode at stage 14 (Fig. 3a, b), and equarin-L is first detected in

the proximal part of the lens vesicle at stage 17 (Fig. 3c, d). Subsequently, at stage 19 when the lens primary fiber cells begin to develop, both equarin-L and equarin-S are strongly expressed in the lens with a high-dorsal-to-low-ventral gradient (Fig. 3e, f). Further frozen sections analyzed with whole-mount in situ hybridization confirmed this expression pattern (Fig. 3g). As well as in the lens, both equarin-L and equarin-S mRNAs were expressed at stage 19 in the isthmus, the cranial neural tube (especially rhombomeres r4 and r6), the dermatome, the dorsal region of the neural tube, the vitelline vein, and the anterior pore of the neural tube (Fig. 3e).

Equarin expression in the lens was further examined by whole-mount in situ hybridization at subsequent stages. During E4.5 to E10, both equarin-L and equarin-S are expressed in the lens equatorial region with a high-dorsal-to-low-ventral gradient (Fig. 4a–h). Two circles are visible in the lens, which correspond to the annular pad and the newly formed fiber cells around the equatorial region (Fig. 4k–l). In situ hybridization of sections showed that equarin transcript persists in the equatorial region (transition zone), where the differentiation of lens epithelium cells into fiber cells occurs (Fig. 4i–l).

Equarin expression in the lens was compared with the expression of known chicken lens markers at E3.5 (Fig. 5). Equarin transcript was located in the transition zone of the lens equator where lens epithelium

cells differentiate into lens fiber cells (Fig. 5a). β-Crystallin protein is expressed in the lens fiber cells (Fig. 5b). Pax6 protein is expressed in the lens epithelium cells and newly formed fiber cells in the equatorial region (Fig. 5c). Chicken connexin56 (Fig. 5d) transcripts were detected in fiber cells; connexin43 (Fig. 5e) in epithelium cells; and connexin45.6 (Fig. 5f) transcripts were detected in both epithelium and fiber cells.

3.3. Equarin proteins

To characterize the equarin proteins, we subcloned equarin cDNAs into expression vectors with His and myc tags at their C-termini, or six myc tags at their N-termini, and transfected these cDNAs into COS-7 cells. Western blot analysis of the supernatant and extracts of the transfected COS-7 cells showed that equarin proteins were present in both the cell supernatants and the cells, indicating that equarin molecules are secreted proteins. Equarin-S products tagged with N-terminal myc were identical to those of equarin-L tagged with N-terminal myc (Fig. 6). The largest band of approximately 165 kDa (star in Fig. 6) and several smaller bands were detected in the supernatant by western blotting, and corresponding bands were detected in whole-cell lysates (Fig. 6). However, when tagged at the C-terminus, the products differed in molecular weight not only between equarin-L and equarin-S, but also from those with N-terminal myc tags

(Fig. 6). Equarin-S was expressed as a single band of approximately 120 kDa in the cell supernatant and as several bands in whole-cell lysates, with the largest principal band approximately 80 kDa. Equarin-L was detected as several bands in whole-cell lysates, with the largest band approximately 150 kDa, but no bands were detected in the supernatant.

We further analyzed equarin proteins expression in transiently transfected COS-7 cells using immunocytochemistry with antibodies directed against the N- or C-terminal tags. Equarin proteins were detected in the extracellular regions of COS-7 cells (Fig. 7). Both secreted proteins with N- or C-terminal tags were strongly expressed around the cells.

3.4 Misexpression of equarin in embryonic lens and cornea did not alter their histology

To investigate if equarin is involved in lens fiber cell differentiation in vivo, we misexpressed equarin into chick presumptive lens placode at stage 10-11 by in ovo electroporation and observed the lens fiber cell differentiation using lens markers at E3.5. In our study, more than 80 chick embryos were undergone in ovo electroporation. Misexpression of equarin by in ovo electroporation was confirmed by both GFP signals in the lens (Fig. 8b, c) and in situ hybridization using equarin antisense probe (Fig. 8d). Both were consistent. Equarin was misexpressed not only in lens

epithelium cells and later lens fiber cells but also in partial cornea epithelium cells at E3.5 chick embryos. This is consistent with the fact that they are all originated from ectoderm. In the lens, equarin was found to be misexpressed in both lens epithelium cells and fiber cells covering 1/4 to 1/2 of the lens. However the eqarin-misexpressed regions of the lens and cornea epithelium showed normal morphology. We further examined these regions using the lens and cornea markers. Tsukushi is a novel molecule particularly expressed in lens epithelium and we used it as a lens epithelium marker. β-Crystallin, Sox1 and CP49 are strongly expressed in chick lens fiber and therefor were used as lens fiber markers. As shown in Fig. 8, both the lens epithelium and fiber markers showed normal expression pattern.

3.5 Equarin did not alter the lens fiber cell differentiation in vitro

Embryonic chick lens culture mimicked the lens fiber cell differentiation in vivo (Le and Musil, 2001; Menko et al., 1984). Explants of the lens epithelium were reported to undergo limited proliferation when cultured in the presence of 20% FCS, showing a thickened explants and cell migration around the edge of the explants. Thickening of the explant corresponds to the elongation of lens epithelium cells during fiber cell differentiation. The cell migration supposed to mimic the lens epithelium

cell proliferation. In our experiment, the explant supplied with 20% FCS (positive control) thickened and cells migration spread out after culture for one week (Fig. 9Bb). However the explants cultured with equarin-conditioned media (Fig. 9Bc) showed the same flatness and the cells around the explants migrated as less as the explants in negative control (Fig. 9Ba) despite of the various concentration of equarin protein in the medium. The explants treated with MAO of equarin and Xenopus Tsukushi (data not show) showed no difference showing as flat as the explants in the negative control after culture for one week (Fig.9Bd).

DCDML culture mimicked lens development in vivo, forming lens-like structure known as lentoids and showing a series of biochemical changes including production of a lens-fiber-cell specific protein, CP49 (Le and Musil 1998). To observe the effect of equarin on the DCDML culture of lens fiber cell differentiation, an equarin-expression construct was transfected into the DCDML culture one day after plating. The DCDML cultures transfected with empty vector cultured with and without presence of 20% FCS were used as positive and negative controls respectively. After incubation for further six days, the DCDML cells were harvested and were analyzed by immunoblotting to recognize the lens fiber cell specific protein, CP49. The immunoblotting showed that the positive control DCDML culture (with the presence of 20% FCS) showed a band of 49 kDa protein, but the equarin-treated and negative DCDML cultures showed no such

band (Fig. 10). The transfection of equarin into DCDML cells was confirmed by immunoblting recognizing the myc tag of equarin protein in the cell extracts (data not shown). The lentoid structure was not obvious in both the control and equarin-treated cultures (data not shown). These data showed that overexpression of equarin in lens cell culture of DCDML did not improve the lens fiber cell differentiation in vitro.

3.6 Effects of equarin on eye morphogenesis

To address the role of equarin in normal eye development, we injected synthetic equarin mRNAs into 4–8-cell stage Xenopus embryos. We observed effects on eye morphogenesis in approximately 75% (195 of 259) of embryos injected with > 200 pg equarin-S mRNA, and in 24% (55 of 231) of embryos injected with > 1000 pg equarin-L mRNA. Therefore, it seems that equarin-S has a stronger effect on eye morphogenesis than does equarin-L. Short-axis embryos were observed in 65% of embryos examined after injection with equarin-S mRNA (data not shown). In stage 46 embryos, eye morphogenesis is normally complete (Fig. 11a). However, on the injected side of the embryo, the optic fissure remained wide open, and its borders did not tend to contact each other (Fig. 11b). Moreover, in dorsal view, a thick optic stalk was seen to connect the eye and the forebrain on the injected side (Fig. 11c). Transverse sections of the injected

embryos showed that morphogenesis of the optic cup was strongly affected on the injected side, because the ventral retina apparently did not encircle the lens correctly (Fig. 11d, f). Instead, the ventral retina was connected to the brain (Fig. 11d, f). However, this abnormally folded retina seemed to undergo neural differentiation in the same way as the normal retina (Fig. 11e), because it showed both an inner plexiform layer and a photoreceptor layer (Fig. 11f). The lens of both uninjected and equarin-injected eyes showed normal morphology (Fig. 11e, f).

