



## Visual arrestin modulates gene expression in the retinal pigment epithelium: Implications for homeostasis in the retina



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### ABSTRACT

The retinal pigment epithelium (RPE) is essential for maintaining retinal homeostasis by removing and recycling photoreceptor outer segment (POS) in membranes. It also produces and secretes growth factors involved in retinal homeostasis. Arrestin 1 (ARR1) is specifically expressed in photoreceptors (PRs) and a vital molecule for keeping visual cycle between PRs and RPE. In the present study, we showed the expression of ARR1 was decreased by form-deprivation (FD) in retina of rat. The ARR1 was detected in the RPE of the controls but not in the RPE of FD, which indicates RPE phagocytes POS containing ARR1. Furthermore, we overexpressed ARR1 in cultured human RPE and revealed the ARR1 upregulates bFGF expression and downregulates TGF- $\beta$ 1, - $\beta$ 2 and bone morphogenetic protein-2 (BMP-2). The upregulation of bFGF by ARR1 directly works for PR survival and the downregulation of TGF- $\beta$ s by ARR1 inhibits epithelial mesenchymal transition (EMT) of RPE, which is the underlying mechanism of keeping retinal homeostasis. Our results also indicate the regulation of ARR1 expression in RPE might become a novel therapeutic option for various ocular diseases.

### 1. Introduction

The retinal pigment epithelium (RPE) includes polarized cells that form a monolayer at the interface between photoreceptors (PRs) and the extracellular matrix structure termed the Bruch's membrane. The RPE has high phagocytosis activity, enabling the removal and recycling of photoreceptor outer segment (POS) membranes, which is essential for maintaining retinal homeostasis. The inability to phagocytose causes retinitis pigmentosa (RP), leading to irreversible retinal degeneration [1]. The RPE also produces and secretes a variety of growth factors, such as basic fibroblast growth factor (bFGF) and transforming growth factor beta (TGF- $\beta$ ), which have important roles in retinal homeostasis [2,3]. bFGF is predominantly synthesized and secreted by the RPE and is a physiologically relevant neurotrophic factor [4]. bFGF pathways play key roles in PR neurogenesis and have neuroprotective effects against light-induced PR cell loss [5,6]. Furthermore, bFGF rescues PR cell from death in the Royal College of Surgeons (RCS) rat model, with impaired POS phagocytosis leading to RP [7]. TGF- $\beta$  is also important for retina homeostasis. TGF- $\beta$  is a multifunctional cytokine, regulating pivotal biological responses, such as differentiation,

apoptosis, migration, immune cell function, and extra cellular matrix (ECM) synthesis [8]. Three TGF- $\beta$  isoforms (TGF- $\beta$ 1, - $\beta$ 2, and - $\beta$ 3) are expressed in RPE cells, which mainly express TGF- $\beta$ 1, - $\beta$ 2, and BMP-2 [9,10]. TGF- $\beta$  is a major driver of myofibroblastic transformation by the epithelial mesenchymal transition (EMT) of RPE cells, a hallmark of epiretinal membrane (ERM) or proliferative vitreoretinopathy (PVR) pathology [11,12]. Therefore, abundant TGF- $\beta$  expression in the RPE results promotes EMT and disease development [13].

Arrestin 1 (ARR1) shuts off the G-protein coupled receptor (GPCR)-light-activated cascades of phototransduction in rod and cone PRs. ARR1 is highly expressed in rod PRs, pinealocytes, and mouse cone PRs [14]. The phenotype of *Arr1*<sup>-/-</sup> mice demonstrates the necessity of this protein for rod phototransduction shutoff and light adaptation [15]. These mice must be dark-reared to prevent light-dependent degeneration of the rods [16]. These findings suggest that ARR1 prevents retinal death from the deleterious effects of light and maintains retinal homeostasis. Despite extensive studies of the effects of ARR1 in rod PRs, the molecular mechanism underlying ARR1 functions in the retina is not fully understood.

In the present study, we found that *Arr1* is predominantly expressed

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in PRs and ARR1 expression is decreased by form-deprivation (FD) in the RPE. ARR1 was expressed in the RPE of control rats but not in FD rats, suggesting phagocytosis by RPE cells from PRs in control rats. Furthermore, ARR1 altered *bFgf* and *Tgf-β* expression in the RPE, thereby influencing retinal homeostasis.

