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TITLE:

Cone-enriched cultures from the retina of chicken embryos to study rod to cone cellular interactions

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KEYWORDS:

Photoreceptors, Inherited retinal degenerations, High content screening, Cell survival, Rod-derived cone viability factor, Therapy.

SUMMARY:

We describe a method to obtain primary cultures of cone photoreceptors from the retina of chicken embryos and its use for high content screening.

ABSTRACT:

Human daytime vision relies on the function of cone photoreceptors in the center of the retina, the fovea. Patients suffering from the most prevalent form of inherited retinal degeneration, retinitis pigmentosa, lose night vision because of mutation driven loss of rod photoreceptors, a phenomenon followed by a progressive loss of function and death of cones leading to blindness. Geneticists have identified many genes with mutations causing this disease, but the first mutations identified questioned the mechanisms of secondary cone degeneration and how a dominant mutation in the rhodopsin gene encoding for the visual pigment expressed exclusively in rods can trigger cone degeneration.

This result of transplantations in a genetic model of the disease led to the concept of cell interactions between rods and cones and of non-cell autonomous degeneration of cones in all genetic forms of retinitis pigmentosa.

Cones comprise 5% of all photoreceptors in humans and only 3% in the mouse, so their study is difficult in these species, but cones outnumber rods in bird species. We have adapted 96-well

plates to culture retinal precursors from the retina of chicken embryos at stage 29 of their development. In these primary cultures, cones represent 80% of the cells after in vitro differentiation. The cells degenerate over a period of one week in the absence of serum. Here, we describe the methods and its standardization.

This cone-enriched culture system was used to identify the epithelium-derived cone viability factor (EdCVF) by high content screening of a rat retinal pigmented epithelium normalized cDNA library. Recombinant EdCVF prevents the degeneration of the cones.

INTRODUCTION:

The retina of vertebrate species is dual, with rod photoreceptors for dim light vision and cone photoreceptors for daylight, color and acuity vision. Primate visual acuity relies on a region at the center of the retina, called the fovea, that is enriched in cones, but overall, cones represent only 5% of all photoreceptors. Consequently, the analysis of the cones in primate retina and especially the culture of cones are technically difficult. All other mammalian species have no fovea and the percentage of cones is low for rodents that are most commonly used in retinal research. This is not the case for avian species, for which cones dominate the retina of these well seeing bird species. Dinosaurs, which have dominated the ecosystem when the mammals first appeared during evolution, are at the phylogenetic origin of birds[1]. As a consequence of such competition between dinosaurs and early mammals, the mammals are mostly nocturnal with retinas dominated by rods. Only later during evolution did the diurnal vision of some mammalian species, among which primates belong, become an evolutionary advantage. Nevertheless, the ancestral period remains as an atavism of the nocturnal bottleneck in the evolution of mammalian vision[2,3].

While studying retinal cell differentiation, Adler and Hatlee showed that photoreceptors represented approximately 70% of the retinal differentiated cells in cultures derived from chicken at the embryonic day (ED) 6 or stage 29[4]. Because of the prevalence of cones in the chicken retina, cultures of retinal cells from ED6 chicken embryos have been developed as cone-enriched cultures[5].

The importance of cone-mediated visual acuity for human is a truism. People affected by genetic or aging diseases that alter cone function are greatly handicapped. This has promoted a very large body of studies on inherited retinal degenerations (IRD) with the objective of finding treatments for these blinding diseases[6,7]. The first success, obtained using a recombinant adeno-associated vector (AAV) for the therapy of a severe form of IRD Leber congenital amaurosis (LCA), is a proof of concept for gene therapy[8]. The identification of the genes whose mutations trigger IRD opens the possibility of curing these diseases using gene therapy. Nevertheless, these diseases are resulting from mutations in more than 200 distinct genes[9]. Even in the case of autosomal recessive forms of IRD, when the reintroduction of the normal copy of the morbid gene could restore visual function, the economic cost of each individual development favors the most prevalent ones in detriment to the less common ones and to those for which the genetic origin remains unknown. This fact led researchers to think about more general therapies. Apoptotic cell death appeared as a common pathway, and a therapeutic target of these diseases that progress by the degeneration of photoreceptors, including for autosomal dominant

forms[10,11]. However, the successes of such an approach are missing. For the most common form of IRD, retinitis pigmentosa (RP), the common pathway is the secondary loss of function ultimately followed by the degeneration of cones[12,13]. Preventing the loss of cone function will preserve central vision of the fovea independently of the causative mutations[14].

In the early stage of RP, the loss of rods triggers a reduction in the expression of rod-derived cone viability factor (RdCVF), encoded by the nucleoredoxin-like 1 (*NXNL1*) gene, which interrupts the metabolic and redox signaling between rods and cones[15]. The administration of a recombinant AAV encoding the two products of the *NXNL1* gene, the trophic factor RdCVF and the thioredoxin enzyme RdCVFL, could theoretically prevent cone vision loss in all genetic forms of RP[16]. We have shown that the *NXNL1* gene product, RdCVFL, is expressed in chicken cone-enriched cultures[17] and where it plays a protective role[18]. RdCVF and the *NXNL1* gene were identified by high content screening of a retinal cDNA library using the survival of cells from a cone-enriched culture as readout[19]. We screened the equivalent of 210,000 individual clones of the library using 8 parallel tests for each clone. This represents a very large number of tests requiring easy access to the biological material, the retinas of chicken embryos. We found that it was relatively easy to obtain embryonated chicken eggs on a weekly basis because they are widely produced for the agro-industry for egg-laying hens and meat-producing chickens. After careful standardization of the cone-enriched cultures, the system provides an easy, robust and reproducible way of testing thousands of molecules for their ability to preserve cone viability. These cells are also amenable to genetic manipulations[20] that benefit to the study of signal transduction and to biochemical analyses[21-23].

Retinal researchers have developed alternative methods as the use of the cone cell line 661W[24-26]. Nevertheless, the identity of this cell line remains controversial[27,28]. The 661W cells were cloned from retinal tumors of a transgenic mouse line that expresses the SV40 large T antigen under control of the human interphotoreceptor retinol-binding protein promoter. SV40 large T antigen mediates cellular transformation and immortalization. As the consequence, signaling pathway identified using 661W cells must be reported into the context of a transformed and immortalized cell line which is distinct in many ways from cones in situ. In that respect, the cone-enriched culture system is composed of primary neurons, the cones that are more physiologically relevant.

While it is possible to obtain a pure culture of photoreceptors using vibratome sectioning of the mouse retina, the very low percentage of cones in the outer retina of rodents makes this approach unsuited for producing cone-enriched cultures[29]. The pig retina contains no fovea but has a region called *area centralis* that is very enriched in cones[30]. The high proportion of cones in the retina diurnal rodents, as *Arvicanthis ansorgei* and *Psammomys obsesus*[31,32], offers a possible solution but requires the breeding of such exotic species. Adult pig eyes, collected from local slaughterhouses, can be used to produce a mixed culture of rods and cones that have been used to study photoreceptor survival[33]. An elegant solution is to pre-purify cones from the pig retina using panning with peanut agglutinin (PNA) lectin, which selectively binds to cones[34]. Nevertheless, this method is difficult to implement on a large scale because of its complexity.

Human induced pluripotent stem cells (iPS) offers the most promising approach to obtain a cone photoreceptor cell population that can be used for retinal transplantation but that can also be adapted to cone-enriched culture[35,36]. Since the transcription factor NRL is required for rod-photoreceptors[37], the *Nrl-/-* mouse has a retina dominated by short wave cones (S-cones). The inactivation could be used to produce S-cone-enriched preparation by differentiation of human from iPS[38,39]. Another possible approach is to promote cone differentiation using thyroid hormone signaling[40]. While novel methods for producing cone-enriched cultures from human iPS are emerging, chicken embryos provide a current proven method[19].

