Differentiation of Induced Pluripotent Stem Cells to Lentoid Bodies Expressing a Lens Cell-Specific Fluorescent Reporter

Taruna Anand¹,2☯, Thirumala R. Talluri¹,2☯, Dharmendra Kumar¹,3, Wiebke Garrels⁴, Ayan Mukherjee¹, Katharina Debowski⁵, Rüdiger Behr⁵, Wilfried A. Kues¹*

¹ Friedrich-Loeffler-Institut, Institut für Nutztiergenetik, Neustadt, Germany, ² ICAR-National Research Center on Equines, Hisar, India, ³ ICAR-Central Institute for Research on Buffalos, Hisar, India, ⁴ Medical School Hannover, Hannover, Germany, ⁵ German Primate Center, Göttingen, Germany

☯ These authors contributed equally to this work.
* wilfried.kues@fli.bund.de

Abstract

Curative approaches for eye cataracts and other eye abnormalities, such as myopia and hyperopia currently suffer from a lack of appropriate models. Here, we present a new approach for in vitro growth of lentoid bodies from induced pluripotent stem (iPS) cells as a tool for ophthalmological research. We generated a transgenic mouse line with lens-specific expression of a fluorescent reporter driven by the alphaA crystallin promoter. Fetal fibroblasts were isolated from transgenic fetuses, reprogrammed to iPS cells, and differentiated to lentoid bodies exploiting the specific fluorescence of the lens cell-specific reporter. The employment of cell type-specific reporters for establishing and optimizing differentiation in vitro seems to be an efficient and generally applicable approach for developing differentiation protocols for desired cell populations.

Introduction

Age-related cataracts are one of the most prevalent ocular conditions resulting from the failure of specific cell types and represent the major eye disease in humans [1]. But a systematic approach to study human cataracts is hampered by the lack of appropriate models [2]. Therefore, in vitro systems for studying lens formation and disease mechanisms represent an alternative for ophthalmological research.

The understanding of lens morphogenesis and the involved cellular and molecular events serves as key in defining the general mechanisms of cell specification and gaining a better understanding of lens function. The eye lens originates from a single progenitor lineage, which comprises both the posterior lens fiber cells and the anterior lens epithelial cells [2]. In mammals, the lens progenitor cells originate from a vesicle at the lens placode [3,4] and the lens fiber cells terminally differentiate to ultimately contributing to the three-dimensional structure of the lens. This includes a massive up-regulation of lens-specific genes, such as alpha- and beta-crystallins [5,6]. Expression of alphaA crystallin (Cryaa) is initiated in the cells of the
inverting lens placode and later on is restricted to the lens [5,6]. The Cryaa represents 20–40% of the crystallin content in the lens [7–9], and a molecular understanding of its temporally and spatially regulated expression in the lens is an important issue of cellular differentiation in general. Knock-out or loss-of-function mutations of the Cryaa gene have been shown to result in the formation of cataracts [10,11] and in apoptosis of lens epithelial cells [12], clearly indicating its pivotal role for lens function. Genetic studies in humans suggested a causative correlation between Cryaa mutations and cataract formations [13–20]. Previously, embryonic stem (ES) cells have been used to differentiate into lentoid bodies \[2,21\] and retinal cells \[21\] \textit{in vitro} by using co-culture techniques with stromal cells \[21\] and by sequential supplementation of the culture medium with Noggin, fibroblast growth factor 2 (FGF2) and Wnt-3a \[2\]. Induced pluripotent stem (iPS) cells were used to generate retinal pigmented epithelium \[22–24\] and recently, the generation of lens progenitor cells from iPS cells of cataract patients and healthy donors \[25\], and the derivation of corneal epithelial cells from human iPS cells was achieved \[26, 27\]. In addition, an iPS cell-based disease model for ectodermal dysplasia and impaired corneal differentiation has been described \[28\].

Here, we conducted a proof-of-principle study for the differentiation of murine iPS cells to lens cells. We exploited the cell-type specific expression of the Cryaa promoter for the generation of a transgenic mouse model with expression of a vital fluorophore reporter, tdTomato, in the eye lens. Fetal fibroblasts derived from these mice were reprogrammed to iPS cells, and the suitability of the reporter to follow differentiation into lens cells via lentoid body formation \textit{in vitro} was assessed. We hypothesized that the derivation of iPS cells from a transgenic mouse line carrying the Cryaa-tdTomato construct can be used to follow differentiation into lens cells \textit{in vitro} (Fig 1). This approach will facilitate the controlled development of more efficient protocols for lens cell-differentiation, and will aid to improve differentiation protocols with human cells.

**Results**

**Generation and characterization of cryTom mouse line**

A \textit{piggyBac} (PB) transposon (pCryTom) was designed, consisting of the alphaA-crystallin promoter, tdTomato cDNA and a SV40 poly adenylation sequence, flanked by PB inverted terminal repeats (ITR). Murine zygotes were treated by co-injection of pCryTom plasmid and pBP helper plasmid into the cytoplasm \[29,30\]. A total of 20 injected zygotes were transferred by surgical embryo transfer into the oviduct of one surrogate mother. One out of the delivered 8 pups was confirmed to carry the transposon construct by Southern blotting (S1 Fig). Importantly, the transgenic founder showed eye-specific expression of the tdTomato transposon (S1 Fig). The monomeric transposon was inherited in a Mendelian fashion and transgenic F1 and F2 offspring exhibited an identical phenotype (Figs 2 and 3).