## 2. Materials and methods

### 2.1. Ethics statement

All experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Guiding Principles for the Care and Use of Animals of the Tokyo Medical and Dental University and complied with the ARRIVE guidelines. This study was approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Permit Number: A2017-356C, A2018-250A).

### 2.2. Preparation of form-deprived model in rats

Six male Wistar rats (CLEA Japan, Tokyo, Japan) at 3 weeks old were used to create the form deprivation (FD) model. Rats were housed under standard humidity and temperature conditions under a 12-h dark/light cycle. The left eyelids of the rats were sutured with 6–0 nylon for 2 weeks to create FD eyes in the continuous dark state. The right eyes were left open and used as controls. All procedures were performed under general anesthesia by an intraperitoneal injection of anesthetic (0.375 mg/kg medetomidine (Nippon Zenyaku Kogyo, Fukushima, Japan), 2.0 mg/kg midazolam (Sandoz, Yamagata, Japan), and 2.5 mg/kg butorphanol (Meiji Seika Pharma, Tokyo, Japan)) and topical anesthesia with 0.4% oxybuprocaine (Santen, Osaka, Japan). Two weeks after suturing, rats were euthanized by an intraperitoneal injection of pentobarbital (100 mg/kg) (Kyoritsuseiyaku, Tokyo, Japan) and the eyes were enucleated.

### 2.3. Immunohistochemistry (IHC)

The eyes were enucleated, fixed in Carnoy's solution (ethanol: chloroform: glacial acetic acid = 6:3:1) on ice, and soaked in ethanol. After dehydration, they were placed in xylene. Finally, the eyes were embedded in paraffin and 10-μm sections were made. Before immunohistochemical staining, the sections were deparaffinized and rehydrated with xylene and ethanol. They were soaked in 10 mM sodium citrate buffer (pH 6.0). Heat-induced epitope retrieval was performed using a microwave. The sections were washed with phosphate-buffered saline (PBS) (Life Technologies, Carlsbad, CA, USA), blocked with 5% normal goat serum (Vector Laboratories, Inc., Burlingame, CA, USA) in PBS, and incubated overnight with a mouse monoclonal antibody to ARR1 (MCA-S128, dilution 1:100; EnCor Biotechnology, Gainesville, FL, USA) in a humidified chamber at 4 °C. They were then washed with PBS and incubated with appropriate fluorophore-conjugated secondary antibodies for 1 h in the dark at 25 °C. After washing three times with PBS, the sections were mounted with Vectashield mounting medium with DAPI (H-1200; Vector Laboratories) and observed using a confocal microscope (TCS SP8; Leica, Wetzlar, Germany).

### 2.4. In situ hybridization (ISH)

The eyes of 8-week-old Wistar rats (CLEA Japan) (n = 3) reared under a 12-h dark/light cycle were enucleated and fixed with G-Fix (Genostaff, Tokyo, Japan), embedded in paraffin on CT-Pro20 (Genostaff) using G-Nox (Genostaff), and sectioned at 6 μm.

ISH was performed using the ISH Reagent Kit (Genostaff) according to manufacturer's instructions. Hybridization was performed with antisense and sense probes at concentrations of 250 ng/ml in G-Hybo-L (Genostaff) for 16 h at 60 °C. After hybridization, the sections were

incubated with anti-digoxigenin AP conjugate (1:2000; Roche Diagnostics, Mannheim, Germany) with G-Block in TBST at room temperature. Coloring reactions were performed with NBT/BCIP solution (Sigma-Aldrich, St. Louis, MO, USA) overnight followed by washing in PBS. Sections were counterstained with Kernechtrot stain solution (Muto Pure Chemicals, Tokyo, Japan), and mounted with G-Mount (Genostaff).

### 2.5. Cell culture

Human RPE (hRPE) cells (Lonza, Walkersville, MD, USA) were cultured in RPE cell basal medium with RteGM SingleQuots (Lonza) containing 2% fetal bovine serum (FBS), l-glutamine, fibroblast growth factor (FGF), and GA-1000 in a 37 °C humidified atmosphere with 5% CO<sub>2</sub> according to the manufacturer's recommendations. All experiments were performed using fourth passage cells.