The cone-enriched culture was instrumental in the identification of RdCVF by expression cloning[19]. This system was also used successfully to demonstrate that RdCVF stimulates glucose uptake and its metabolism by aerobic glycolysis[22]. Furthermore cone-enriched culture was used to validate the protective role of RdCVFL, the second product of the *NXNL1* gene[23]. More recently, this system was used to demonstrate the existence of protecting molecules secreted by retinal pigmented epithelial cells transduced with OTX2[41].

PROTOCOL:

The protocol was approved by the Committee on the Ethics of Animal Experiments of the University Pierre and Marie Curie and the French ministry of research (Permit Number: APAFIS#1028 2015070211275177). The animal experiments were performed under the following authorization: “Certificat d'autorisation d'expérimenter sur les animaux vertébrés A-75-1863. Préfecture de Police de Paris (November 9th 2011-November 8th 2016)”.

1. Incubation of fertilized eggs

1.1. Collect weekly fertilized eggs (strain I 657, red label), obtained naturally, at an industrial hatchery.

1.2. Maintain the fertilized eggs at 17 °C (their biological zero) in the laboratory after they are "laid" by the hen.

1.3. For each culture, incubate seven fertilized eggs for 24 hours at 20 °C and then 136 hours at 37 °C with intermittent reversion of the inclination (a progressive movement for 2 hours from one side to its opposite, a 4-hours cycle) of the eggs in a humidified chamber.

2. Recovery of the chicken embryos

2.1. Wash the surface of the seven eggs with disinfectant (e.g., Pursept A express).

2.2. To break the eggshell, make a hole in the top of the shell with large straight pliers. Then cut the shell to remove the hat from the egg as a soft-boiled egg.

2.3. Gently extract each embryo from the eggshell with curved forceps, and then transfer it to

a Petri dish containing sterile phosphate buffer saline (PBS) previously heated to 37 °C. Gently, remove the envelope that surrounds the embryos (the chorion or chorioallantoic membrane).

2.4. Verify the stage of development of each embryo by visual comparison to Hamburger and Hamilton[42].

2.5. Select two embryos at the 29th stage of development (**Figure 1**). The wings bend at the elbows. The collar stands out visibly. The bill is more prominent than at the 28th stage.

2.6. Enucleate the eyes of these selected embryos and transfer them in CO_2-independent medium (Life technologies).

CAUTION: It is very important that the embryo is at stage 29, and not at stage 28 or 30 (**Figure 1**); that is why it is necessary to incubate at least 7 eggs, even if only two will be finally used. The chorion is very thin, so hard to distinguish, but it is very close from the embryo, so it has to be removed without touching the embryo.

3. Dissection of the retinas

3.1. Decapitate and enucleate the selected embryos with curved forceps.

3.2. Transfer the four eyes into CO_2-independent medium. This medium contains 0.9 mM $CaCl_2$ and 0.65 mM $MgCl_2$.

3.3. Position the eye with the cornea face down, the optic nerve facing the experimenter. Drill a hole in the optic nerve using two straight forceps.

3.4. Insert a branch of each forceps between the retina and the pigment epithelium (**Figure 2**). Pull on each forceps and rotate the eye to detach the epithelium from the retina. Remove the cornea followed by the lens and the vitreous.

3.5. Transfer the four retinas in a Petri dish containing Ringer's medium at pH 7.2.

CAUTION: Make sure that only the retina remains and remove any traces of vitreous and remaining retinal pigmented epithelium.

4. Preparing the retinal cell suspension

4.1. Cut the four retinas in very small pieces using two straight pliers.

4.2. Wash the retinal pieces twice with Ringer's medium.

4.3. After the second wash with Ringer's media, let the pieces of retina fall on the bottom of the tube and remove the Ringer's media. Treat the retinal pieces for 20 minutes at 37 °C with a

solution of trypsin (0.25% w/v).

4.4. Disperse the solution after 10 minutes by successive suction. Discharge using a Pasteur pipette and check for dissociation of the retinal pieces. Stop the reaction by adding culture media supplemented with 10% inactivated foetal calf serum.

4.5. Incubate the cell suspension with 0.05 mg of DNase I. Dissociate the cell clusters and the DNA by successive suction and discharge using a Pasteur pipette immediately after adding the DNase.

4.6. Wash the retinal cell suspension twice with chemical defined culture medium (CDCM): an equal volume of Dulbecco's Modified Eagle Medium and M199 media supplemented with 100 µg/mL linoleic acid/BSA, 0.86 µM insulin, 0.07 µM transferrin, 2.0 µM progesterone, 0.28 µM prostaglandin, 0.29 µM Na_2SeO_3, 182 µM putrescine, 3 mM taurine, 4.7 µM cytidine 5'-diphosphocholin, 2.7 µM cytidine 5'-diphosphoethanolamine, 0.55 µM hydrocortisone, 0.03 µM triiodothyronine, 1 mM sodium pyruvate and 20 µM gentamycin.

5. Retinal cell seeding

5.1. Treat two black 96-well culture plates with transparent bottom for 2 hours at 37 °C with poly-L-lysine at 32.25 µg/cm².

5.2. Rinse these plates twice with M199 culture medium. Resuspend the cell pellet in 1 mL of CDCM.

5.3. Add to an aliquot of 10 µL of the cell suspension trypan blue to stain the living cells. Add the cells suspension specimen to a haemocytometer (cell counting-chamber of Malassez).

5.3.1. Under a microscope, count the stained cells for four rows of the haemocytometer (i.e., 40 squares), and then calculate the average cell number for one row. Calculate the concentration of cells of the suspension by applying the following method: Number of cells/ml of suspension = average number of cells in a row (10 squares) x 10 the total number of squares of the haemocytometer x dilution with trypan blue x 1,000 (to express the result as cells/ml).

5.4. Bring the cell suspension to two concentrations (5.6×10^4 cells/mL and 1.12×10^5 cells/mL) corresponding to the two plating densities (1×10^5 cells/cm^2 and 2×10^5 cells/cm^2) using CDCM.

5.5. Seed 50 µL of the two cell suspensions into the two pretreated black 96-well culture plates. Distribute the cells in the plates with a multichannel pipette from the right of the plate to the left, homogenizing between each column, so that the distribution of the cells is homogeneous.

5.6. Add 50 µL of the library of molecules (e.g., the conditioned media from a cDNA library, see below) to be screened using a predefined pattern (**Table 1**).

5.7. Incubate the plates for seven days at 37°C under 5% CO_2 with no change of media.

6. Counting viable cells

6.1. To each well of the plate, add 2.7 µM of calcein AM and 0.3 mM of ethidium homodimer.

NOTE: Calcein penetrates the cells that are impermeable to large molecules as ethidium homodimer. In the cytoplasm of living cells, calcein is hydrolyzed by endogenous esterases, becomes fluorescent by emitting at 520 nm when excited at 485 nm. Ethidium homodimer bind to DNA on dead cells. Ethidium DNA-homodimer binding causes red fluorescence to be emitted at 635 nm after excitation at 520 nm.

6.2. Incubate the plates for 1 hour at room temperature in the absence of light.

6.3. Read the fluorescence on an automated plate reader composed of an inverted microscope equipped with a mercury lamp with two excitation filters at 485 and 520 nm, two filters of emission at 520 and 635 nm, an objective (x10), a motorized stage controlled by a processor and a charge-coupled device camera (CCD). This counting platform is controlled by the Metamorph software[19]. It makes it possible to acquire fluorescence images of living cells and dead cells simultaneously in each well of the 96-well plate, starting from wells A1 towards well A12, then B12 towards B1, C1 towards C12 and so on (**Table I**).