During fetal development, the first expression of the reporter was found in the eye lens of day 12.5 fetuses (Fig 2), and lenses of older fetuses showed increasing fluorescence intensities. Likely, an increased cell number, increased expression level, and protein accumulation contributed to the increased reporter intensity. No ectopic expression of the reporter construct was found by whole mount imaging of fetuses, indicating that the reporter construct faithfully mirrors differentiation of the eye lens.

In accordance with the known expression pattern of the Cryaa gene ([5], \url{www.genevestigator.com}), the highest expression level of the cryTom was found in the adult eye lens, and reduced levels were detected in retina and ciliary muscle (Fig 3). In postnatal lens samples the full size tdTomato and three to four smaller products were consistently found in immunoblots using a polyclonal antibody (Fig 3). The smaller products most likely represent degradation products of
tdTomato, which seemed to be removed at a slow rate in mature lenses. Western blotting of the endogenous alphaA crystallin showed that the expression of this lens protein was not affected by the transgenic status (Fig 3).

The apparent accumulation of smaller “degraded” products of tdTomato raised the question, whether the presence of these ectopic protein products may interfere with the highly ordered organisation of crystallin proteins in the lens. Therefore the light transmittance properties of wild type and cryTom lenses were assessed. Fig 4 shows a representative image of adult lenses recorded while illuminated from below in a stereomicroscope. A quantitative determination suggested that the transgenic lens indeed showed a reduced light transmittance in comparison to a non-transgenic lens.

Reprogramming of cryTom fetal fibroblasts to induced pluripotent stem (iPS) cells

Heterozygous matings were initiated to isolate fetuses of day 11.5 of gestation, which were used to derive fetal fibroblasts. The fibroblasts were genotyped by PCR for the presence of the
cryTom construct. As expected both cryTom-positive and cryTom-negative fibroblast populations did not express the reporter. Reprogramming of the fibroblasts to iPS was done by a non-viral approach employing a *Sleeping Beauty* (SB) transposon system as described previously [31,32]. Seven to nine days after co-electroporation of SB helper plasmid and multi-cistronic reprogramming transposon, encoding the murine cDNAs of Oct4, Sox2, Klf4 and c-Myc separated by sequences coding for the self-cleaving 2A peptides, initial colonies appeared. Around day 18 post electroporation, individual colonies were picked and expanded. None of these iPS

---

**Fig 2. Exclusive expression in eye lens during fetal development.** (A) Murine fetus at day 10.5 p.c., A’ corresponding fluorescence image. (B) Murine fetus at day 11.5 p.c., B’ corresponding fluorescence image. (C) Murine fetus at day 12.5 p.c., C’ corresponding fluorescence image, note the onset of tdTomato expression in the forming lens area (arrow). (D) Higher magnification of the d12.5 fetus, overlay, D’ brightfield and D” fluorescence images. (E) Murine fetus at day 13.5 p.c., E’ corresponding fluorescence image. (F) Murine fetus at day 14.5 p.c., F’ corresponding fluorescence image. Size bars = 1 mm.

doi:10.1371/journal.pone.0157570.g002
cultures did express the cryTom reporter, supporting the notion that lens-exclusive expression of cryTom is maintained under in vitro culture conditions.

The iPS cells expressed typical features of pluripotent cells (Fig 5). They were alkaline phosphatase positive and showed the typical colony growth of murine pluripotent cells. They showed an up-regulation of the stemness genes Oct4, Sox2, Nanog, Utf2 and Rex1.

**Differentiation to lentoid bodies in vitro**

Then we assessed, whether the cryTom construct can be utilized to establish and to follow differentiation into the lens cell lineage in vitro. Therefore mitotically-inactivated NTERA-2, P19 and STO cells were used as feeder cells, respectively. The NTERA-2 cells represent a committed human neuronal precursor line, and the P19 is a murine embryonic carcinoma cell line with differentiation potential into all three germ layers. We assumed that NTERA-2 and P19 may provide a suitable niche for differentiation of the iPSs towards the ectodermal direction, including lens cell differentiation. Critical factors for ectodermal differentiation may be surface epitopes and the secretion of paracrine factors, like BMP4. STO cells, a murine embryonic fibroblast line, served as control.

One day after seeding of the iPS cells on the different feeders, the stem cell medium was switched to a DMEM-based medium without LIF. The proliferation of iPS cells slowed down under these conditions. The cultures were split three days later and again seeded on the respective feeders. Around day 28 after seeding on feeders, the first tdTomato-positive cells were
identified in the cultures with NTERA-2 and P19 feeders, but not in co-culture with STO cells. In some cases, individual cells expressed the reporter (Fig 6), in other cases the positive cells grouped to form lentoid bodies. The lentoid bodies also showed a changed light refraction in the brightfield view (Fig 6). At day 45 of the differentiation the cultures were used for molecular analyses, a mean of 5–10 lentoid bodies/well were counted at this time point. Expression analyses indicated that the co-cultures with NTERA-2 cells up-regulated the \textit{tdTomato} and the endogenous \textit{Cryaa} transcripts, but also other crystallin genes, like \textit{Cryf}. Importantly, key regulator genes of lens differentiation, like \textit{Pax6} and \textit{Prox1} were also detected by RT-PCR (Fig 6).