### 2.6. Gene transfection

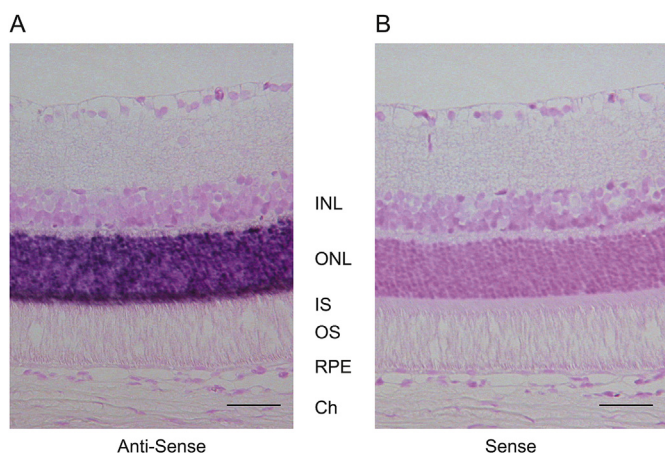
hRPE cells were seeded in twelve-well plates until reaching 90–100% confluence. The SAG expression vector (RC220057; Origene, Rockville, MD, USA) and control vector (PS100001; Origene) were transfected into hRPE cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and OptiMEM Medium (Life Technologies) according to the manufacturers' instructions. Seventy-two hours after transfection, total RNA was isolated.

### 2.7. Real-time polymerase chain reaction (PCR)

Total RNA was isolated from cultured hRPE cells using the AllPrep RNA/Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using ReverTra Ace qPCR RT master mix with gDNA remover (Toyobo, Osaka, Japan) according to the manufacturer's instructions. PCR was performed using Kapa SYBR Fast qPCR Master Mix (Kapa Biosystems, Wilmington, MA, USA) using a LightCycler 480 II system (Roche). The following primer sequences were used: *Arr1* forward, 5'-CGTACCCCT TTCTCTGACG-3' and reverse, 5'-CGGATCAGTAATCGCACGGA-3' (NCBI accession number: NM\_000541.4), *Tgf-β1* forward, 5'-GGGACT ATCCACCTGCAAGA-3' and reverse, 5'-GGGTTTCCACCATTAGCAGC-3' (NM\_000660.6), *Tgf-β2* forward, 5'-GTGCTCTGTGGGTACCTTGA-3' and reverse, 5'-GCGCTGGGTTGGAGATGTTA-3' (NM\_001135599.3), *Bmp-2* forward, 5'-GCTGTCTTCTAGCGTTGCTG-3' and reverse, 5'-CTGTTTCA GGCCGAACATGC-3' (NM\_001200.3), *bFgf* forward, 5'-AAGGAGTGTG TGCTAACCGT-3' and reverse, 5'-TTCTGCCCAGGTCCTGTTTT-3' (NM\_002006.5), *β-actin* forward, 5'-GGATCAGCAAGCAGGATATG-3' and reverse, 5'-AGAAAGGGTGTACGCAACTAA-3' (NM\_001101.4).

### 2.8. Western blot analysis

ARR1 protein expression was analyzed by western blotting. After rat eyeballs were enucleated, the retina and sclera were isolated. Protein lysates were extracted using RIPA lysis buffer (Thermo Scientific, Carlsbad, CA, USA) supplemented with protease inhibitor (Sigma-Aldrich), according to manufacturer's protocol. Briefly, all lysates were quantified and equal volumes were loaded on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) before transferring to PVDF membranes (Invitrogen). Membranes were blocked with 3% skim milk and incubated with anti-ARR1 primary antibodies (1:1000; EnCor) overnight at room temperature. Bound proteins were detected using peroxidase-conjugated ECL anti-mouse IgG (GE Healthcare UK Ltd., Little Chalfont, UK). Proteins were visualized using a chemiluminescence substrate (Bio-Rad, Hercules, CA, USA) and imaging system (DNR, Never Yamin, Israel). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. The intensities of protein bands were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).



**Fig. 1.** In situ hybridization of *Arr1* mRNA in the rat retina  
 A: Digoxigenin (DIG)-labeled anti-sense mRNA probe. *Arr1* antisense-specific staining was detected in purple. B: DIG-labeled sense mRNA probe.  
 INL: inner nuclear layer, ONL: outer nuclear layer, IS: inner segment, OS: outer segment, RPE: retinal pigmental epithelium, Ch: choroid. Scale bar, 50  $\mu$ m.

### 2.9. Statistical analysis

Student's *t*-tests were used to determine whether differences in gene expressions were significant. A value of  $p < 0.05$  was considered significant. Each experiment was performed at least three times.