6.4. Calculate the average area A of a single cell using the 18 wells of the negative controls (**Table I**).

6.5. Count the cells in each well of the plate and apply the following empirical formula A x 29 / 20.7 to prevent that cell doublets (grouping of two cells) are counted. Score the protective effect of the cones by molecules as the ratio between the average cell number in the 4 wells where we tested the molecule versus the average cell number in the 18 wells of the negative control (see **Table 1** and **Supplementary Figure 1**).

6.6. Combine the results of the plate seeded at 1 x 10^5 cells/cm^2 with the one seeded at 2 x 10^5 cells/cm^2 to evaluate the potential protection (viability ratio) by each molecule screened.

REPRESENTATIVE RESULTS:

We describe here how the cone-enriched culture system can be used to identify novel cone protecting proteins. We used this protocol to screen a normalized cDNA library made of choroid and retinal pigmented epithelium from 400 eyes of 8 weeks old Long-Evans rats[43].

This library contains 6.0 x 10^6 independent colonies forming units (CFUs) and has an average cloned insert size of 2.1 kilobase (kb), with greater than 99% of recombinant clones. Pools of 100 clones from that library were transiently transfected (0.1 µg of plasmid DNA) into COS-1 cells and the conditioned medium (CM) of COS-1 were harvested after incubation for 48 hours in DMEM

without serum. Membranes or exosomes were not removed by ultracentrifugation. Fifty microliters of each CM were added to 4 wells of two 96-well plates. one seeded at 2 x 10^5 cells/cm^2, the other one at 4 x 10^5 cells/cm^2. CM from COS-1 cells transfected with the empty vector pcDNA3.1 used to construct the library was used as negative control (**Table 1**). A total of 2,112 sets of 100 clones corresponding to 211,200 individual clones were evaluated in four culture wells and for two seeding conditions of cone-enriched cultures. The two conditions correspond to two slightly different inoculation densities which makes it possible to assess the protective activity more precisely, for a total of 1,689,600 culture wells.

Among the 42 pools of clones with a ratio greater than 2, pools 0080 and 0073 have viability ratio 16 and 14 times higher after 7 days of culture than the negative control, pcDNA3.1 (**Supplementary Figure 1**). This analysis is essential to identify the pools of interest. Each selected pool of 100 clones was subdivided into 16 sets of 10 clones from their glycerol stock. These sub-pools were prepared and tested according to the same method in a second round of screening (i.e., a total of 3,200 culture wells). The sub-pool 0073-09 gave the strongest viability ratio (**Supplementary Figure 2A**) and was subdivided to produce 16 individual clones that were tested in a third round of screening on cone-enriched cultures. The clone 0073-09-37 clone clearly stands out from the others with a viability ratio equal to 2.5 (**Supplementary Figure 2B**). The y axis has a different scale even if the seeding density was the same than in Supplementary figure 2A. We have seen this commonly when the assays are repeated weekly for months. After analysis, these results confirm that clone 0073-09-37 has a robust and reproducible effect on cone survival. The test was repeated independently (**Figure 3A**), and the insert of 1.8 kb was sequenced (**Figure 3B**).

A bioinformatic analysis revealed that the clone 0073-09-37, that we named epithelium-derived cone viability factor (EdCVF), contains three open reading frames (ORFs), the one the most upstream (ORF1) encodes for 84 residues of the C-terminal part of the rat protein zinc finger protein-180 (ZFP180, NP_653358) of 727 amino acids[44]. The other two ORFs (ORF2 and ORF3) are much less well conserved in mice and absent in other mammals. When tested independently, only ORF1 exerts a protective effect on the cones (**Supplementary Figure 3**). ORF1 was produced as a glutathione S-transferase (GST) fusion protein (**Figure 4A**). The EdCVF protein was purified and the GST tag removed (**Figure 4B**). EdCVF is able to prevent cone degeneration in the cone-enriched culture system (**Figure 4C**).

FIGURE AND TABLE LEGENDS:

Figure 1: Chicken embryos at stages 28th, 29th and 30th of development. Arrows W: wing, C: collar and B: bill. Scale bar 1 cm.

Figure 2: Dissection of the retina of the chicken embryo

Figure 3: **Epithelium-derived cone viability factor (EdCVF), clone 0073-09-37. A.** Increased viability on cone-enriched culture. **B.** The sequence of the cDNA clone 0073-09-37. The underlined sequence GAATTC is the EcoRI restriction site used to construct the library. The two

codons GTG in bold are uncommon translation initiation sites for EdCVF originating from the vector. Statistical analysis by Student's test.

Figure 4: Recombinant EdCVF activity. **A.** Sequence of EdCVF in the fusion with glutathione S-transferase (GST). **B.** The purified recombinant EdCVF protein. **C.** Trophic activity of GST-EdCVF on cone in culture. Statistical analysis using Tukey's test.

Supplementary Figure 1: **Ratio of the average cell number for a cDNA pool and the negative control, the conditioned medium of COS-1 cells transfected with the empty vector pcDNA3.1 during the first round of screening.**

Supplementary Figure 2: **Number of cells alive. A.** The second round of screening with sub-pools of 0073 pool. **B.** The third round of screening with isolated clones. CN: the conditioned medium of COS-1 cells transfected with the empty vector, pcDNA3.1.

Supplementary Figure 3: Trophic activity of the three open reading frames of the isolated clone 0073-09-37. Statistical analysis using Dunnett's test.

Table 1: Plan of the 96-well plate for high content screening

DISCUSSION:

Among the many parameters that might limit the production of a cone-enriched culture from chicken embryos, the first critical step is to accurately identify the stage of development of the embryos in the hatched eggs. It has been observed that culture of cells from the retinas of embryos at ED8 (34^{th} stage) produces only 35% photoreceptors, with the remaining 65% are made of other neurons[4]. Whatever the logistic applied to get the hatched eggs, it is necessary to fine-tune the temperature and the incubation time, and to examine carefully the embryos as compared to the reference pictures of all stages of development[42,45].

Originally, the cone-enriched culture system was developed using the White Leghorn strain[4]. The white color of the eggs of that strain is not particularly appreciated in France, so we used a strain of chicken that produce brown eggs. We utilized the I 657 strain, which is made by crossing I 66 roosters with JA57 hens[5]. We were able to reproduce the characteristics of the original cultures. This shows that the genetic background of the chicken is not critical to obtain cone-enriched cultures.

We have not tested the effect of individual removal of the supplements in the culture medium, but have observed that insulin plays a critical role in accordance to the effect of insulin on the survival of cones in the *rd1* mouse, a model of autosomal recessive RP[46]. Triiodothyronine (T3) may also participate in the differentiation of retinal precursor cells of the chicken embryo into cones according to the role of thyroid hormone receptor in retinal cell fate during development[40]. Consequently, the cone-enriched culture system cannot be used to identify insulin by expression cloning[46].

The cone-enriched culture system relies on the culture of primary neurons and is much more appropriate tha methods relying on the use of immortalized cells as the cell line 661W[24-26].

The method described here can be modified by performing a prior electroporation with plasmid DNA[20]. Before preparing the retinal cell suspension, the entire retina is placed in the chamber of a custom-made electroporator with 120 µL of 0.5 µg/µL of plasmid DNA in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Five pulses of 15 V for 50 ms each are applied separated by 950 ms interval[22]. Attempts to deliver interfering RNA (RNAi) using the replication competent avian splice (RCAS) retroviruses into cone-enriched cultures were unsuccessful[47]. This is certainly due to the fact that in the absence of serum and in cultures of low density, retinal precursor cells are not replicative, a requisite for the propagation of retroviruses.