To determine whether equarin could affect the expression of the dorso-ventral marker molecule, we assayed the expression of Xpax2, normally confined to the ventral developing retina, in equarin injected Xenopus embryo. At stage 31, Xpax2 is specifically expressed in the ventral area of the prospective optic cup and the prospective optic stalk on the control side (Heller and Brandli, 1997; Barbieri et al., 1999; Fig. 11g). Remarkably, Xpax2 expression is maintained in the prospective optic stalk but not in the ventral area of the prospective optic cup on the injected side (Fig. 11h).

4. Discussion

In the present study, we used SST molecular cloning from an E6 chick lens cDNA library to identify a novel molecule that is possibly involved in eye development. Equarin-L and equarin-S, alternatively spliced products of the same gene, were identified and analyzed in detail. Our data suggest that equarin-L and equarin-S are soluble molecules expressed in the lens equatorial region, and are involved in eye development.

4.1 Equarin is expressed in the lens with high-dorsal-to-low-ventral polarity

The asymmetric development of the retina has been studied extensively at the molecular level. While retinoic acid and sonic hedgehog have been implicated in the regulation of ventral retinal development (Hyatt et al., 1992; Zhang and Yang, 2001), bone morphogenetic proteins (BMPs) have been described as regulators of dorsal retinal development (Koshiba-Takeuchi et al., 2000). In Xenopus, the dorsal-specific expression of type III deiodinase, an enzyme that inactivates thyroid hormone, is critical for the development of the asymmetric growth and differentiation of

the retina at metamorphosis (Marsh-Armstrong et al., 1999). Therefore, the asymmetry of the eye lies not only in its anatomical polarity with the choroid fissure and the optic nerve in the ventral eye, but also in the regionally differentiated expression of many signaling molecules and transcription factors (Adler and Belecky-Adams, 2002).

The lens is also a polarized structure, consisting of a mitotic epithelial layer and terminally differentiated lens fiber cells. Several signaling pathways, such as those involving fibroblast growth factor (FGF) (Chow et al., 1995; McAvoy and Chamberlain, 1989), insulin-like growth factor (IGF-1) (Beebe et al., 1980; Beebe et al., 1987), and transforming growth factor (TGF)-β ligands (de Iongh et al., 2001), have been implicated in the regulation of this polarity. Although much is known about the proximal–distal polarity of the lens, little is known about its dorso-ventral asymmetry. Recently, Faber et al. (2002) suggested the presence of nasal–temporal polarity in a histological study, showing that primary fiber differentiation in the ventral lens is faster on the temporal side than on the nasal side. Using mice expressing a dominant-negative form of the BMP receptor, they confirmed that, in the lens, primary fiber differentiation is inhibited only on the nasal side of the lens on the ventral side (Faber et al., 2002). We have demonstrated that equarin is expressed in the lens with a high-dorsal-to-low-ventral gradient, not only during the embryonic early stage, but also during the postnatal stage. To our knowledge, equarin is the

first molecule to be reported that is expressed in the lens with dorso-ventral polarity. However, equarin mRNA injected into Xenopus embryos affected retinal development rather than lens development. Therefore, during the continuous growth of the lens throughout life, equarin may maintain the distinct polarity of the retina, as well as that of the lens.

4.2 Microinjection of equarin mRNA into Xenopus embryos caused ventral eye malformation

The development of the vertebrate eye involves complex and reciprocal interactions between derivatives of the head surface ectoderm and the forebrain neuroectoderm (reviewed by Jean et al., 1998). In general, lens formation in higher vertebrates requires the presence of the optic vesicle in vivo (Harrington et al., 1991; Hyer et al., 1998). Equarin is expressed exclusively in the lens during eye formation, from the early stage until the postnatal period, with a high-dorsal-to-low-ventral gradient at the equatorial region. Microinjection of equarin mRNA into the dorso-animal blastomere of Xenopus embryos, where the eye and anterior brain are to develop, caused abnormal formation of only the ventral optic cup without altering the dorso-ventral patterning within the optic cup (Fig. 11). There is a possibility that the expression of unknown molecule(s) that interact with equarin differs between the dorsal and ventral optic cups, resulting in

varied responses to the ectopic equarin.

One plausible explanation for the abnormality of the ventral optic cup is that equarin affects on the cell migration. Cells in the ventral retina, where the choroid fissure forms, move into position from the optic stalk region during optic cup formation (Holt, 1980). In normal eye development, the asymmetric expression of equarin with a high-dorsal-to-low-ventral gradient might not influence cell movement. However, when equarin is ectopically expressed throughout the anterior region by microinjection, cell movement is inhibited, resulting in abnormal morphogenesis of the ventral retina, although neural differentiation occurs normally (Fig. 11). The expression of Xpax2, normally expressed in the optic stalk and the ventral retina (Heller and Brandli, 1997; Barbieri et al., 1999), was observed in the prospective optic stalk but not in the prospective ventral optic cup on the injected side, thus suggesting that the ventral tissue is contiguous with the neural tube (Fig 11).

Cell death and/or cell proliferation are supposed to be involved in the normal formation of the optic cup, especially in the ventral region (Schook, 1980; Navascues et al., 1985; Martin-Partido et al., 1988). However, the size of the retina and the number of the retinal cells are almost the same between the control and injected sides (Fig. 11), therefore, cell proliferation might not be influenced by the equarin overexpression. Identification of an equarin ligand or receptor in the retina will open the way to explaining our

observation.

4.3 A novel molecule equarin may belong to the same family as *Urb* and SRPX

Equarin shows relatively high homology and structural similarity to mouse *Urb* (Fig. 1a and Fig. 2b). Mouse *Urb* is a newly identified gene, the expression of which is highly upregulated in bombesin-receptor-subtype-3-deficient mice. It is therefore thought to be involved in the regulation of body weight and energy metabolism (Aoki et al., 2002). However, its function remains unclear. Mouse *Urb* mRNA is expressed in the anterior olfactory nucleus, the dorsal endopiriform nucleus, and the choroid plexus of the brain, and in the digestive system, liver, and other tissues in the mouse; however, its expression does not overlap that of the equarin in the chick embryo. Therefore, the low homology between *Urb* and equarin in regions other than the consensus repeat domains, together with their different patterns of mRNA expression, indicates that they belong to the same gene family but are not homologs.

The consensus domains identified in both equarin-L and equarin-S are also homologous to SRPX. SRPX is abundantly expressed in the human retina and is deleted in patients with retinitis pigmentosa (Meindl et al., 1995; Dry et al., 1995; Nangaku et al., 1997). A rat homolog of *SRPX* (*drs*)

is identified as a gene downregulated by v-src (Pan et al., 1996). SRPX, a member of the sushi-repeat-containing protein family, consists of three sushi domains (short consensus repeats [SCR] of 60 amino acids) with six conserved cysteine residues, a sushi-like domain with two conserved cysteines located between the second and third sushi domains, and a fifth domain following the third sushi domain that contains two cysteine residues (Fig. 2b). The consensus repeat domains in the equarin and *Urb* share about 30% homology with the fifth domain of SRPX, with the same consensus residues. The conservation of the consensus repeat domains in the chick equarin, SRPX, and mouse and human *Urb* suggests that they belong to the same gene family, and are thought to belong to the sushi-repeat-containing protein superfamily, as discussed by Aoki et al. (2002).

4.4. Equarin is modified after translation, cleaved, and secreted

By immunoblotting analysis of transiently transfected COS-7 cells, we found that the molecular weights of both expressed equarin-L and equarin-S proteins were much greater than those predicted from their amino acid sequences. It suggests that the equarin proteins are modified, for instance, by post-translational glycosylation because each contains several potential N-glycosylation sites (Fig. 1a). Furthermore, we identified four potential O-linked GlcNAc glycosylation sites (serine residues 60, 330, 378,

and 499) using a computational prediction method (Gupta et al.. 1999). However, the glycosylation of the equarin proteins requires verification.

The size disparity between the equarin proteins tagged at the N- and C-termini suggests that the endogenous proteins are cleaved after translation. As shown in Fig. 1, several proteinase cleavage sites occur within both equarin amino acid sequences. Because the two equarin proteins with N-terminal tags were identical in both the supernatant and whole-cell lysates when analyzed by immunoblotting, despite the difference in the lengths of their cDNAs, we predict that one of the cleavage sites is located in the sequence common to equarin-L and equarin-S. If this is the only cleavage site in the equarin proteins, we can predict the relative molecular weights of the equarin proteins by combining the masses of the products with the C- or N-terminal tags. In this way, we predicted that the molecular weights of equarin-L and equarin-S proteins before cleavage are approximately 315 kDa and 285 kDa, respectively.