## 3. Results

### 3.1. Confirmation of the localization of *Arr1* mRNA in the retina

We examined *Arr1* mRNA expression in the retina of rats by ISH using paraffin sections (Fig. 1A and B). *Arr1* antisense-specific staining was detected in the outer nuclear layer and PR layer of the retina, but the signal was not detected in the RPE layer (Fig. 1A). These results revealed the specific expression of *Arr1* mRNA in PR cells in the rat retina.

### 3.2. Distribution of ARR1 in the FD model

Next, we examined the distribution of ARR1 in the retina by IHC. ARR1 was expressed in the outer nuclear layer, the PR layer, and the RPE (Fig. 2A). ARR1 was detected in the RPE of control eyes but was decreased in the RPE of the FD model (Fig. 2B and C). In summary, although ARR1 is normally localized in both PR cells and the RPE, its expression in the RPE was decreased by FD.

### 3.3. ARR1 expression level in the retina of the FD model

We examined ARR1 expression in the whole retina of the FD model and control eyes by Western blot analyses. Although ARR1 was observed in both groups, the expression level was significantly decreased by 70% in the FD model relative to controls ( $p = 0.04$ ) (Fig. 3A and B). These results showed that ARR1 expression in the retina was suppressed under FD conditions.

### 3.4. Expression changes of TGF- $\beta$ superfamily members and bFGF in the hRPE in vitro

Previous experiments suggested that ARR1 expression in the whole retina is suppressed by FD condition. We further examined whether ARR1 affects the expression levels of growth factors in the RPE in vitro using hRPE cells. An *Arr1*-specific vector was transfected into the hRPE as a model with normal light stimulation, and a control vector was

transfected as a model of the continuous dark state, like FD. We evaluated *Arr1*, *Tgf- $\beta$ 1*, *Tgf- $\beta$ 2*, *Bmp-2*, and *bFgf* expression by PCR. In the *Arr1* group, *Arr1* levels were 87-fold higher than levels in the control ( $p < 0.001$ ) (Fig. 4A). *Tgf- $\beta$ 1*, *Tgf- $\beta$ 2*, and *Bmp-2* levels decreased by 30%, 46%, and 37% ( $p = 0.05$ , 0.002, and 0.007) (Fig. 4B, C and D). *bFgf* expression increased significantly by 93-fold ( $p < 0.001$ ) (Fig. 4E).