We developed the cone-enriched culture system to identify trophic factors that promote cone survival using expression cloning[19]. In order to make it feasible, we performed a first step of high content screening using conditioned medium from pools of 100 clones. Even if the cDNAs from the library are expressed under the control of a strong CMV promoter after transfection of COS-1 cells, it does not offer a guaranty that all the proteins encoded by individual cDNAs reach a concentration sufficient to be scored positive by the viability assay. This is a major limitation. In that sense, any screening is not really exhaustive. In addition, even if membranous proteins were not removed from the conditioned medium, the configuration of the assay is unfavorable to the identification of non-diffusive factors. An alternative would be to screen individual clones after having obtained the sequence of the cDNAs in order to avoid duplications in assaying many times the same candidate protein. This was initiated by sequencing the retinal cDNA libraries we used[43]. While rational, this approach has also its limitations. The bioinformatic analysis of the cDNA sequences will irresistibly impose, beside the reduction of the redundancy, the prioritization of screening certain clones based on knowledge. This will not be detrimental if finally, the whole library would be screened even if the time required to do it will be significantly lengthened. But invariably, the identity of the sequence will influence our way of looking at the results. This will not be neutral since the interpretation of the sequence will naturally be in competition with the experimental data.

The identification of EdCVF also show that high content screening entails technical limitations. From the first round of screening, we identified two pools with high activity (**Supplementary Figure 1**). The pool 0073 led to the successful identification of EdCVF, while pool 0080 did not conduct to such finding. We have not solved the problem that could result from the loss of the active clone during the preparation of sub-pools. Alternatively, it is not excluded, even if not statistically favorable, that among the cDNAs of pool 0080, two proteins were acting synergistically and their activity could not be observed as individual clones.

The identification of molecules protecting cones by screening small molecules is a future application of the cone-enriched culture system. Such molecules will be invaluable for the treatment of retinal pathologies for which gene therapy is not the most appropriate approach as age-related macular degeneration.

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DISCLOSURES:

TL, J-AS and VF hold a patent on the use of EdCVF to treat retinal degenerations [WO2009071659 (A1). Jun. 12 2007].

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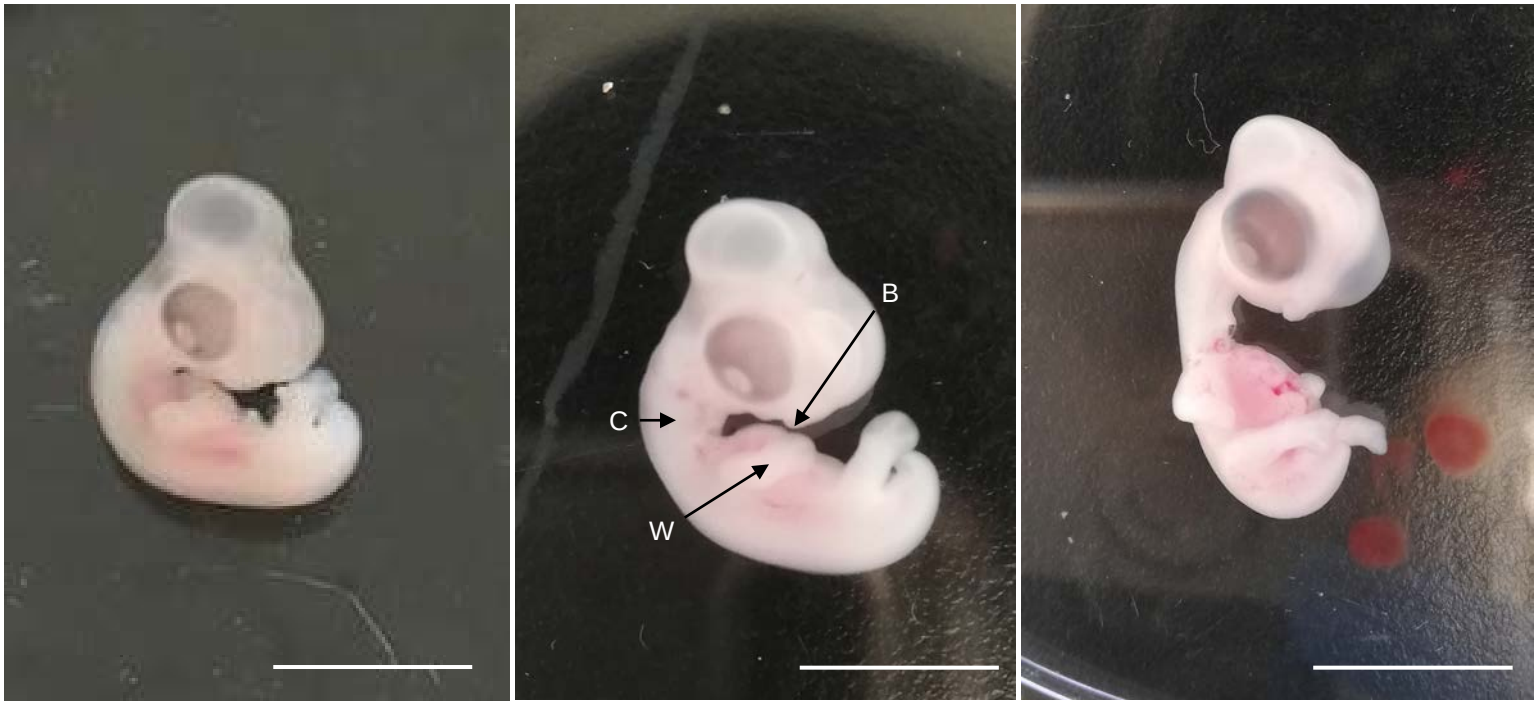
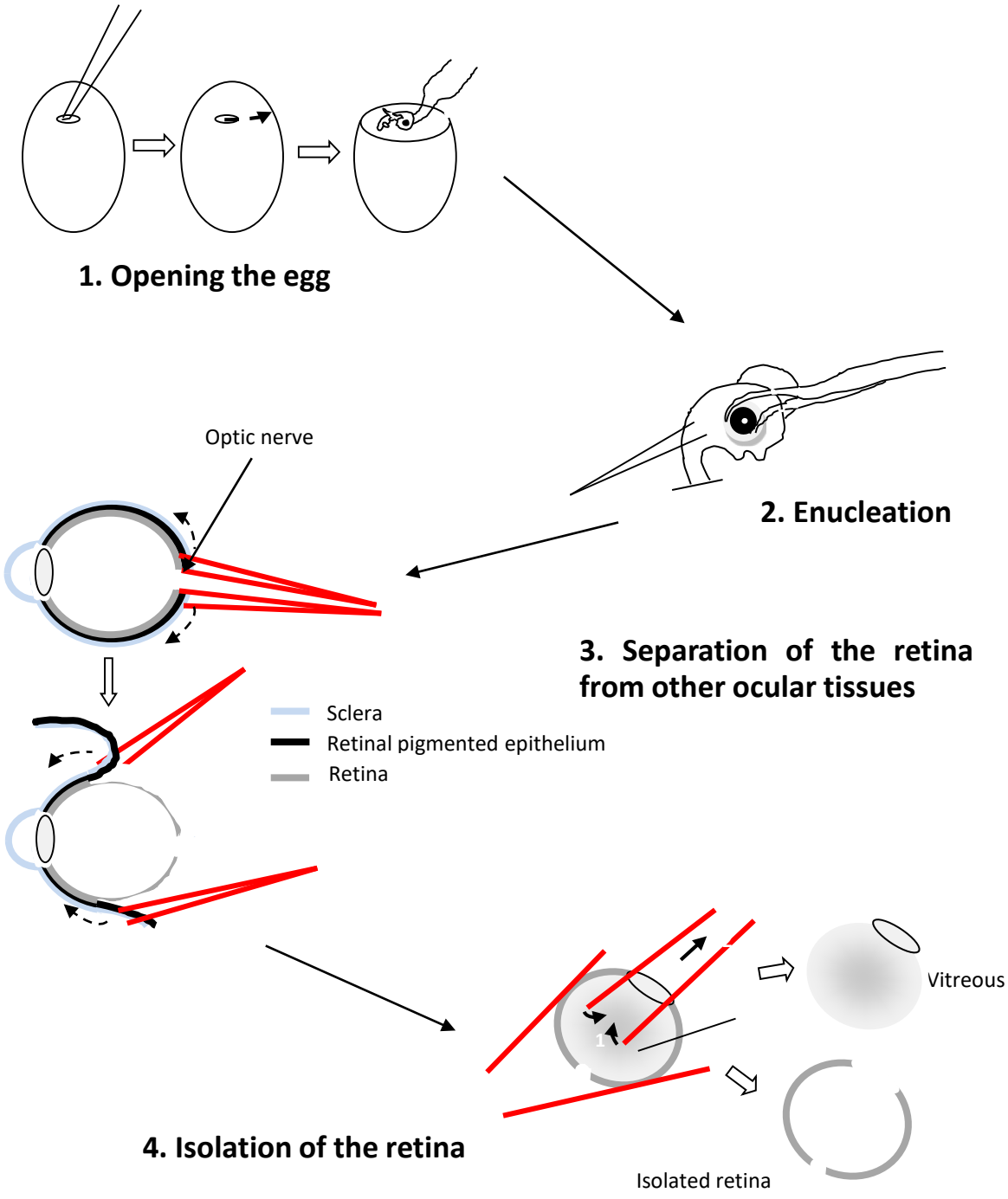
Figure 1