Because the equarin proteins have several protease cleavage sites, and because they were tagged only at their N- and C-termini, it is possible that some fragments were not labeled. Therefore, the estimated molecular weights of the equarin proteins are minimum values. Antibodies directed against different parts of the equarin proteins will help us determine their molecular sizes and their localizations in vivo.

When both forms of equarin were tagged at the C-termini, equarin-L

protein showed no detectable band in the supernatant with immunoblotting. However, immunocytochemistry using an antibody against the C-terminal tag showed clear evidence that the C-terminal end of equarin-L was also secreted. Therefore, it is possible that the C-terminal part of equarin-L protein was cleaved and that the cleavage site is too close to the C-terminus for detection by immunoblotting. One proteinase cleavage site occurs between amino acid residues 907 and 908 (Fig. 1a). Alternatively, because the hydrophobicity of the C-terminal half of equarin-L is high (Fig. 1b), the solubility of the C-terminal part of the molecule should be very low, resulting in the precipitation and depletion of equarin-L in the supernatant.

As we could not detect in the cell lysate fractions any bands larger than those detected in the supernatant, we infer that the equarin proteins are cleaved soon after translation. Furthermore, secreted proteins are not retained in high amounts by the expressing cells in general. However, the bands of both equarin proteins were detected strongly in the cell lysates. This is consistent with the heavy precipitation of equarin proteins, as revealed immunohistochemically. Our samples may have included both cell fractions and proteins that were secreted and then precipitated.

Immunocytochemical studies showed that both equarin proteins are detected in the extracellular region close to the cells transfected with equarin cDNAs (Fig. 7). Extracellular matrix (ECM) molecules are secreted molecules that are immobilized outside cells, and include the

collagens, laminin, fibronectins, syndecan, and others. Some of these have restricted and developmentally-regulated distributions and important functions in embryonic development. The fact that equarin proteins were localized to areas of intercellular contact raises the possibility that the equarin molecules might constitute ECM proteins.

4.5 Equarin and lens fiber cell differentiation

After lens induction, the presumptive lens ectoderm elongate to form lens placode at chick embryonic stage 13-14, which then invaginate to form lens vesicle at stage 15-16. The primary lens fiber cell differentiation begins from the proximal part of the lens vesicle around chick embryonic stage 16-17, with the cells elongating to fill the enclosed cavity. The distal part of the lens vesicle remains cuboidal single layer as lens epithelial cells, which continuously proliferate at the equator region and differentiate into secondary lens fibers around the older layers. The process of differentiation from lens epithelial cell to fiber cell is ongoing in most animal well into adulthood (Piatigorsky, 1981). Equarin is first detected in chick lens placode at stage 14 where the cells are about to evaginate and pinch off to form lens vesicle and then in the proximal part of lens vesicle at stage 17 where the cells are about to elongate to form primary lens fiber cells. Later its expression persists in the lens equator region where lens fiber cell

differentiation occurs. The expression pattern suggested that equarin might involved in lens cell elongation and lens fiber cell differentiation.

To testify these hypotheses, we first carried out in ovo electroporation to misexpress equarin into chick presumptive lens ectoderm at stage 10-11. We observed the embryonic lens development at E3.5 when the primary lens fiber cell differentiation has started. The GFP signal as a tracer and in situ hybridization probed for equarin mapped the misexpression location in about half of the lens and partial cornea cells. However, no abnormalities of histology or lens marker expression were observed in the equarin-misexpressed region of E3.5 chick embryo.

Lens cell culture has long been used for analysis of lens fiber cell differentiation in vitro. To observe if equarin is involved in lens fiber cell differentiation, we further conducted lens cultures of explant and DCDML culture. As for lens epithelia explants, we used equarin-containing media as a gain-of-function analysis and used MAO of eqaurin as a loss-of-function analysis. However, neither thickness nor cell migration changes were observed, comparing to the positive and negative controls. As for DCDML cultures, we transfected equarin-expression plasmid DNA into DCDML cells as gain-of-function treatment. Lens fiber cell differentiation maker, CP49 protein, was not detected in the treated group, whereas it was detected in the positive group by immunoblotting.

During lens fiber cell differentiation a series of events occur,

including the arrest of DNA synthesis and cell division; cell elongation; initiation of synthesis of the different crystallins and differential genes expression (Piatigorsky, 1981). Equarin is one of the genes accompanying this processing. However, our data did not show any changes in histology or marker expression after misexpression of equarin in chick embryos and lens cultures. This reason might be because the limited amount of the misexpression conducted by both in ovo electroporation and transfection or treatment with equarin-containg medium in vitro. Another explanation is that equarin function in the lens may need the presence of other co-factors. Further experiments concerning these events during lens fiber cell differentiation will help us to understand the function of equarin during lens development.

5. Conclusion

In this study, we analyzed two novel alternatively spliced transcripts of secreted molecules, equarin-L and equarin-S, which were isolated from a chick E6 lens cDNA library. In the eye, these molecules are expressed exclusively in the lens. In vitro protein analysis showed that they are modified, cleaved, and secreted after translation. Microinjection of equarin mRNA into Xenopus embryos led to abnormal eye development, which suggests that equarin plays an important role in eye formation.

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7. Figures and Legends

a.

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equarin-S     1 MNWMPALSLALLWTAWLVCGSEKTGRLAERGSHGVRKVPQSHRAPSSLLRRSGASLKNLSPSPQHPVTKRDSSVPPKAPA
equarin-L     1 ...............................................................................

equarin-S    81 NLLKEESRSQPRSVGTRTRRLQRLTAAAKYSKSEMIKDEGISTASQSRAVRFPSGSSSPNVLASFAGKNRVWVISAPHAS  1
equarin-L    81 ...............................................................................  1

equarin-S   161 EGYYRLMMSLLKNDVYCELAERHIQQIVLFHEEGEEGGKVRRITNEGKILEQPLDPALIPKLMSFLKLEKGKFGMVLLKK  2
equarin-L   161 ...............................................................................  2

equarin-S   241 TLQVEERYPYPVRLEAMYEVIDQNPIRKIEKMRQKGFIQTCKAAGVEGQVVEDDNNGGSTQSIPGGGHVQVSAGGRKEEP  3
equarin-L   241 ...............................................................................  3

equarin-S   321 RRSSNQPTRTKTVRKPMTTTVATPLPTVRTTTLPTTTTATRATTRTVTTASRPTTTTTPLPTTQRTWTTKSHTTTEYHRL  4
equarin-L   321 ...............................................................................  4

equarin-S   401 PASPEVTTPRVMASEDFYSPVWKANRRDRQRGHPEKHLAATRKPSKGGRYESFTEVPTAPSVHYTKASMSRFKDNRTDRK  4
equarin-L   401 ...............................................................................  4

equarin-S   481 DYNHRDLNVTPGQHKPTKTKPPKKKTQEKILSNEYEDKYDPSKPASPHLEEEIAVGSIPPKKGKESKKHERMDKPEKKKK  5
equarin-L   481 ...............................................................................  5

equarin-S   561 KDRPDKLHKSEKQSKKDKAEKKSKQDKDRSKKKQEGEQNRE                                        6
equarin-L   561 ..............................NKK.SRTENEDFPKPNKKPFLQPPRKSVANLLDYFEGKRRLILITTPK  6

equarin-L   641 ADNTMYVQQRDEYLESFCKMATRKISVITIFGTMNNSSMKIDHFQLDNEKPMKVIEDEDLVDQQLISELRKEYGMTYNDF  7

equarin-L   721 FMVLTDTDMKVKQYYEVPIAMKSVFDLIDTFQSRIKDMERQKKEGIVCKEDKKQSLESFLSRFRWRRRLVVISAPSDEDW  8

equarin-L   801 AYSQQLAALSGQACNFGLRHITILKLLGVGEDIGGVLELYPINGSATVDREDIPANLVKDIRNYFQISPEYFSMLLVGKD  8

equarin-L   881 GNVKSWYPSPMWSMAIVYDLIDSMQLRRQEMTIQQSLGMQCPEDEYGGYGYHSYHQGYQEGYQDDYRHHGSYHHGYPY     9
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b.