**Discussion**

Here, we generated a transgenic mouse line carrying an \textit{alphaA crystallin}-promoter driven \textit{tdTomato} reporter (cryTom), and generated an iPS cell line using a transposon-mediated approach. The generated iPS cell line was exploited in a proof-of-concept study for directed differentiation to lens cell lineage in a co-culture system. The cryTom mouse line was generated by \textit{in ovo}-transposition, co-injecting the cryTom-transposon and a \textit{piggyBac} helper plasmid. A detailed characterization of the cryTom mouse line suggested that the reporter faithfully mirrored the spatial and temporal expression pattern of the \textit{Cryaa} gene. Induced PS cells could be derived from cryTom fibroblasts; upon exposing the cryTom iPS cells to a differentiation protocol, expression of the \textit{tdTomato} reporter was resumed, thus allowing simple identification and vital recording of lentoid body growth \textit{in vitro}.

For differentiation we used a novel co-culture system with the human NTERA-2, a committed neuronal precursor line [33] and P19, a murine embryonic carcinoma line [34]. Previously,
in vitro differentiation to lens cells was achieved by coculture [21], but also by supplementation of the culture media with defined growth factors, such as Noggin, FGF2 and Wnt-3a [2]. We hypothesized that the NTERA-2 and the P19 cell form “niches” for ectodermal differentiation of the cryTom iPS cells. It has been shown that the supplementation with retinoic acid can promote the expression of ectodermal characteristics of P19, but also of NTERA-2 [35,36]. This may be an approach to increase the directing effects of the feeder cells. Apparently, the spontaneous capabilities of mitotically inactivated NTERA-2 and P19 are sufficient to direct the differentiation of murine iPS cells into the lens cell lineage. The cryTom reporter construct allowed the unambiguous identification of onset of tdTomato expression as a faithful indicator of lens cell differentiation. Importantly, the detailed characterization of the spatio-temporal pattern of the cryTom reporter confirmed the exclusive expression in lens cell progenitors and mature lens cells.

The design of cell type specific-promoter sequences driving fluorescent reporters, such as EGFP or mCherry gained reasonable interest in genetics over the last years. Examples are the Oct4 promoter-EGFP cassette [37], as well as a multitude of other constructs [38–40]. Recently, the development of hyperactive transposon systems made transposase-catalyzed gene integration an attractive alternative [41,42] to commonly employed random integration, or homologous recombination approaches. Here, we employed a piggyBac transposase catalyzed integration of the cryTom reporter in ovo, using a simplified plasmid microinjection technique in murine zygotes [29,43,44]. The main advantage of the transposase-catalyzed approach is that integration will take place at transcriptional permissive loci in the genome allowing for promoter-dependent expression [43,45]. Thus avoiding the generation of multiple founders.
and screening of those with appropriate expression patterns [46]. Indeed, from the first embryo transfer we obtained a single transgenic founder, which faithfully showed the expected phenotype. Importantly, the transposition of the cryTom reporter in zygotes supersedes the necessity to include any selection marker, such as an antibiotic resistance. It has been shown before that antibiotic selection markers and regulatory elements of secondary expression cassettes can exert massive effects on the primary expression construct, e.g. via promoter interference [47,48].

Importantly, the cryTom mice show a reduced light transmission through their lenses, a feature, which is also found in cataracts. Whether this is due to characteristics of the tdTomato protein itself, the accumulation of degraded products of tdTomato, or an unspecific interference of the ectopic protein with the highly ordered organisation of crystallins in the lens.

Fig 6. Characterization of in vitro-formed lentoid bodies. (A) Lentoid bodies with tdTomato expression derived from a co-culture of cryTom iPSc on NTERA-2, A’ corresponding brightfield view. Bar = 50 micrometer. (B) Individual tdTomato-positive cells derived from a co-culture of cryTom iPSc on P19, B’ overlay; B’’ corresponding brightfield view. Bar = 50 micrometer. (C) Lentoid body with tdTomato expression derived from a co-culture of cryTom iPSc on P19, C’ overlay; C’’ corresponding brightfield view. Bar = 50 micrometer. (D) Expression analyses of co-cultures by RT-PCR. The endogenous murine Cryaa gene could be detected in P19 co-cultures. The endogenous lens-specific CryF transcript could be detected in NTERA-2/iPS and P19/iPS co-cultures, but also in P19 cells. CoN, co-culture of NTERA-2 and iPSc; CoP19, co-culture of P19 and iPSc; N, NTERA-2; P19, P19 cells; eye, positive control; -RT, without reverse transcriptase; H2O, no template. (E) Immunodetection of tdTomato protein. CoN, co-culture of NTERA-2 and iPSc; CoP19, co-culture of P19 and iPSc; N, NTERA-2; P19, P19 cells. (F) Expression analysis of co-cultures for key regulatory genes, Pax6 and Prox1, of lens differentiation by RT-PCR. CoN, co-culture of NTERA-2 and iPSc; CoP19, co-culture of P19 and iPSc; F, fibroblasts.