Click here to access/download;Figure;Figure_1_12_11_2020.pdf

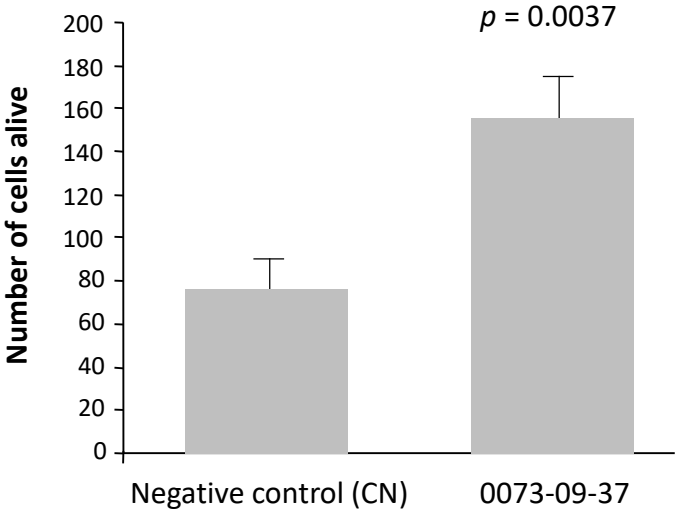


Stage 28 Stage 29 Stage 30

Figure 2



A



B

TCCAGT**GTGGTG**GAATTCTGCAGATGGAAGACATTCCGGCAGAGCTCCTGCTTTACCCAGCATCAGAGGACTC
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CCAGCGAACCCACACTGGGGAAAAGCCCTACAAATGCAGCCAGTGTGGCAAAGCCTTTATTAGCAGTTCTAAG
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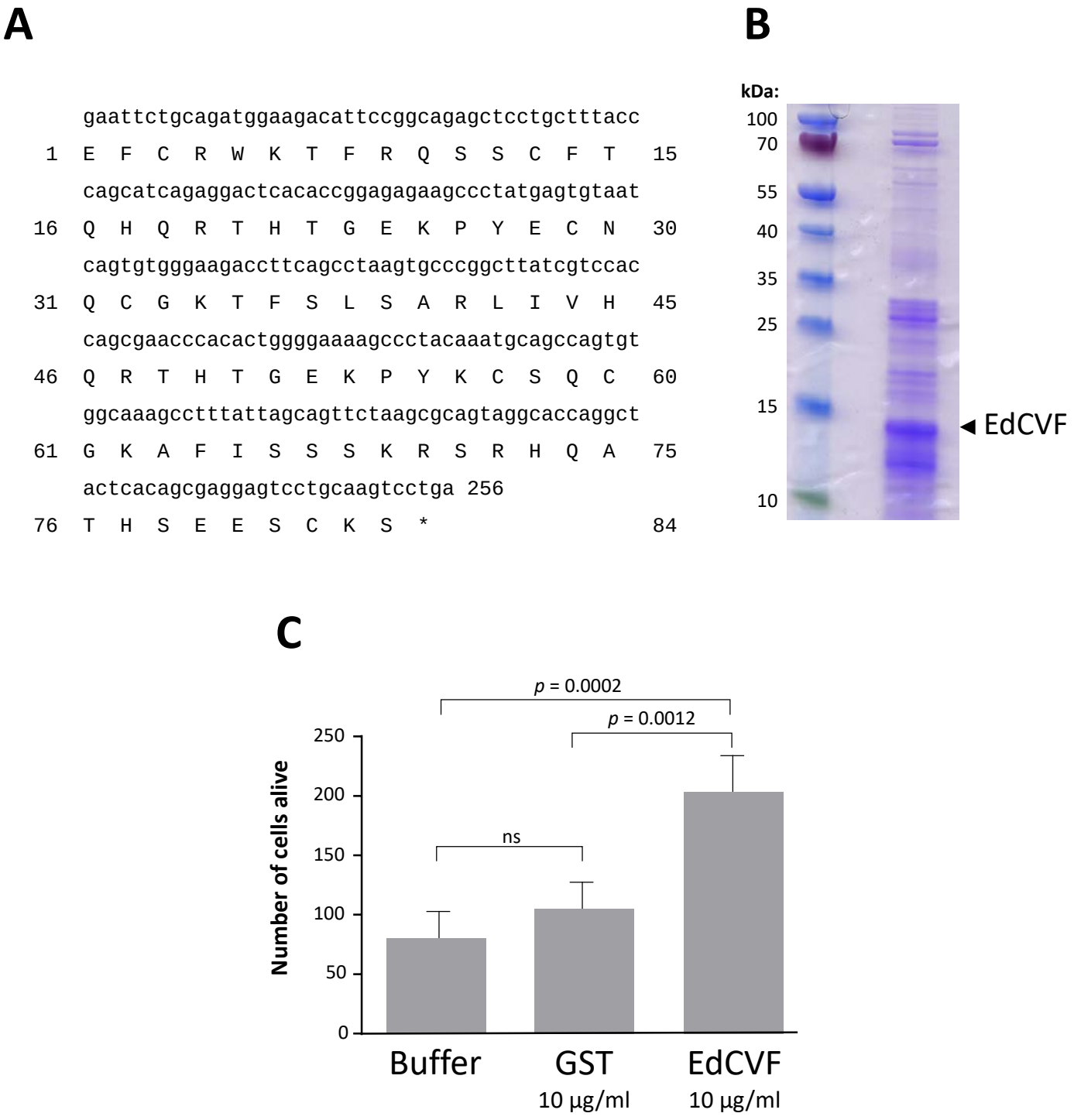