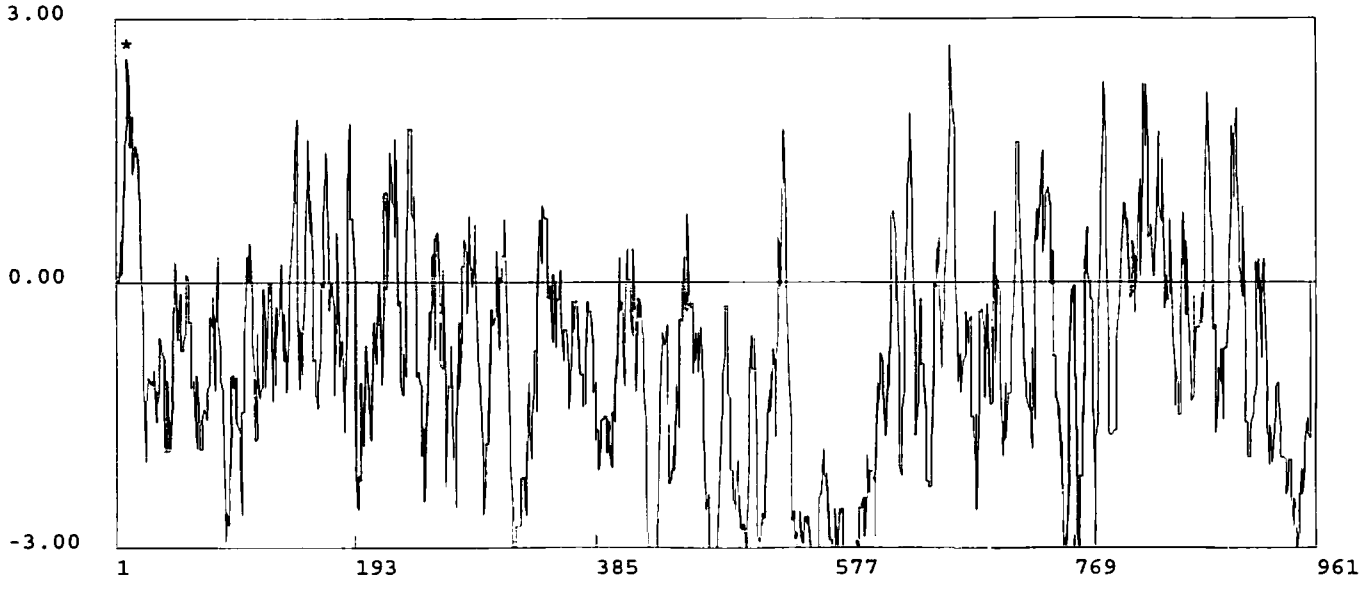

Fig. 1. Alignment of the deduced amino acid sequences of equarin-S and equarin-L. (a) Dots replace residues in equarin-L that are identical to the corresponding residues in equarin-S. The sequence in italics at the N-terminus indicates the putative signal sequence. Consensus repeat domains are underlined with solid lines. The Thr-rich region (328–395) is underlined with a wavy line. The Lys-rich region is underlined with a broken line. Dibasic protease-susceptible sites are marked with stars. Potential N-glycosylation sites are indicated by circles. (b) A Kyte–Doolittle hydrophilicity/hydrophobicity plot (range to average seven amino acids) of equarin-L. Star indicates the signal sequence.

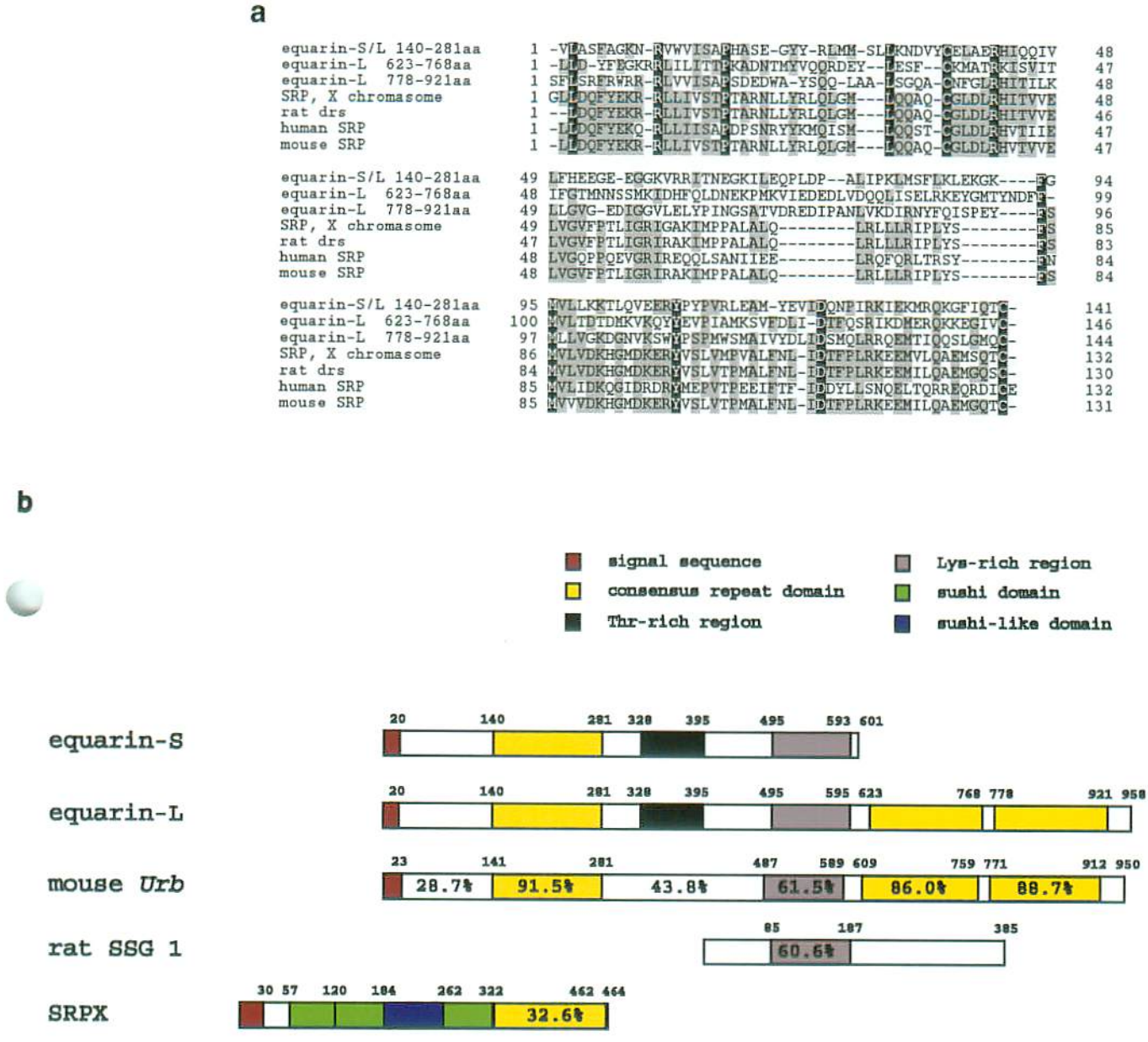

Fig. 2. Comparison of the primary structure of the equarin with those of known proteins. (a) The consensus repeat domains of the equarin-S and equarin-L, and comparable domains in human sushi-repeat-containing protein encoded on the X chromosome (SRPX), the product of the rat *drs* gene, human SRP, and mouse SRP were aligned to yield maximum identity. Amino acids identical in all seven sequences are indicated by black shading. Amino acids identical in more than four sequences are shaded in gray. (b) Schematic representation of the domain structure of equarin-S, equarin-L, mouse *Urb*, rat SSG1, and SRPX. The value given for each domain represents the percentage amino acid identity with the corresponding domains in equarin-L. The value given for the consensus domain in SRPX represents the percentage identity with the common consensus domain of equarin-S and equarin-L.