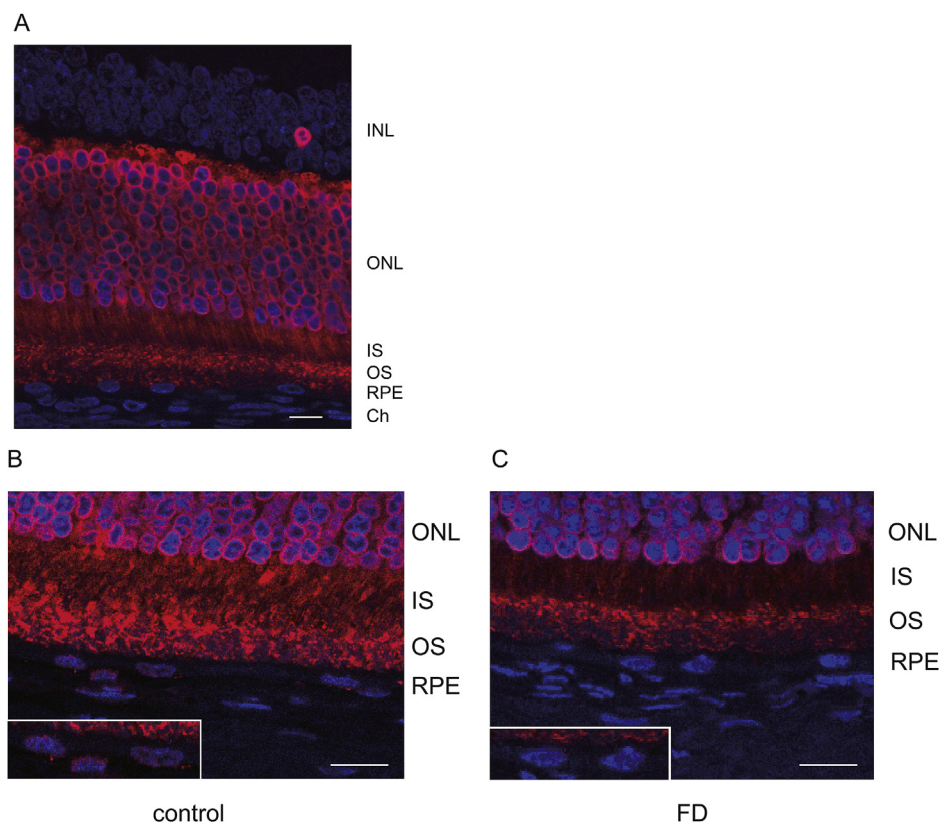
## 4. Discussion

In the present study, we demonstrated that *Arr1* is also specifically expressed in the PRs of rats as well as human and mouse [14]. Our IHC analysis demonstrated that ARR1 expression in PRs is decreased by FD, indicating that light stimulus up-regulates *Arr1* expression in PRs. Previous studies have shown that mammalian retinal physiology and function are under the control of a retinal circadian clock, rod disk shedding, and rhodopsin gene expression [17,18]. Vancura et al. demonstrated that ARR1 is under circadian regulation in mouse PRs [19].

Light exposure is an important factor for the maintenance of the mammalian circadian clock [20]. Our results clearly demonstrate that *Arr1* expression in PRs of rats is regulated by light, consistent with the functions of ARR1 in the visual cycle. We also showed that *Arr1* is expressed in the RPE under a normal light cycle but not in FD eyes. Interestingly, ARR1 predominantly localizes to rod inner segments and the outer plexiform layer under dark-adapted conditions; after light exposure, ARR1 is translocated to rod outer segments [21]. These characteristics of ARR1 not only help to maintain a healthy visual cycle but also promote ARR1 intake by the RPE via phagocytosis. These findings led us to hypothesize that *Arr1* functions in retina homeostasis and normal visual function by modulating gene expression in the RPE.

Two known retinal diseases are caused by mutations in *Arr1*, retinitis pigmentosa (RP) and Oguchi disease type 1 [22,23]. RP is a well-known inherited retinal degenerative disease associated with more than 100 mutations, including *Arr1* mutations. RP is a retinal dystrophy characterized by retinal pigment deposits and a primary loss of rods, followed by a secondary loss of the PR and finally RPE atrophy. Although it is unclear why *Arr1* abnormalities cause RP, it is thought that normal retina homeostasis cannot be maintained without ARR1. Oguchi disease type 1 is a congenitally inherited non-progressive disease of the retinal rods characterized by stationary night blindness [23]. The mutations disrupt the recovery phase of phototransduction and lead to extremely slow dark-adaptation of rod PRs [24]. It is possible that ARR1 works to maintain retinal homeostasis and function via the RPE, in addition to maintaining a visual cycle. However, the correlation between ARR1 and the RPE is unclear.

In the present study, we demonstrated that ARR1 is expressed in the RPE via POS phagocytosis and this expression is decreased by FD. PRs have a highly evolved phenotype, requiring cell-cell interactions. A potential source of interactions is cells within the retina (Muller, horizontal, bipolar, etc.) and the RPE [25,26]. PR-RPE interactions are vital for the normal functioning of the retina because POS phagocytosis by the RPE is necessary for maintaining healthy PRs [1,27]. The RPE is a monolayer of highly specialized cells located between the PR and the vascular choroid, influencing the structure and function of the retina by secreting numerous growth factors [9,10,28]. These soluble growth factors secreted from the RPE could modulate viability in various retinal cell types, specifically photoreceptors. We found that the over-expression of *Arr1* significantly increases the expression of *bFGF* and decreases the expression of *TGF- $\beta$ 1*, *TGF $\beta$ 2*, and *BMP-2* in the hRPE in vitro. In our knowledge, it has not been unknown a direct interaction between *bFGF* and TGF super family in retina, these molecules seem to work independently for keeping retinal homeostasis. *bFGF* is a neurotrophic factor capable of promoting survival in various cells of the retina, including PRs [29,30]. Previous studies have demonstrated that *bFGF* is prominently expressed in the RPE [2]. The RPE is also the site