Table I

	1	2	3
A	1	1	1
B	NC	3	3
C	6	NC	6
D	8	8	NC
E	11	11	11
F	13	13	14
G	16	16	16
H	18	18	19

NC negative controls

1-20 positions of the pools tested in quadruplicate

4	5	6	7	8	9	10
1	2	NC	2	2	2	3
4	4	4	NC	4	5	5
6	6	7	7	NC	7	7
9	9	9	9	10	NC	10
NC	11	12	12	12	12	NC
14	NC	14	14	15	15	15
16	17	NC	17	17	17	18
19	19	19	NC	20	20	20

11	12
3	NC
5	5
8	8
10	10
13	13
NC	15
18	NC
NC	20

Table of Materials

Click here to access/download;Table of Materials;Table_of_Materials_13_11_2020.xlsx

Name of Material/Equipment	Company	Catalog Number
96 black plates (Clear Button with lid Tissue culture treated)	Corning	3603
Calcein AM	Thermo-Fisher scientific	C1430
CCD Camera	Photometrics	CoolSnap FX HQ
CDPC (Cytidine 5'-diphosphocholine sodium salt dihydrate)	Sigma-Aldrich	C0256
CO_2 Independant	Thermo-Fisher scientific	18045-054
Curved forceps	Dutscher	005093
DMEM Media	Thermo-Fisher scientific	41966-029
DNAse	Sigma-Aldrich	D4263
Eggs incubator	FarmLine	M08 01 3100
Ethidium Homodimer	Thermo-Fisher scientific	E1169
Fœtal bovine serum	Thermo-Fisher scientific	10270-098
Gentamycin	Thermo-Fisher scientific	15710-049
Hydrocortisone	Sigma-Aldrich	H0880
ITS (insulin Transferine selenium)	Sigma-Aldrich	I1884
large straight pliers	Dutscher	005074
Linoleic acid	Sigma-Aldrich	L8384
M199 medium	Thermo-Fisher scientific	31150-022
Metamorph software	Metamorph	
Microscope	NIKON	Eclipse TE2000
Motorized stage	Martzauzer	Mutlicontrol 2000
Optical filter switch	Shutter Instrument company	Lambda 10-2
PBS 1X	Thermo-Fisher scientific	14190-086
Poly-L-lysine	Sigma-Aldrich	P6282
Progesterone	Sigma-Aldrich	P7556
Pursept A express	Fisher scientific	11814110
Putriscine	Sigma-Aldrich	P5780
Sodium pyruvate	Sigma-Aldrich	S8636
straight forceps	Dutscher	005092
Taurine	Sigma-Aldrich	T8691
Triiodothyronine	Sigma-Aldrich	T6397
Trypan blue	Thermo-Fisher scientific	15250-061

Trypsine 0.25 %	Thermo-Fisher scientific	25200-056

Comments/Description

Dear Nam Nguyen,

We have revised our manuscript, JoVE61998 "Cone-enriched cultures from the retina of chicken embryos to study rod to cone cellular interactions," and addressed all the critics.

Sincerely,

Thierry

_____

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

2. As you have described other models in use, please add a comment in the Introduction on whether chicken embryo retinas contain the RdCVF, NXNL1 gene, and RdCVFL that you have cited to be important in RP.

We have added the following sentence and its citations on line 95: We have shown that the *NXNL1* gene product, RdCVFL, is expressed in chicken cone-enriched cultures[17] and where it plays a protective role[18].

3. Unfortunately, there are sections of the manuscript that show overlap with previously published work. Please revise the following lines: 90-92, 345 (whole…)-348 (…electroporator).

Revised lines 90-92: In RP, the loss of rods through the action of causative mutations results in the reduction in the expression of rod-derived cone viability factor (RdCVF), encoded by the nucleoredoxin-like 1 (*NXNL1*) gene. This causes the interruption of the metabolic and redox signaling between rods and cones.

Revised lines 345-348: the entire retina is placed in the chamber of a custom-made electroporator with 120 µl of 0.5 µg/µl of plasmid DNA in 10 mM Tris-HCl pH 8.0, 1 mM EDTA. Five pulses of 15 V for 50 ms each are applied separated by 950 ms interval.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Pursept A express (Fisher Scientific), Life Technologies, all products from, including the name, “Thermo Fisher Scientific”, Sigma-Aldrich, Corning, Nikon TE 2000, Martzauzer, Coolsnap, Metamorph software, BTX Harvard apparatus, ECM 830;

Done

5. If required for embryos and also because you mention that you used Long-Evans rats, please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s animal research ethics committee.

We have added on line 291: The protocol was approved by the Committee on the Ethics of Animal Experiments of the University Pierre and Marie Curie and the French ministry of research (Permit Number: APAFIS#1028 2015070211275177). The animal experiments were performed under the following authorization: “Certificat d'autorisation d'expérimenter sur les animaux vertébrés A-751863. Préfecture de Police de Paris (November 9th 2011-November 8th 2016)”.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Done

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add

references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Done

8. 5.10: What do you mean by "library of molecules"? Please mention where you have described or listed these molecules and why you are using them.

We have modified the sentence : 5.10 Add 50 µl of the library of molecules (e.g. the conditioned media from a cDNA library, see below) to be screened using a predefined pattern (Table1).

9. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Done

10. The Representative Results section is confusing; please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

We have added this introducing sentence of line 292 : We describe here how the cone-enriched culture system can be used to identify novel cone protecting proteins.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

Done

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol

b) Any modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Done

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references. Please do not abbreviate journal names.

We used https://www.jove.com/files/JoVE.ens

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Leveillard et al. describe a new technique to culture cone photoreceptors from the chick retina. This protocol will benefit studies of retinal degeneration primarily affecting photoreceptors, such as RP, and will facilitate the study of molecular affectors of cone health. The authors have demonstrated success in using these cultures to identify EdCVF as a cone photoreceptor protective element. I recommend several modifications to the manuscript/protocol listed below:

Major Concerns:

There are several spelling and grammatical errors throughout the manuscript, some of which make the sentences and ideas difficult to understand. Please re-read the manuscript and make sure to clarify and fix those errors.

Done

You fail to explain how your protocol will facilitate studies of photoreceptor degeneration and the novelty of this approach. Therefore, I suggest you add a paragraph at the end of the introduction discussing how the technique addresses the challenges discussed and some of the benefits and potential applications of this protocol.

We have added the following paragraph on line 137 : The cone-enriched culture was instrumental in the identification of RdCVF by expression cloning[1]. This system was also used successfully to demonstrate that RdCVF stimulates glucose uptake and its metabolism by aerobic glycolysis[2]. Furthermore cone-enriched culture was used to validate the protective role of RdCVFL, the second product of the *NXNL1* gene[3]. More recently, this system was used to demonstrate the existence of protecting molecules secreted by retinal pigmented epithelial cells transduced with OTX2[4].

Many of your figures and/or their legends need more detail. Here are some points that need to be addressed:

Figure 1: add arrows/various shapes to point out the wings, collar, and bill- briefly mention in figure legend the characteristics you are looking for at E29

Done

Figure 4B: this is a western blow showing the EdCVF protein at ~15kDa - this should be clearly stated. Also, what are the other bands in this lane (not necessary to publish, but I am interested to know)?

Done. The other bands are unknown *E. coli* proteins.

Supp. Figure 1: The graph is not very informative and would benefit from a title on the x-axis and an explanation of the y-axis title and units.

As indicated in the revised figure, the x axis is the Identifier of the pools of 100 clones and the y-axis is revised to: Viability activity (ratio of cells alive to cell s alive in the negative control). For having the expertise in expression cloning, I know that this graph carries an essential information. We have added the following sentence on line 321: This analysis is essential to identify the pools of interest.