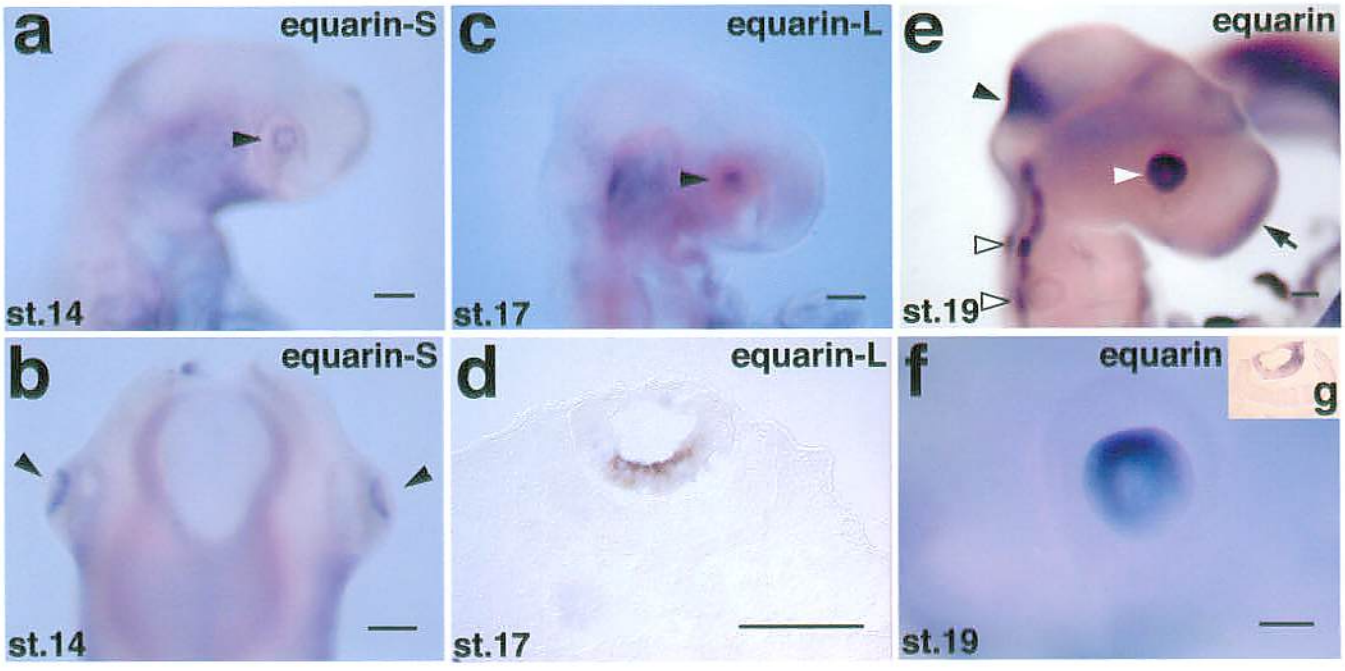

Fig. 3. Localization of equarin-S and equarin-L mRNAs during early lens development. (a) Equarin-S is first detected in the lens placode at stage 14 (arrowheads in a and b). (b) Dorsal view of (a). (c) Equarin-L is first detected in the lens vesicle at stage 17 (arrowhead). (d) Coronal section of the eye at stage 17 (proximal is downwards, dorsal towards the right). Note that equarin-L is localized on the proximal side of the lens vesicle. (e) Lateral view of stage 19 embryo. The distributions of equarin-S and equarin-L transcripts are identical. Note that equarin transcripts are strongly expressed in the lens (white arrowhead) with a high-dorsal-to-low-ventral gradient (e and f). Equarin transcripts are also expressed in the isthmus (arrowhead), the rhombomeres (particularly strongly at r4 and r6; open arrowhead), and the anterior pore of the neural tube (arrow). (f) High magnification of the eye shown in (e). (g) Coronal section of the eye in (e) with the same orientation as (d). Scale bars: 50 μm.

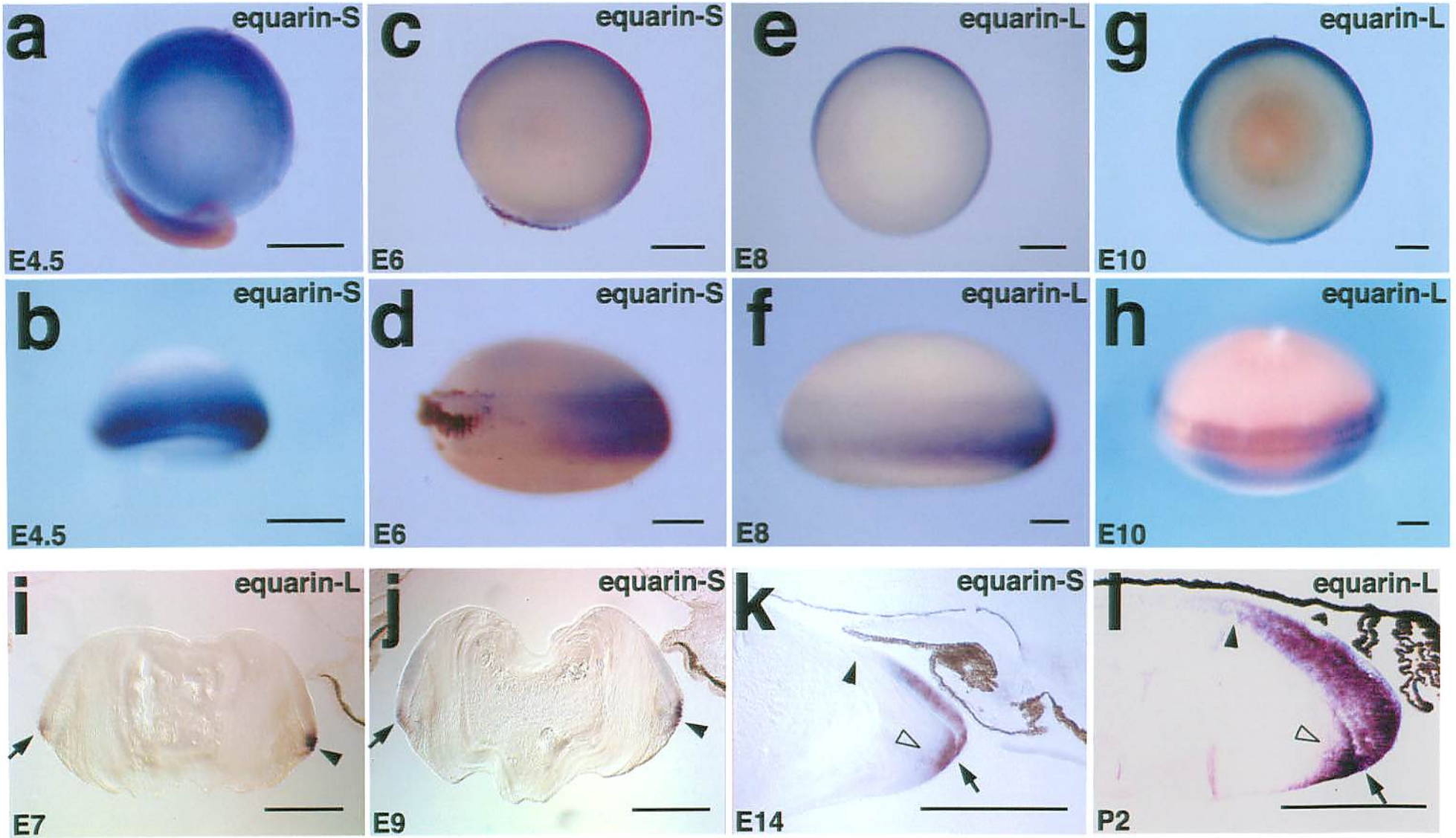
a
equarin-S
E4.5
c
equarin-S
E6
e
equarin-L
E8
g
equarin-L
E10
b
equarin-S
E4.5
d
equarin-S
E6
f
equarin-L
E8
h
equarin-L
E10
i
equarin-L
E7
j
equarin-S
E9
k
equarin-S
E14
l
equarin-L
P2