doi:10.1371/journal.pone.0157570.g006
warrants further investigations. Here, we conducted a proof-of-principle study in murine cell cultures. The mouse model allowed to thoroughly investigate, whether the reporter expression faithfully reflect the temporal and spatial pattern of the endogenous gene. For human iPS cells the transduction in ovo is of course not a realistic option, however, the here gained evidence may allow to transpose existing human iPS cell lines with the reporter construct and still exploit the reporter for optimized differentiation approaches. The main advantage of the here described system is that it allows to optimizing the differentiation conditions. These optimized protocols may then be applied to human cells.

The present data show that it is possible to employ two different transposon systems, here PB and SB, to perform complex genetic modification. The current data support the notion that the cell type-specific reporter approach is instrumental for the development, validation and optimization of differentiation protocol of murine iPS cells into the lens cell lineage. The specific fluorescence of the tdTomato reporter will allow the development of semi-, or high throughput approaches for the rapid testing of media supplements. We speculate that the obtained knowledge can be translated to optimize lens cell differentiation of human iPS cells and thus to advance the growth of patient-specific lentoid bodies. Likely, the cell type-specific reporter approach is also adaptable for in vitro tracking of other cell types.

**Materials and Methods**

**Ethics statement**

Animals were maintained and handled according to the German laws for animal welfare, and genetically modified organisms. The experiments were approved by the authorititative external ethics committee of the LAVES (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, AZ 33.9-42502-04-09/1718).

**Plasmid construction**

A Cryaa promoter-tdTomato plasmid was gifted by T. Xu (Yale) [6]. The Cryaa promoter-tdTomato cassette was released by restriction with MluI and AflII and ligated in compatible sites between 5’ and 3’ piggyBac ITR’s, resulting in pTTcryTom (cryTom) plasmid consisting of the alphaA-crystallin promoter, tdTomato cDNA and a SV40 poly adenylation sequence, flanked by PB ITRs (S1 Fig). The PB transposase plasmid was described before [42], and essentially contained a cytomegalovirus, immediated early promoter driven hyperactive PB transposase cDNA. The SB reprogramming transposon carrying the murine cDNAs of Oct4, Sox2, Klf4 and cMyc separated by sequences coding for self-cleaving 2A peptides, and the SB transposase helper plasmid were described before [32,49].

**Generation of PB transgenic founder mouse**

The NMRI mice were bred and maintained in an air-conditioned animal quartier at 20°C and 60% humidity with 12 hour light and 12 h dark cycles. For zygote flushing, NMRI females of 5–6 weeks of age were superovulated by i.p. injection of 10 units PMSG and 10 units hCG in a 46–48 h interval. The treated animals were then mated with fertile males. Females with a copulation plug were identified the next day, sacrificed by CO₂, and subsequently the isolated oviduct was flushed with M2-medium. Zygotes with two polar bodies were treated by cytoplasmic injection (CPI) of an equimolar mixture of pTTcryTom and PB helper plasmid [44,45]. A total of 20 treated zygotes were surgically transferred into the oviduct of a surrogate mother, resulting in the birth of 8 offspring of which one was transgenic for the cryTom transposon. The transgenic founder was used to establish a stable line by mating with a wild type animal. The
offspring were phenotyped by whole body excitation with a green LED flood light, and images were recorded with a digital camera and an appropriate emission filter.

**Fluorescence microscopy**

For fluorescence microscopy of cell cultures, a Zeiss Axiovert 35M microscope equipped with fluorescence optics was used. For specific excitation of tdTomato a filter block with excitation of 530–570 nm and emission of 590–610 nm were used. Alternatively, images were obtained by an Olympus BX 60 (Olympus, Hamburg, Germany) fluorescence microscope equipped with a high resolution digital camera (Olympus DP71).

For imaging of tissue biopsies an Olympus SZ16 stereomicroscope with epifluorescence optics was used. The light transmittance of lenses was also measured with the stereomicroscope. Therefore wildtype and transgenic lenses were isolated, and placed side by side under the stereozoom microscope. Normalized grey scale images were kept while illuminated from below. With the Olympus CellF software histograms (relative light transmission) of identically treated lenses (dotted lines in Fig 4A) were determined and plotted.

**Genotyping by PCR and Southern blotting**

Southern blots and PCR reactions of genomic DNA were done according to standard procedures. In brief, for Southern blot detection of the transposon copies, the genomic DNA was digested with NcoI. Hybridisation with a tdTomato probe (1.6 kb fragment generated by BamHI and MfeI digest of pTTcryTom) resulted in constant internal fragments of ~ 0.5 and 0.7 kb and variable external fragment(s) of > 2.1 kb per integration. To assess for PB plasmid sequences, the blots were hybridized with a PB probe, generated by labelling the whole helper plasmid.