Supp. Figure 2: Again, both graphs would benefit from a title on the x-axis.

In the revision A: Identifier of the sub-pools of 10 clones, and B: Identifier of the isolated clones

The phrase, "The clones were first selected…" is not necessary if you tell the reader what each of the numbers means by adding a title.

We have removed the sentence in then figure legend.

Table 1: Please provide more detail in the legend as to what is being shown here. For example, what is NC?, and what do each of the numbers 1-20 represent?

We have added NC = negative controls and 1-20 positions of the pools tested in quadruplicate in the Excel file.

Minor Concerns:

Introduction, line 60: Sentence wording is not clear - do you mean that later mammals are mostly nocturnal? The word mammals needs to be added to this sentence in this case.

Done

Step 1.2: you use the word "hatching", but the eggs do not hatch. They are maintained in the lab after they are "laid" by the hen.

Done

Step 1.3: please provide more detail on the "intermittent reversion" that you mention here. How exactly should the eggs be handled and approximately how often?

We have added: inclination (a progressive movement for 2 hours from one side to its opposite, a 4-hour cycle).

Step 2.4: for the envelope that surrounds the embryos - are you referring to the chorion?

Yes, inserted

Step 4.3: is the trypsin in Ringer's medium?
We have made the following modification: 4.3 After the second wash with Ringer's media, let the pieces of retina fall on the bottom of the tube and remove the Ringer's media, add trypsin.
Step 4.5: how long should the cell suspension be treated with DNase I?
The revised text is: Immediately after adding the DNAse, dissociate the cell clusters and the DNA by successive suction and discharge using a ground-breaking Pasteur pipette.
Steps 5.4-5.7: These steps could be shortened to one step that instructs the reader to perform cell counting with trypan blue. I think it's safe to assume anyone performing cell culture has adequate knowledge of how to perform cell counts and calculate cell density.
We have put the whole description under step 5.4.
Step 5.11: how often should the media be changed?
We have added: No change of media during 7 days.
Step 6.5: please provide more detail of your calculation of the protective effect of a molecule, or provide an annotated equation.
We have added the following : (see Table I and supplementary figure 1).

Reviewer #2:
Manuscript Summary:
The authors described the usefulness of cone-enriched retinal primary culture for molecular screening of cone survival. There is much valuable information contained in this manuscript, and a detailed summary of this methodology would be very useful for reproducing experiments. The following minor points reduce misunderstanding of this manuscript.

Minor Concerns:
1) It might be better for the authors to describe that this reconstruction experiment across the species was worked well with no problem about species difference in the text because it is proved in other publications by the same authors.
We have added at the end of the introduction: The cone-enriched culture was instrumental in the identification of RdCVF by expression cloning[1]. This system was also used successfully to demonstrates that RdCVF stimulates glucose uptake and its metabolism by aerobic glycolysis[2]. Furthermore cone-enriched culture was used to validate the protective role of RdCVFL, the second product of the *NXNL1* gene[3]. More recently, this system was used to demonstrate the existence of protecting molecule secreted by retinal pigmented epithelial cells transduced with OTX2[4].

2) line 156
"2.4 Gently, remove the envelope that surrounds the embryos."
Envelope that surrounds the embryos should be "chorioallantoic membrane".
Done. We have moved the rest of the paragraph in the Caution, lines 183-185.
3) line 176
"3.2 Transfer the four eyes into CO2-independent medium.
~
3.7 Remove the cornea followed by the lens and the vitreous."
Since it is generally known that the removal of pigment epithelium from the neural retina is very easy in Ca++ Mg++ free medium (at least in many labs that this reviewer knows of and in the labs where this reviewer worked), this reviewer would like the authors to describe whether the authors used Ca++ Mg++ free medium in this step or not.
The formulation of the CO_2 Independent Medium (Thermo-Fisher scientific 18045054) is confidential. But the vendor informed us that it contains 110 mg/ml of calcium chloride dihydrate and 131 mg/ml of magnesium chloride hexahydrate. We have added on lines 191-192: This medium contains 0.9 mM $CaCl_2$ and 0.65 mM $MgCl_2$.

4) line 199
"4.3 ~ Disperse the solution after 10 minutes ~"
line 207
We have introduced this as section 4.4
"4.6 ~ Dissociate the cell clusters and the DNA by successive suction and discharge using a ground-breaking Pasteur pipette."
4.7 in the revised manuscript: 4.7 Dissociate the cell clusters and the DNA by successive suction and discharge using a ground-breaking Pasteur pipette immediately after adding the DNAse.
It may be somewhat difficult to imagine the difference between "disperse" and "dissociation through pipetting." The additional description of "Disperse the solution (4.3)" may be helpful.
We have modified the section 4.4 (in the revised manuscript): 4.4 Disperse the solution after 10 minutes by successive suction and discharge using a ground-breaking Pasteur pipette and check for dissociation of the retinal pieces.
5) line 290~
The authors used conditioned medium (CM) recovered from transfected COS-1 cells, but did not describe whether membranes or exosomes were removed (e.g., ultracentrifugation). The reviewer would like to know if the CM may possibly contain membranes.
We have added the following sentence: Membranes or exosomes were not removed by ultracentrifugation on lines 315-316.
The reviewer would also like to read a discussion of whether it is possible to screen for non-diffusive factors (e.g., membranous) using this method, if possible.
We added the following sentence on lines 398-400: In addition, even if membranous proteins were not removed from the conditioned medium, the configuration of the assay is unfavorable to the identification of non-diffusive factors.
6) Increasing the resolution of the photos and furthermore, comparing them with the different stages in Figure 1 (e.g., HH stage 28 and/or 30) may help the reader's understanding because the authors emphasize the importance of the embryonic stage (HH stage 29).
We provide in the revised figure, the three stages of development.
7) In Supplemental Figure 2, the y-axis in A is very different from that in B and the other graphs (Figure 3, Figure 4 and Supplemental Figure 3). Was the seeding density in A different in other experiments? If so, this should be stated in the manuscript.
We have added the following sentences on lines 332-3334: The y axis has a different scale even if the seeding density was the same than in Supplementary figure 2A. We have seen this commonly when the assays are repeated weekly for months.

Reviewer #3:
Manuscript Summary:
The paper describes an improved and modified method to obtain chicken retinal cultures enriched in cone photoreceptors. This is particularly interesting as authors showed that the model is suitable for genetic manipulation and testing for specific genes involved in photoreceptor survival, which is important for discovery of therapeutic strategies to treat retinal degenerations.

Major Concerns:
I have no concerns since the method is well described and the results are clear. Maybe the authors could explain better that the cultures can also be obtained using fetal serum, and this can be used as a first screen for tests since is less expensive.
We wrote previously : **Retrospectively, the thought that the RdCVF factor could not have been identified if we had realized the cone-enriched cultures in the presence of serum gives vertigo Léveillard et al., Oxidative medicine and cellular longevity 2017, 8475125 (2017).**
It would also be interesting to demonstrate the feasibility of electroporation procedures.
The electroporation procedure was successfully used in Ait-Ali et al., Cell 161, 817 (2015) and Mei et al., Antioxidants & redox signaling 24, 909 (2016).