Fig.4

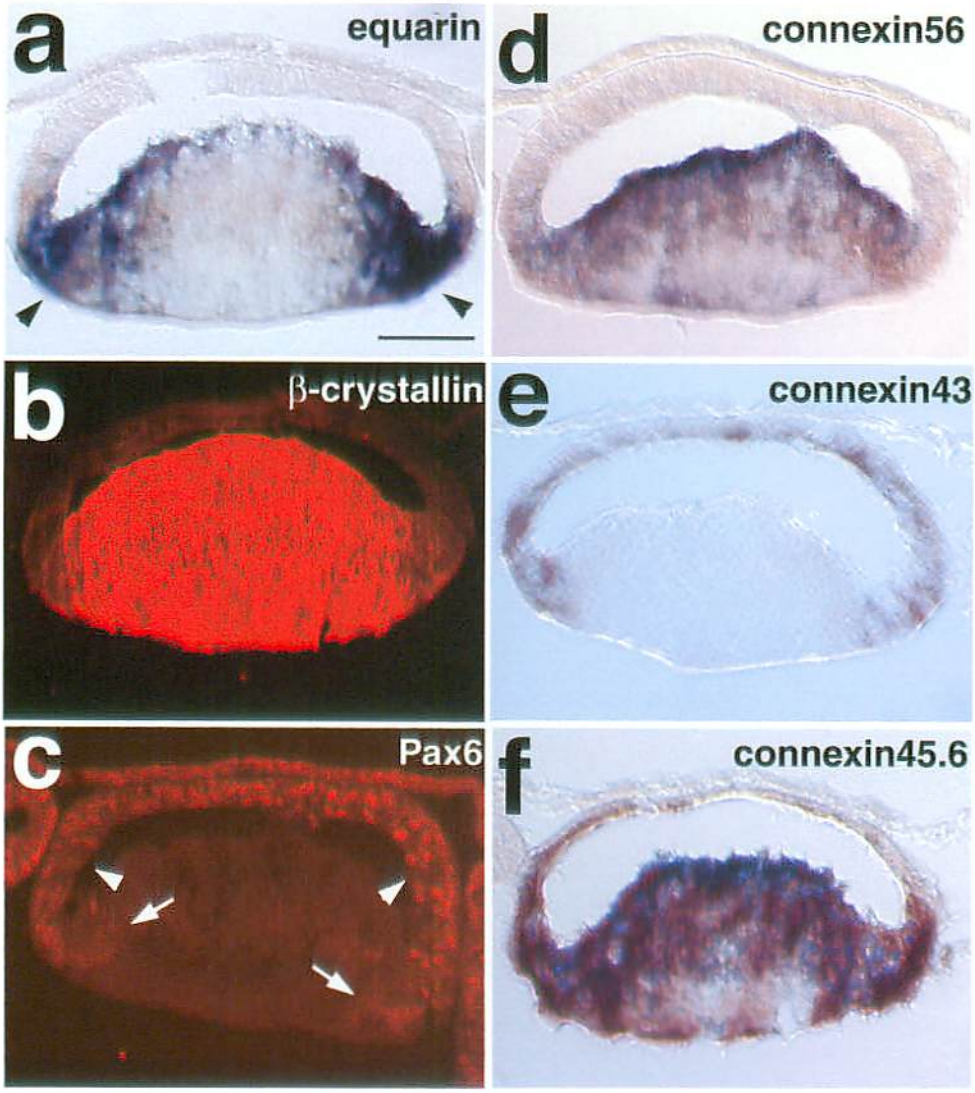

Fig. 5. Comparison of equarin expression with the expression of other known lens markers in the chick E3.5 lens. All panels are oriented proximal side down with dorsal towards the right. (a) Equarin is localized in the lens transition zone (arrowhead) where lens epithelium differentiates to lens fiber cells. The level of dorsal expression of equarin is relatively higher than the ventral level. (b) β-Crystallin is expressed in the lens fiber cells. (c) Pax6 is expressed in the lens epithelium (between arrowheads) and newly differentiated fiber cells (between arrowhead and arrow). (d) Connexin56 is mainly expressed in the lens fiber cells. (e) Connexin43 is expressed in the lens epithelium cells. (f) Connexin45.6 is expressed in both the lens epithelium and fiber cells. β-Crystallin and Pax6 were detected immunohistochemically using anti-β-crystallin and anti-Pax6 antibodies, respectively. Connexin and equarin transcripts were detected by *in situ* hybridization. Scale bar: 100 μm.

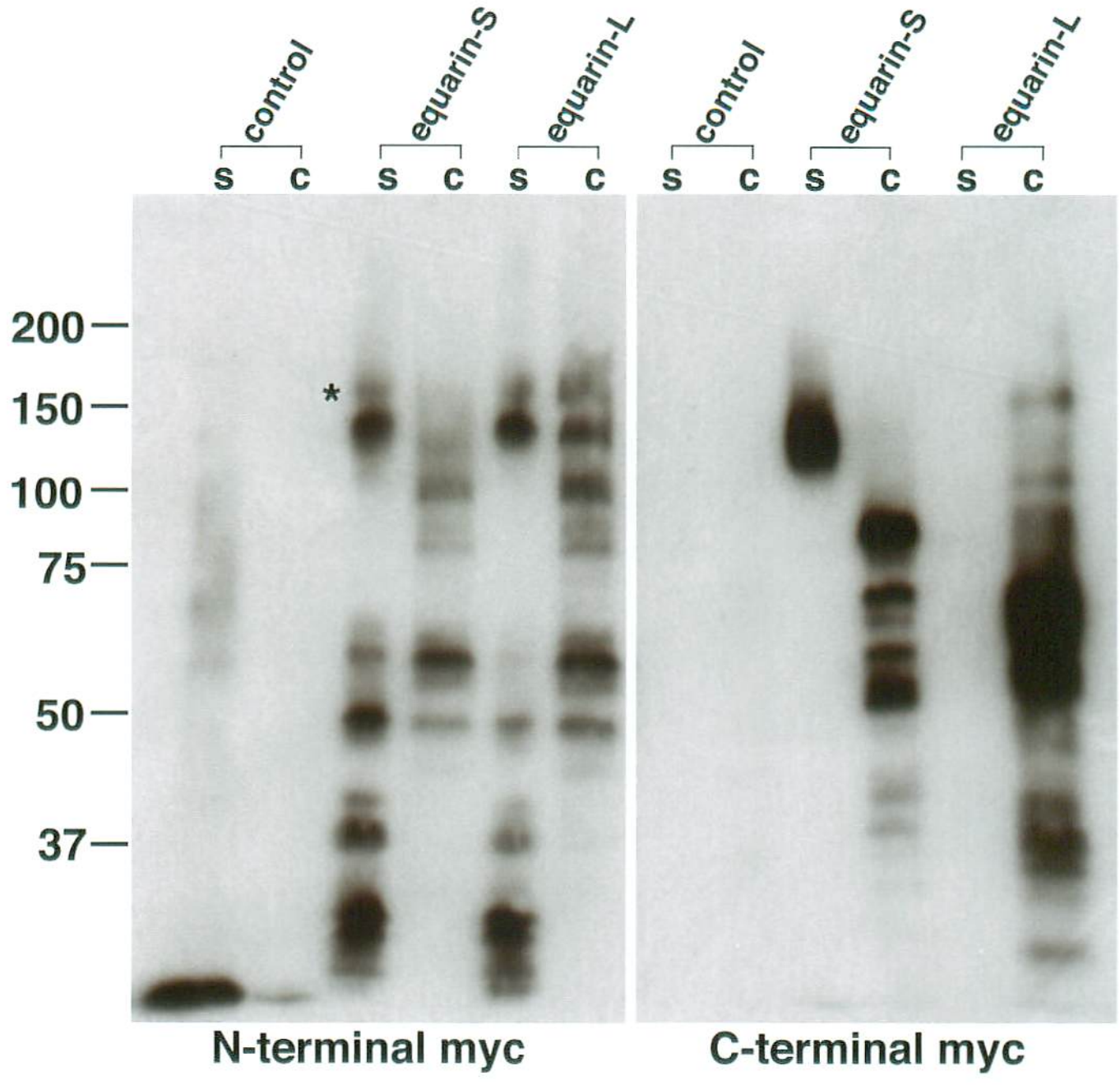

Fig. 6. Western blotting analysis of equarin-S and equarin-L proteins. Whole-cell lysates and cell supernatants were harvested 48 h after transfection. Equarin proteins were detected with anti-myc antibody directed against the myc tag at the N- or C-termini. The vector without cDNA was used as the control. Star (*) indicates the largest band detected in the supernatant via the N-terminal tag. The molecular masses (kDa) of the protein standards are indicated on the left. S: supernatant. C: whole-cell lysates.