**Preparation of primary cell cultures**

Fetuses of specific developmental stages were recovered from non-transgenic females mated with cryTom hemizygous males. The day of detection of a copulation plug was counted as day 0.5. Primary cells were derived from fetal tissue as described [49] and cultured in DMEM supplemented with 10% fetal calf serum and antibiotics. Fetal fibroblasts were cultured in high-glucose DMEM supplemented with 10% heat-inactivated fetal calf serum (PAA, Pasching, Austria), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells at passage 3 were used for electroporation with transposon plasmids. A Biorad electroporator with square wave function was used for electroporation. For feeder cells, primary murine embryonic fibroblasts (MEFs) were grown to subconfluency and inactivated with 10 μg/ml mitomycin C (Sigma) followed by thorough washings.

**iPS cell generation and cultivation**

Induced pluripotent stem cells were cultured in ES cell medium consisting of DMEM/F12 supplemented with 20% knock-out serum replacement (Millipore), 1 mM L-glutamine, 0.1 mM non-essential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1000 units/ml LIF (Santa Cruz) in a humidified atmosphere consisting of 5% CO2 in air at 37°C.

The iPS cells were maintained on gelatinized plates, or plates seeded with inactivated MEFs feeders and enzymatically (trypsin/EDTA) subpassaged every second or third day. For gelatinization, the intended culture dishes were wetted with sterile 1% gelatin in PBS and allowed to
dry immediately before sub-passaging. Alternatively, the iPS cells were passaged on MEF feeders seeded the day before.

**In vitro differentiation assays**

NTERA-2 [33] and P19 [34] cells were obtained from the Deutsche Sammlung für Mikroorganismen (DSMG, Braunschweig) and cultured in high-glucose DMEM medium supplemented according to the description in “Preparation of primary cell culture”. The cultures were split in 1:6 to 1:8 ratios in 2–3 day intervals. STO cells were treated identically. For mitotic inactivation, NTERA-2, P19 and STO cells were grown to subconfluency, respectively, and incubated in fresh medium containing 10 μg/ml mitomycin C for 3 hours followed by thorough washings with PBS.

For ectodermal differentiation, iPS cells were trypsinized and re-suspended in regular ES cell medium for generation of embryoid bodies (EBs). To induce EB formation, the hanging-drop method was used and drops of 20 μl containing 600 cells were pipetted onto the lids of 10 cm culture dishes and incubated at 37°C for three days. EBs were washed off the plate with PBS and transferred to 6-well plates seeded with inactivated NTERA-2, P19 or STO cells, respectively. The stem cell medium containing LIF was replaced one day after EB seeding against a DMEM medium containing 1% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% NEAA, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (without LIF). The cultures were inspected in regular intervals of 3–5 days for the appearance of tdTomato-positive cells. As controls, NTERA-2, P19, STO and iPS cells were cultured individually.

**Alkaline phosphatase staining**

Cells were fixed with 4% formaldehyde, washed with Tris-buffered saline (with 0.1% Tween-20) and stained with AP staining solution [32].

**Reverse transcription-PCR**

Total RNA was prepared using TriReagent (Ambion, Germany) according to the manufacturer’s instructions. Isolated total RNA from cell samples was treated with RNase-free DNase (1 U/μg RNA) (Epicentre Biotechnologies, Madison, WI) and 0.5 μg was used for cDNA synthesis. Reverse transcription (RT) was performed in a 20 μl volume consisting of 4 μl of 10x RT buffer (Invitrogen), 4 μl of 50 mM MgCl₂ (Invitrogen), 4 μl of 10 mM dNTP solution (Bioline), 2μl (20 Units) of RNAsin (Applied Biosystems), 2 μl (50 Units) of MMLV reverse transcriptase (Applied Biosystems) and 2 μl hexamers (50 μM) (Applied Biosystems). The samples were incubated at 25°C for 10 minutes for primer annealing and then incubated at 42°C for 1 hour. Finally, the samples were heated to 95°C for 5 minutes. The cDNA was diluted 1:5 and 2 μl (10 ng) were used for PCR amplification. PCR program: activation of the Taq Polymerase for 10 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Primer sequences are listed in S1 Table. As control, the housekeeping genes Gapdh or Papola were amplified.

**Western blotting**

Finely grinded tissues and cells were extracted in RIPA buffer, and 10 microgram of protein per slot was separated on 12% SDS-PAGE gel, blotted to PVDF membrane, blocked in 5% non-fat milk powder and probed with a rabbit polyclonal antibody against mCherry, which is cross-reactive with other red fluorophore variants, such as tdTomato (Thermo) in 1:1000 dilution. This was followed by a secondary anti-rabbit antibody in 1:10 000 dilution (Sigma-
Aldrich). For detection of the endogenous alphaA crystallin an antibody from Santa Cruz (alphaA crystallin, sc-28306) was use at 1: 2000 dilution. For detection an ECL+ kit (GE Healthcare) and an image acquisition system (Vilber Lourmat, Fusion SL 3500) were used.