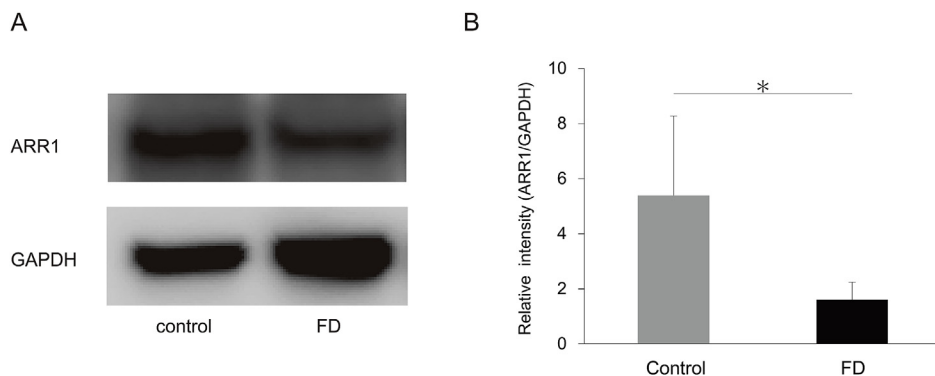


**Fig. 2.** Confocal microscope image of rat retina with ARR1 antibodies  
**A:** Immunohistochemical detection of ARR1 in the normal rat retina. ARR1 was observed in the outer nuclear layer, inner and outer segment, and RPE. ARR1; red, DAPI; blue. **B:** Magnified image of the retina centering on the RPE of control eyes. **C:** Magnified image of the retina centering on the RPE of FD eyes.  
 INL: inner nuclear layer, ONL: outer nuclear layer, IS: inner segment, OS: outer segment, RPE: retinal pigmental epithelium, Ch: choroid, FD: form deprivation. Scale bar, 10  $\mu$ m.

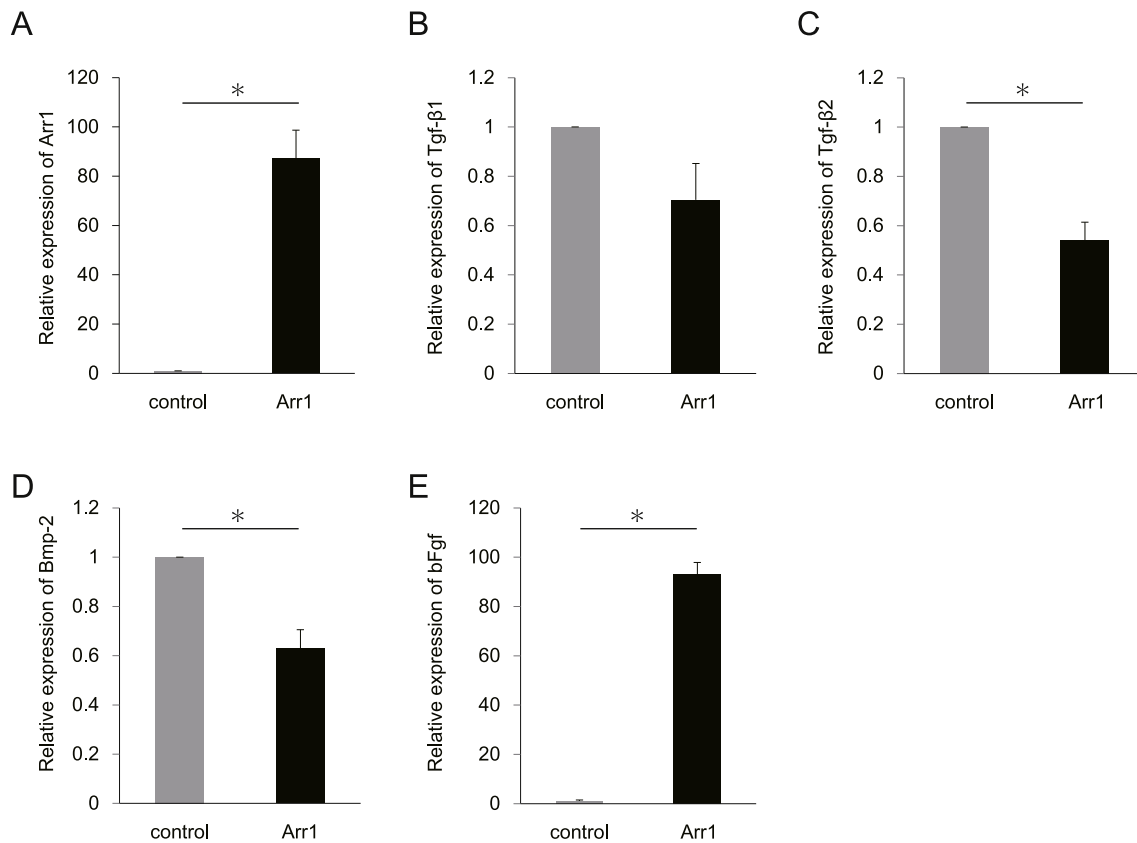
of the primary lesion in inherited retinal dystrophy in the RCS rat model [1,31]. The degeneration of PRs in RCS rats is prevented or slowed by a number of trophic growth factors, particularly bFGF [6]. Intravitreal injection or virus-mediated delivery of bFGF protects PRs from cell death in several animal models of RP [5,7]. Li et al. [32] showed that bFGF expression in the human retina is higher in areas where PRs are in an earlier stage of degeneration than in areas where PRs are severely degenerated. Accordingly, the expression and secretion of bFGF from the RPE are vital for the normal function of the retina, particularly PRs. Interestingly, Walsh et al. showed that bFGF in the retina is up-regulated by light exposure [33]. We demonstrated that ARR1 is down-regulated by FD and the over-expression of *Arr1* in cultured hRPE cells markedly up-regulates *bFgf*. We concluded that ARR1 expression in PRs is regulated by light stimulus to the retina, and the up-regulated ARR1 is phagocytosed by the RPE, thereby increasing the expression and secretion of bFGF and promoting PRs homeostasis. As previously described, RPE cells that undergo dedifferentiation by TGF- $\beta$  are transformed into myofibroblasts with accelerated proliferation and migration, promoting EMT. In the present study, *Arr1* overexpression in cultured hRPE cells decreased the expression of TGF- $\beta$ 1 and - $\beta$ 2 to 70%