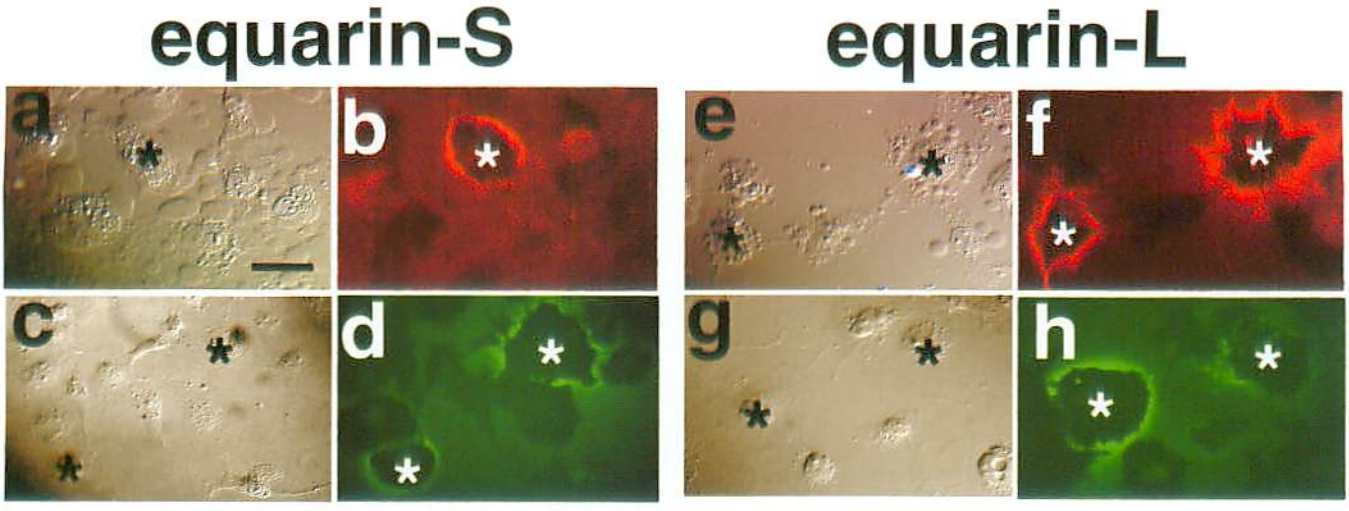

Fig. 7. Expression of equarin proteins in COS-7 cells. Equarin-S (a–d) and equarin-L (e–h) cDNAs were transfected into COS-7 cells, and cells were observed without fixation. Equarin proteins with His tags at their C-termini were visualized with an anti-His antibody conjugated with Alexa Fluor fluorescent dye (b, f). Equarin proteins with myc tags at their N-termini were visualized with anti-myc monoclonal antibody and a FITC-conjugated secondary antibody (d, h). Differential interference contrast (DIC) images of (b), (d), (f), and (h) correspond to (a), (c), (e), and (g), respectively. Note that the extracellular regions around the cells are stained for both equarin-S and equarin-L proteins. Secreted equarin proteins are heavily deposited around the cells (*), especially those proteins tagged at the C-terminus, and are deposited thinly far from the cells. No signals were detected in cells transfected with the control vector (data not shown). Scale bar: 50 μm.

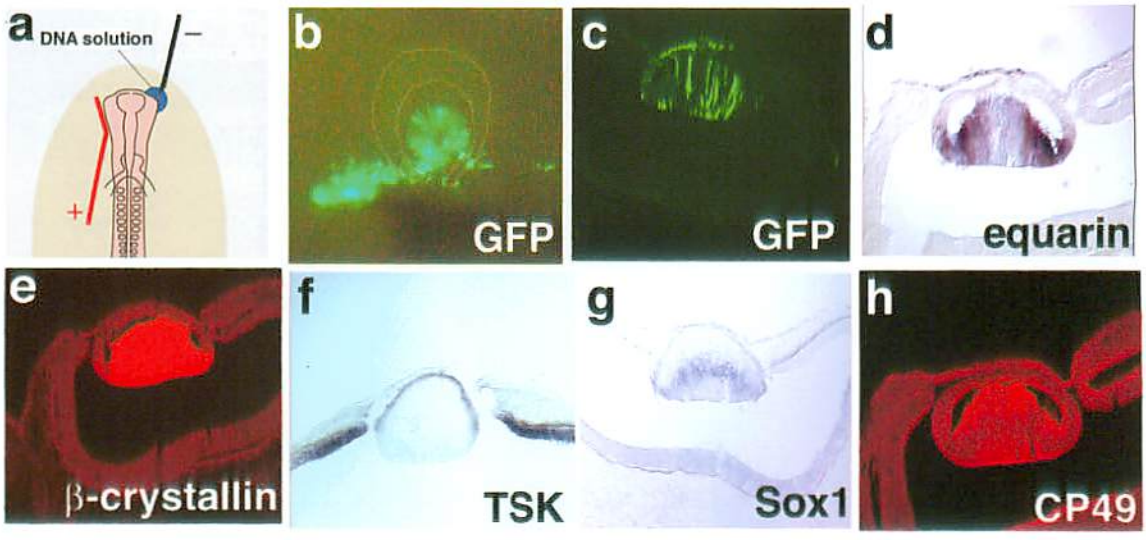

Fig. 8 Misexpression of equarin in chick embryonic lens by in ovo electroporation. After pEF-equarin DNA solution was injected, DNA was incorporated into the right presumptive lens ectoderm using two micro-electrodes at E1.5 (a). Embryos were observed at E3.5. Induction of equarin was confirmed by the GFP signals (b, c) and by in situ hybridization using equarin antisense RNA probe (d). (b) is the GFP expression in lens vesicle and surface ectoderm of the whole mount embryo, optic cup is indicated by dots. (c) is the section of the eye after in ovo electroporation at E3.5. No histological abnormalities of the lens were observed in both whole mount embryos and sections. The lens was further examined using the lens epithelium marker of TSK and the fiber markers of β-crystallin, CP49 and Sox1. All are expressed normally in the lens after electroporation.