Bioinformatic searches
Genevestigator server (www.genevestigator.com) was used to examine expression data of Cryaa. Genevestigator summarises DNA array data of several independent studies indicating highest Cryaa mRNA level in the eye lens, low levels in eyecup, ciliary body and retina, and undetectable levels in other organs.

Supporting Information
S1 Fig. Generation of cryTom founder. (A) Scheme of the cryTom transposon. An expression cassette of the alphaA crystallin (Cryaa) promoter driving tdTomato-cDNA and a poly adenylation sequence is flanked by 5´ and 3´-ITR’s of PB. For generation of transposon mice, the cryTom transposon was co-injected together with a PB expression plasmid (helper plasmid) into the cytoplasm of murine zygotes. N, Ncol site; dotted line, labeled probe for Southern blotting. Drawing not at scale. (B) Founder animal shown under daylight conditions, B’ specific excitation of tdTomato and B’’ overlay of both images. The animal was imaged, while sleeping under a stereomicroscope equipped with epifluorescence. (C) Newborn F1-offspring (two transposon pups and a non-transgenic littermate) shown under specific excitation of tdTomato. Scale bars = 1 cm. (D) Southern blotting of founder, F1 and F2 offspring. The design of the Southern blot predicts two internal fragments of constant size, and a flanking fragment depending of the next neighbouring Ncol site in the genome.

S1 Table. Primers used in RT-PCR.

Acknowledgments
The authors thank M. Ziegler, K. Klingemann, J. Kramer and S. Breitschuh for excellent technical assistance. We thank T. Xu (Yale University School of Medicine) for gifting a Cryaa-tdTomato construct. This work was supported by a grant of the Deutsche Forschungsgemeinschaft (KU 1586/2-1), a NAIP India Fellowship to TA, a DBT CREST Fellowship to DK, and ICAR International Fellowships to TRT and AM. The funding agencies had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author Contributions
Conceived and designed the experiments: WG WAK. Performed the experiments: TA TRT DK WG AM WAK KD. Analyzed the data: TA TRT DK WAK. Contributed reagents/materials/analysis tools: RB KD. Wrote the paper: RB WAK.