and 54%, respectively. Pfeffer et al. demonstrated that TGF- $\beta$ 2 is the predominant isoform in the RPE, with  $\beta$ 2:  $\beta$ 1 ratios of 6:1 [34]. It suggests a greater contribution of TGF- $\beta$ 2 to EMT regulation in the RPE than TGF- $\beta$ 1. Our results emphasize the importance of TGF- $\beta$ 2 expression in the RPE. We showed that the over-expression of *Arr1* in cultured hRPE cells significantly decreased the expression of *BMP-2*. *The correlation between BMP-2 and EMT of the RPE is poorly studied. However, many studies have shown that BMP-2 induces EMT in other epithelial cells in mammals [35,36].* Considering that the RPE is a kind of epithelial cell, BMP-2 should be an important modulator of EMT of the RPE. EMT regulation is necessary to prevent ocular diseases, and our results indicate that ARR1 is a negative regulator of TGF- $\beta$ 1, TGF- $\beta$ 2, and BMP-2 in the RPE. Phagocytosed ARR1 in the RPE alters the expression of various important genes, such as *bFGF*, *TGF- $\beta$ 1*, *TGF- $\beta$ 2*, and *BMP-2*, and maintains homeostasis in the retina.

We demonstrated that ARR1 regulates the expression of various growth factors in the RPE, indicating its importance for the maintenance of retinal homeostasis and healthy vision. It is possible that ARR1 regulates the expression of additional growth factors in the RPE and is associated with many diseases. Our results also indicate that the



**Fig. 3.** Western blot analysis of ARR1 in the whole retina of control and FD  
**A:** Representative images of ARR1 in the retina of control and FD by western blotting. GAPDH was used for normalization of protein loading. **B:** Relative intensity of ARR1 in FD and control. Values for each sample were normalized against GAPDH. Data are expressed as means  $\pm$  SEM. Statistical analyses was performed using t-tests (\* $p$  < 0.05).



**Fig. 4.** Reverse transcription-PCR analyses of Arr1, Tgf- $\beta$  superfamily members and bFgf in control RPE cells and Arr1 gene transfected RPE cells.

A: Arr1 mRNA expression. B: Tgf- $\beta$ 1 mRNA expression. C: Tgf- $\beta$ 2 mRNA expression. D: Bmp-2 mRNA expression. E: bFgf mRNA expression. These mRNA expression levels were normalized using beta actin mRNA expression. Data are expressed as means  $\pm$  SEM. Statistical analyses were performed using t-tests ( $p < 0.05$ ).

regulation of ARR1 in the RPE might become a novel therapeutic option for various ocular diseases. Further studies, such as *Arr1* gene transfer experiments to the RPE in vivo, are warranted to improve our understanding of the molecular mechanisms underlying the roles of ARR1 in the retina.

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#### Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.bbrep.2019.100680>.

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