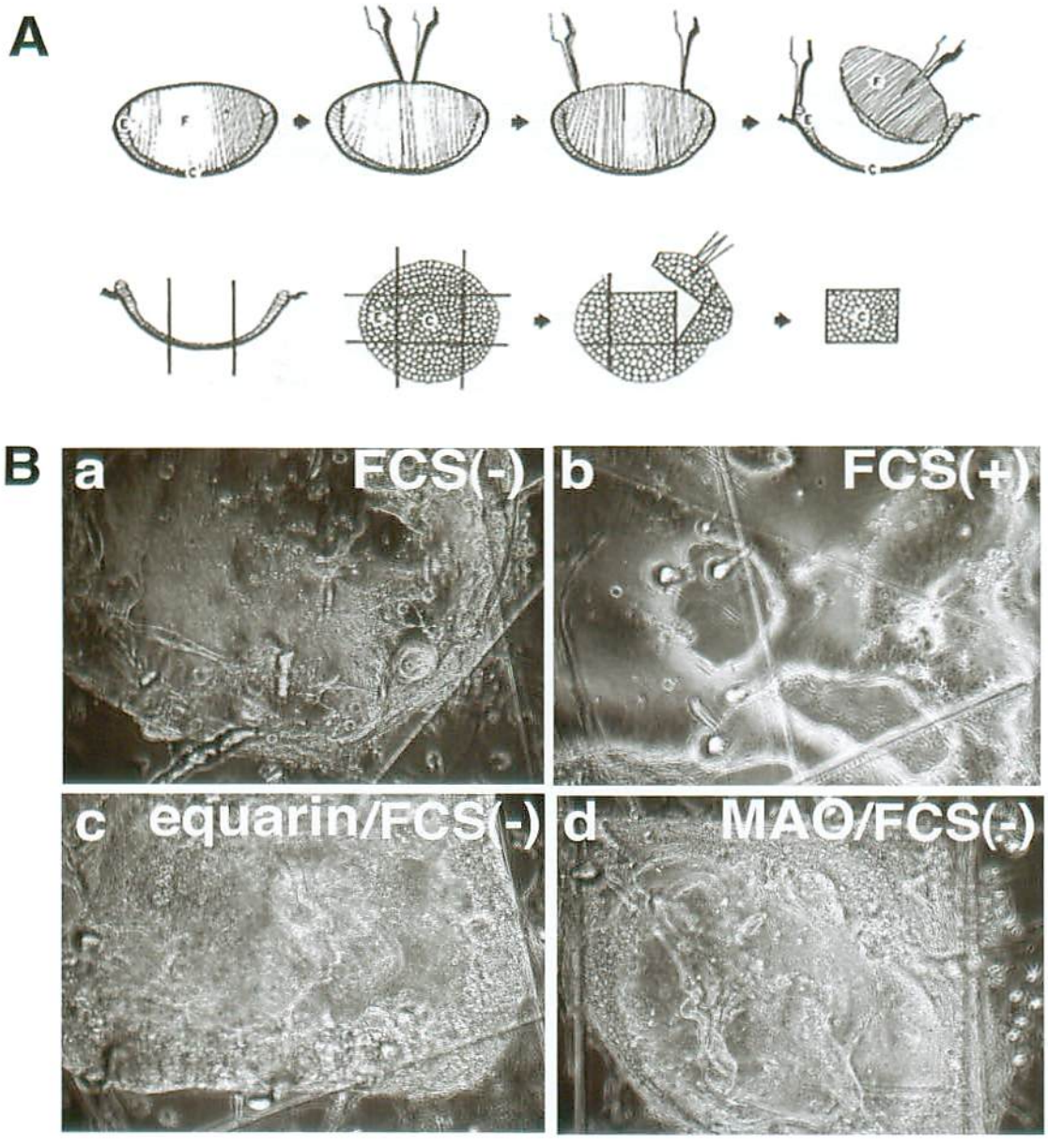

Fig. 9 Equarin did not promote lens fiber cell differentiation as shown in chick lens epithelia explants. (A) Chick lens epithelia explants were prepared from E6 chick lens. (B) Explants were culture for six days. The explants in basic medium of M199 and 0.1% BSA (negative control, a) remained flat and less cells in the periphery migrated out. However the explants cultured with the presence of 20% FCS (positive control, b) became thick and the cells in the periphery expanded. The explants cultured with the presence of equarin-containing media (c) or treated with morpholino antisense of oligonucleotide of equarin (d) remained flat and less cells migrated out like the negative control.

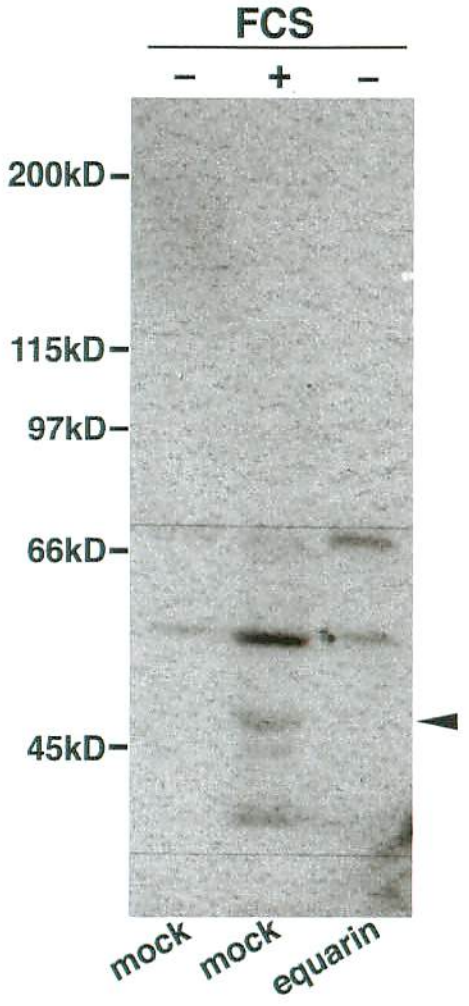

Fig. 10 Western blotting of chick DCDML cell culture one week after transfection. Dissociated cell-derived monolayer (DCDML) cultures were prepared from E10 chick lenses and were transfected one day after plating. DCDML cultures were then supplied with basic culture medium. The DCDML transfected with empty pEF plasmid DNA and cultured with and without the presence of 20% FCS were used as positive and negative control, respectively. Cultures were harvested six days later and immunoblot was performed to recognize lens fiber cell specific protein CP49. The immunoblotting shows that the positive control DCDML culture shows a band of 49 kDa band (arrow), but the equarin-treated and negative DCDML cultures showed no such band.

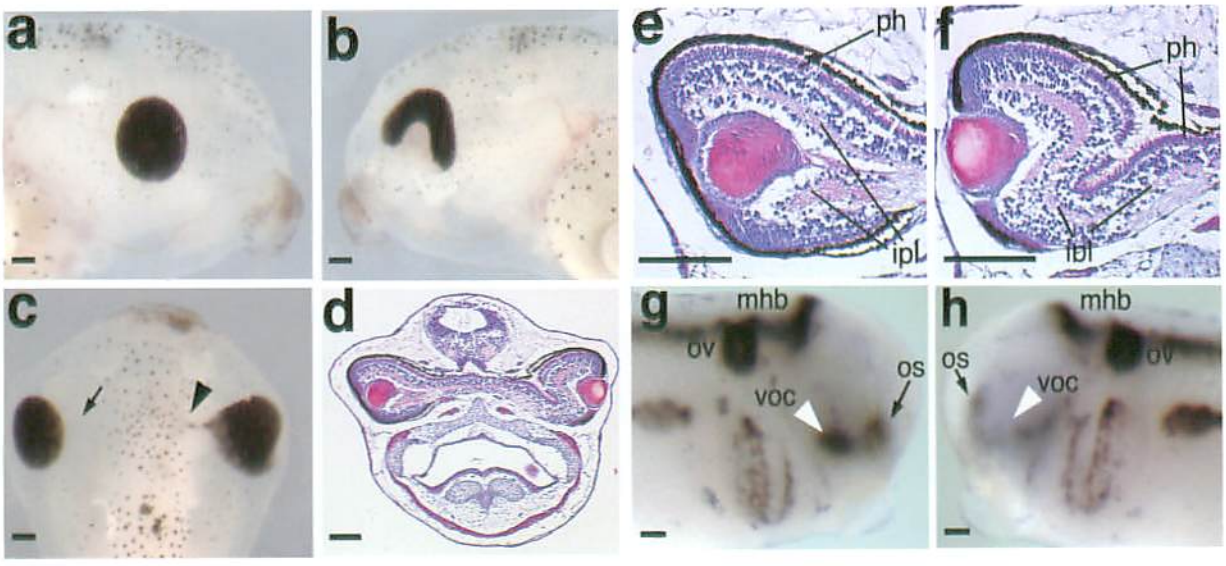

Fig. 11. Effects of equarin-S on eye formation.

(a) Lateral view of the embryo at stage 46 from the uninjected side of the eye. Eye morphogenesis is complete. (b) Lateral view of the embryo at stage 46 from the side of the eye injected with equarin-S mRNA. The optic fissure remains wide open. (c) Dorsal view of the same embryo shown in (a) and (b). On the side injected with equarin-S RNA, the optic cup and the forebrain are abnormally connected by a thick optic stalk (arrowhead). The optic cup and the forebrain are connected by a thin optic nerve on the uninjected side (arrow). (d) Transverse sections of a stage 41 embryo injected with equarin-S mRNA. (e and f) Higher magnification of (d). (e) On the uninjected control side, the formation of the retinal structure is completed normally. The neural retina consists of an inner plexiform layer (ipl) and a photoreceptor layer (ph). (f) On the side injected with equarin-S RNA, defective invagination of the ventral optic cup is evident. Note that the aberrantly folded neural retina still forms an ipl and a ph. (g) Lateral view of the embryo at stage 31 from the uninjected side of the eye. Xpax2 is expressed in the prospective ventral optic cup (arrowhead) and the prospective optic stalk (arrow). (h) Lateral view of the embryo at stage 31 from the side of the eye injected with equarin-S mRNA. Xpax2 is expressed in the prospective optic stalk (arrow) but not in the prospective ventral optic cup (arrowhead). mhb, mid-hindbrain boundary; os, optic stalk; ov, otic vesicle; voc, ventral optic cup. Scale bars: 100 μm.