References
1. Dong LM, Stark WJ, Jefferys JL, Al-Hazzaa S, Bressler SB, Solomon SD, et al. (2009) Progression of age-related macular degeneration after cataract surgery. Arch Ophthalmol 127: 1412–1419. doi: 10.1001/archophthalmol.2009.152 PMID: 19901205
2. Yang C, Yang Y, Brennan L, Bouhassira EE, Kantrow M, Cvekl A(2010) Efficient generation of lens progenitor cells and lentoid bodies from human embryonic stem cells in chemically defined conditions. FASEB J 24: 3274–3283. doi: 10.1096/fj.10-157255 PMID: 20410439
3. Streit A (2004) Early development of the cranial sensory nervous system: from a common field to individual placodes. Dev Biol 276: 1–15. PMID: 15531360
4. Cvekl A, Duncan MK (2007) Genetic and epigenetic mechanisms of gene regulation during lens development. Prog Retin Eye Res 26: 555–597. PMID: 17905638
5. Wolf L, Yang Y, Wawrousek E, Cvekl A (2008) Transcriptional regulation of mouse alpha A-crystallin gene in a 148kb Cryaa BAC and its derivatives. BMC Dev Biol 8: 88. doi: 10.1186/1471-213X-8-88 PMID: 18803847
6. Cornett JC, Landrette SF, Xu T (2011) Characterization of fluorescent eye markers for mammalian transgenic studies. PLOS ONE 6: e29486. doi: 10.1371/journal.pone.0029486 PMID: 22216292
7. Andley UP (2007) Crystallins in the eye: Function and pathology. Prog Retin Eye Res 26: 78–98. PMID: 17166758
8. Bloemendal H, de Jong W, Lubsen NH, Slingsby C, Tardieu A (2008) Transcriptional regulation of mouse alpha A-crystallin gene in a 148kb Cryaa BAC and its derivates. BMC Dev Biol 8: 88. doi:10.1186/1471-213X-8-88 PMID: 18803847
9. Cornett JC, Landrette SF, Xu T (2011) Characterization of fluorescent eye markers for mammalian transgenic studies. PLOS ONE 6: e29486. doi:10.1371/journal.pone.0029486 PMID: 22216292
10. Brady JP, Garland D, Dugas-Tabor Y, Robison WG Jr., Groome A, Warhouse EF (1997) Targeted disruption of the mouse alpha A-crystallin gene induces cataract and cytoplasmic inclusion bodies containing the small heat shock protein alpha B-crystallin. Proc Natl Acad Sci U S A 94: 884–889. PMID: 9023351
11. Xi JH, Bai F, Gross J, Townsend RR, Menko AS, Andley UP (2008) Mechanism of small heat shock protein function in vivo: a knock-in mouse model demonstrates that the R49C mutation in alpha A-crystallin enhances protein insolubility and cell death. J Biol Chem 283: 5801–5814. PMID: 18056999
12. Xi JH, Bai F, Andley UP (2003) Reduced survival of lens epithelial cells in the alphaA-crystallin-knock-out mouse. J Cell Sci 116: 1073–1085. PMID: 12584250
13. Mackay DS, Andley UP, Shiels A (2006) Identification of a novel, putative cataract-causing allele in CRYAA (G98R) in an Indian family. Mol Vis 12: 768–773. PMID: 16862070
14. Graw J, Klop N, Illig T, Preising MN, Lorenz B (2006) Congenital cataract and macular hypoplasia in humans associated with a de novo mutation in CRYAA and compound heterozygous mutations in P. Graefes Arch Clin Exp Ophthalmol 244: 912–919. PMID: 16453125
15. Khan AO, Aldahmesh MA, Meyer B (2007) Recessive congenital total cataract with microcornea and heterozygote carrier signs caused by a novel missense CRYAA mutation (R54C). Am J Ophthalmol 144: 949–952. PMID: 17937925
16. Santana A, Waiswol M, Arcieri ES, Cabral de Vasconcellos JP, Barbosa de Melo M (2009) Mutation analysis of CRYAA, CRYGD, and GJA8 associated with autosomal dominant congenital cataract in Brazilian families. Mol Vis 15: 793–800. PMID: 19999652
17. Bachtold A, Prada MM, Wu L, Neuhann F, Ropers HH, Naudet N, et al. (2007) A new phenotype associated with a novel mutation in the CRYAA gene: nuclear cataract, iris coloboma, and microphthalmia. Am J Med Genet A 143A: 93–100. PMID: 17304622
18. Pauli S, Soker T, Klop N, Illig T, Engel W, Graw J (2007) Mutation analysis in a German family identified a new cataract-causing allele in the CRYBB2 gene. Mol Vis 13: 962–967. PMID: 17653036
19. Santinato A, Waiswol M, Arcieri ES, Cabral de Vasconcellos JP, Barbosa de Melo M (2009) Mutation analysis of CRYAA, CRYGC, and CRYGD associated with autosomal dominant congenital cataract in Brazilian families. Mol Vis 15: 793–800. PMID: 19999652
20. Hikita ST, Rowland TJ, Friedrich AM, Hinman CR, Johnson LV, et al. (2009) Generation of functional retinal pigmented epithelium from induced pluripotent stem cells. Stem Cells 27: 2427–2434. doi: 10.1002/stem.185 PMID: 19658190
21. Maeda T, Lee MJ, Palczewska G, Marsili S, Tesar PJ, Palczewski K, et al. (2013) Retinal pigmented epithelial cells obtained from human induced pluripotent stem cells possess functional visual cycle enzymes in vitro and in vivo. J Biol Chem 288: 34484–34493. doi: 10.1074/jbc.M113.516531 PMID: 24129572
22. Brandl C, Zimmermann SJ, Milenkovic VM, Rosendahl SM, Grassmann F, Milenkovic VM, et al. (2014) In-depth characterisation of Retinal Pigment Epithelium (RPE) cells derived from human induced
25. Qiu X, Yang J, Liu T, Jiang Y, Le Q, Lu Y (2012) Efficient generation of lens progenitor cells from cataract patient-specific induced pluripotent stem cells. PLOS ONE 7: e32612. doi:10.1371/journal.pone.0032612 PMID: 22403680

26. Mikhailova A, Ilmarinen T, Uusitalo H, Skottman H (2014) Small-molecule induction promotes corneal epithelial cell differentiation from human induced pluripotent stem cells. Stem Cell Reports 2: 219–231. doi: 10.1016/j.stemcr.2013.12.014 PMID: 23355677

27. Hayashi R, Ishikawa Y, Ito M, Kageyama T, Takashiba K, et al. (2012) Generation of corneal epithelial cells from induced pluripotent stem cells derived from human dermal fibroblast and corneal limbal epithelium. PLOS ONE 7: e45435. doi:10.1371/journal.pone.0045435 PMID: 23029008

28. Shalom-Feuerstein R, Serror L, Adermark E, Müller FJ, van Bokhoven H, Wiman KG, et al. (2013) Impaired epithelial differentiation of induced pluripotent stem cells from ectodermal dysplasia-related patients is rescued by the small compound APR-246/PRIMA-1MET. Proc Natl Acad Sci U S A 110: 2152–2156. doi:10.1073/pnas.1201753109 PMID: 23355677

29. Iqbal K, Barg-Kues B, Broll S, Bode J, Niemann H, Kues W (2009) Cytoplasmic injection of circular plasmids allows targeted expression in mammalian embryos. Biotechniques 47: 959–968. doi:10.2144/000113270 PMID: 20041849

30. Garrels W, Talluri TR, Ziegler M, Most I, Forcato DO, Schmeer M, et al. (2015) Cytoplasmic injection of murine zygotes with Sleeping Beauty transposon plasmids and minicircles results in the efficient generation of germline transgenic mice. Biotechnol J. 11: 178–184. doi:10.1002/biot.201500218 PMID: 26470758