1 Leveillard, T. *et al.* Identification and characterization of rod-derived cone viability factor. *Nat Genet.* **36** (7), 755-759, doi:10.1038/ng1386, (2004).
2 Ait-Ali, N. *et al.* Rod-derived cone viability factor promotes cone survival by stimulating aerobic glycolysis. *Cell.* **161** (4), 817-832, doi:10.1016/j.cell.2015.03.023, (2015).
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Figure S1

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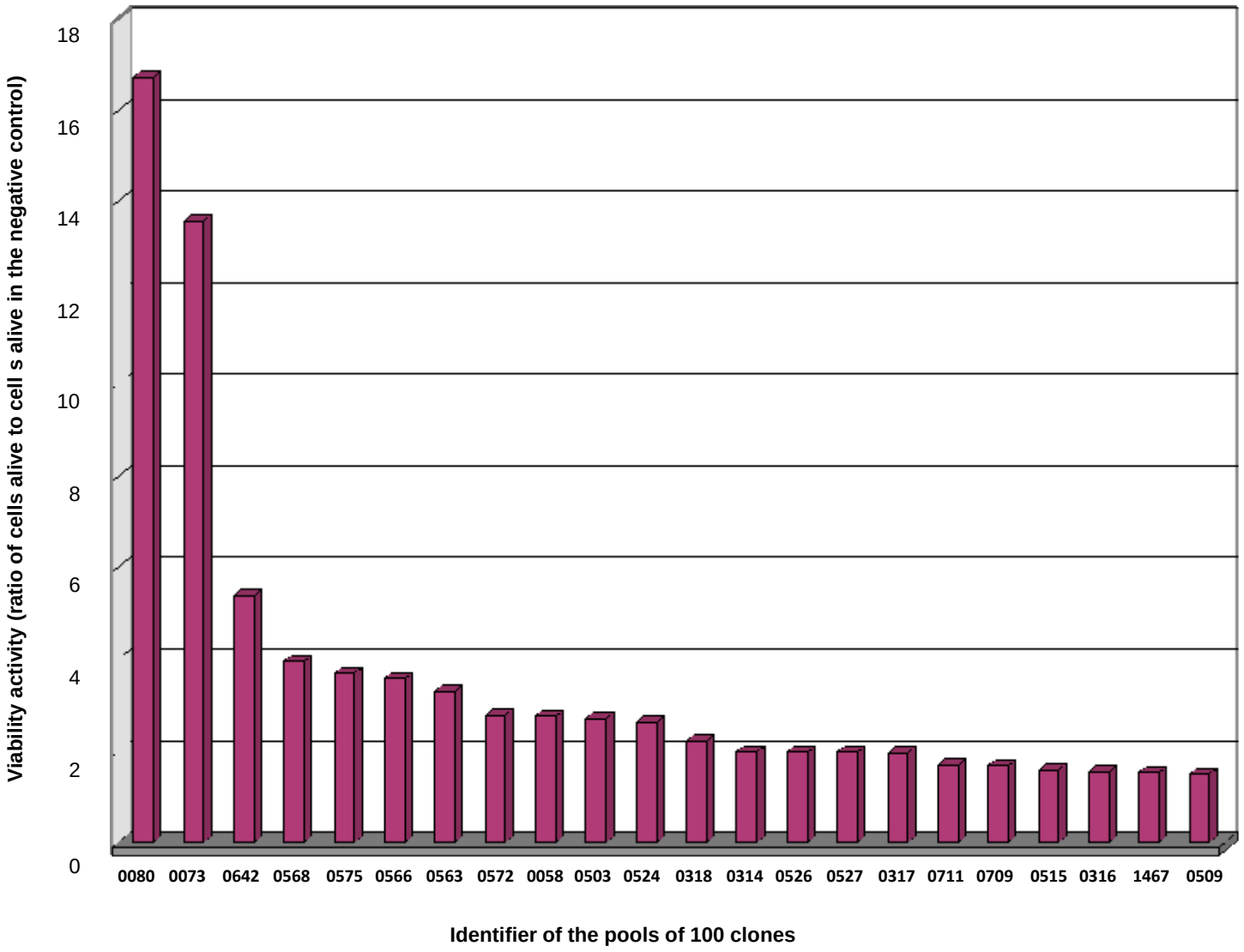

Figure S2

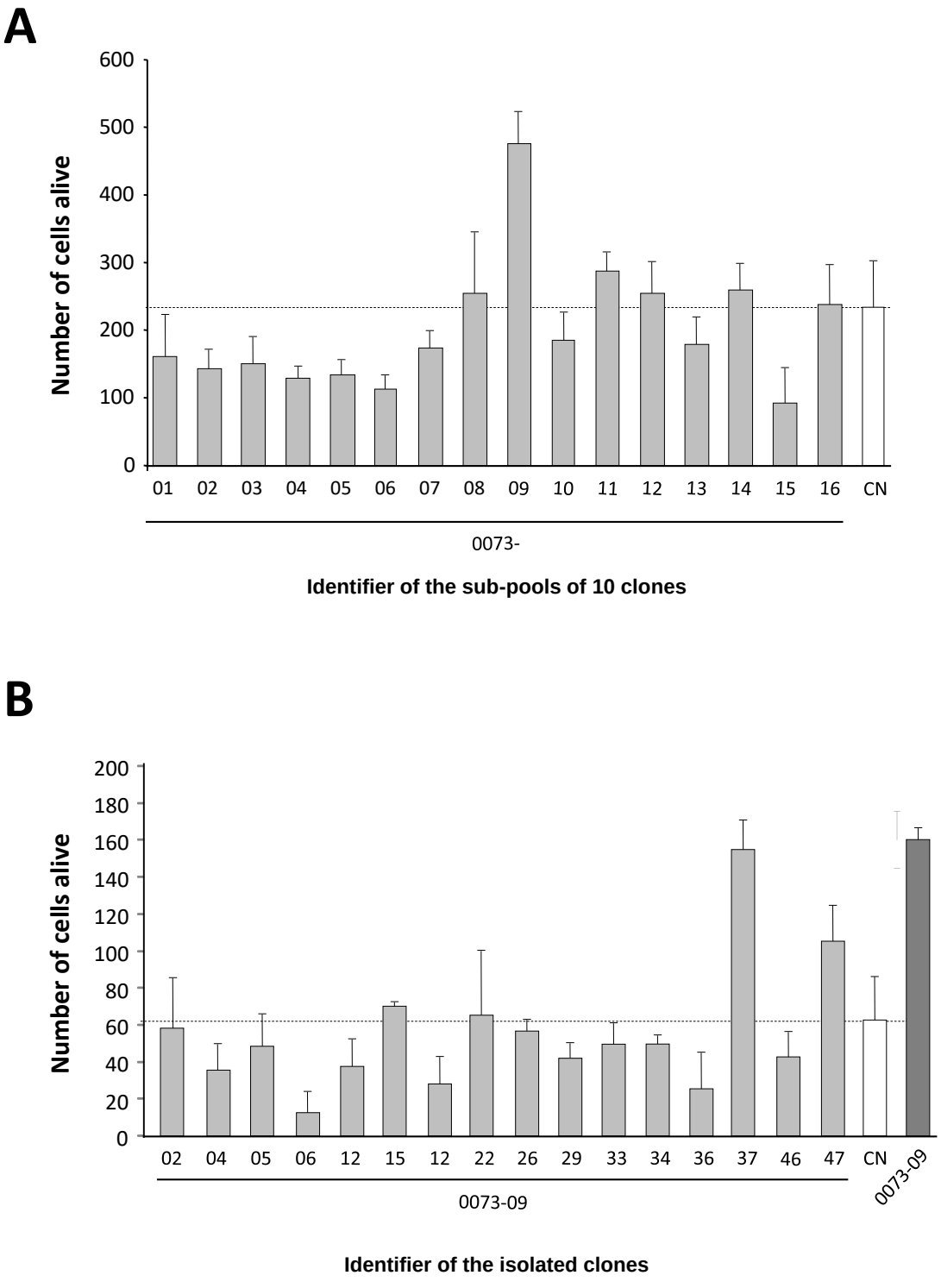

Figure S3

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