31. Grabundzija I, Wang J, Sebe A, Erdei Z, Kajdi R, Devaroj A, et al. (2013) Sleeping Beauty transposon-based system for cellular reprogramming and targeted gene insertion in induced pluripotent stem cells. Nucleic Acids Res 41: 1829–1847. doi:10.1093/nar/gks1305 PMID: 23275558

32. Kues WA, Herrmann D, Barg-Kues B, Haridoss S, Nowak-Imialek M, Buchholz T, et al. (2013) Derivation and characterization of sleeping beauty transposon-mediated porcine induced pluripotent stem cells. Stem Cells Dev 22: 124–135. doi: 10.1089/scd.2012.0382 PMID: 22989381

33. Damjanov I, Clark RK, Andrews PW (1984) Cytoskeleton of human embryonal carcinoma cells. Cell Differ 15: 133–139. PMID:6085562

34. Rossant J, McBurney MW (1982) The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. J Embryol Exp Morphol 70: 99–112. PMID:7124904

35. Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. J Cell Biol 94: 253–262. PMID: 7107698

36. Lee VM, Andrews PW (1986) Differentiation of NTERA-2 clonal human embryonal carcinoma cells into neurons involves the induction of all three neurofilament proteins. J Neurosci 6: 514–521. PMID: 24195705

37. Yoshimizu T, Sugiyama N, De Felice M, Yeom YI, Ohbo K, Masuko K, et al. (1999) Germline-specific expression of the Oct-4/green fluorescent protein (GFP) transgene in mice. Dev Growth Differ 41: 675–684. PMID: 10646797

38. Long Q, Meng A, Wang H, Jessen JR, Farrell MJ, Lin S (1997) GATA-1 expression pattern can be recapitulated in living transgenic zebrafish using GFP reporter gene. Development 124: 4105–4111. PMID: 9374406

39. Tumbar T, Guasch G, Greco V, Bianpain C, Lowry WE, Rendl M, et al. (2004) Defining the epithelial stem cell niche in skin. Science 303: 359–363. PMID: 14671312

40. Wang J, Trowbridge JJ, Rao S, Orkin SH (2008) Proteomic studies of stem cells. StemBook. Cambridge (MA): Harvard Stem Cell Institute Copyright: (c) 2008 Jianlong Wang, Jennifer J. Trowbridge, Sridhar Rao, and Stuart H. Orkin.

41. Mates L, Chu K, Belay E, Jerchow B, Manoj N, Acosta-Sanchez A, et al. (2009) Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. Nat Genet 41: 753–761. doi: 10.1038/ng.343 PMID: 19412179

42. Yusa K, Zhou L, Li MA, Bradley A, Craig NL (2011) A hyperactive piggyBac transposase for mammalian applications. Proc Natl Acad Sci U S A 108: 1531–1536. doi: 10.1073/pnas.1008322108 PMID: 21205896

43. Ivics Z, Garrels W, Mates L, Yau TY, Bashir S, Židek V, et al. (2014) Germline transgenesis in pigs by cytoplasmic microinjection of Sleeping Beauty transposons. Nat Protoc 9: 810–827. doi: 10.1038/nprot.2014.010 PMID: 24625780
44. Ivics Z, Mates L, Yau TY, Landa V, Zidek V, Bashir S, et al. (2014) Germline transgenesis in rodents by pronuclear microinjection of Sleeping Beauty transposons. Nat Protoc 9: 773–793. doi:10.1038/nprot.2014.008 PMID: 24625778

45. Garrels W, Mates L, Holler S, Daida A, Taylor U, Peterson B, et al. (2011) Germline transgenic pigs by Sleeping Beauty transposition in porcine zygotes and targeted integration in the pig genome. PLOS ONE 6: e23573. doi:10.1371/journal.pone.0023573 PMID: 21897845

46. Henikoff S (1998) Conspiracy of silence among repeated transgenes. Bioessays 20: 532–535. PMID: 9723001

47. Tasic B, Hippenmeyer S, Wang C, Gamboa M, Zong H, Chen-Tsui Y, et al. (2011) Site-specific integrase-mediated transgenesis in mice via pronuclear injection. Proc Natl Acad Sci U S A 108: 7902–7907. doi: 10.1073/pnas.1019507108 PMID: 21464299

48. Sakaguchi M, Watanabe M, Kinoshita R, Kaku H, Ueki H, Futani J, et al. (2014) Dramatic increase in expression of a transgene by insertion of promoters downstream of the cargo gene. Mol Biotechnol 56: 621–630. doi: 10.1007/s12033-014-9738-0 PMID: 24526517

49. Talluri TR, Kumar D, Glage S, Garrels W, Ivics Z, Debowski K, et al. (2014) Non-viral reprogramming of fibroblasts into induced pluripotent stem cells by Sleeping Beauty and piggyBac transposons. Biochem Biophys Res Commun 450: 581–587. doi: 10.1016/j.bbrc.2014.06.014 PMID: 24928388

50. Talluri TR, Kumar D, Glage S, Garrels W, Ivics Z, Debowski K, et al. (2015) Derivation and characterization of bovine induced pluripotent stem cells by transposon-mediated reprogramming. Cell Reprogram. 17: 131–140. doi: 10.1089/cell.2014.0080 PMID: 25826726