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Impaired Autophagy in Retinal Pigment Epithelial Cells Induced from iPS Cell of Distal Myopathy with Rimmed Vacuole Patient

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Objective: We generated induced pluripotent stem (iPS) cells from a patient with distal myopathy with rimmed vacuoles (DMRV), in which sialic acids synthesis is reported to be defective. In this study, we examined whether the differentiation to retinal pigment epithelial (RPE) cells and autophagy was affected in the patient derived cells.

Methods: Patient derived iPS cells were established through the transduction of re-programming factors into peripheral mononuclear cells via retrovirus vectors. RPE cells were induced from iPS cells through aggregation culture. Then the autophagy induced by amino acid starvation was estimated by measuring LC3-containing "puncta" structure.

Results: A 3D aggregate culture of patient-derived iPS cells resulted in some irregular shapes, and the aggregate contained large vacuoles filled with lipid droplets and cellular components such as damaged mitochondria. RPE cells induced from patient-derived iPS cells showed impaired autophagy flux under amino acid starvation.

Conclusion: These findings were similar to those of sialidosis patient-derived iPS cells, in which cleavage of terminal sialic acids in oligosaccharide chains is defective. This suggests that the control of both the addition and removal of sialic acids are pivotal for autophagy progression.

Key words: autophagy, iPS cell, retinal pigment epithelium, distal myopathy with rimmed vacuoles

INTRODUCTION

Sialic acids are abundant terminal monosaccharides of a variety of glycoconjugates. Both the addition and cleavage of sialic acids are involved in the control of various cellular functions such as cell-cell interaction, signal transduction, degradation of glycoproteins, and trafficking of cellular components.

We previously generated type I sialidosis patient-derived iPS cells [1]. Sialidosis is a lysosomal storage disease caused by an N-acetyl-lysosomal sialidase (*NEU1*) gene mutation. One of the diagnostic criteria for sialidosis is a macular "cherry-red spot" manifesting as a whitish circular shape in the perifoveal region of the retina, and is an accumulation of substrate within the retinal ganglion cell layer (GCL) [2]. In a previous report, we observed accumulation of lipid droplets and damaged organelles in 3D culture aggregates of patient-derived induced pluripotent stem (iPS) cells, and that induced retinal pigment epithelial (RPE) cells showed impaired autophagy progression [1]. These observations indicate that cleavage of terminal sialic acids is pivotal for the proper development of stem cells and homeostasis of differentiated cells.

The enzyme UDP-N-acetylglucosamine 2-epimerase/N-acetylmannosamine kinase (GNE), which is highly conserved in mammalian species, initiates sialic acid biosynthesis [3]. It catalyzes the conversion of UDP-N-acetylglucosamine (UDP-GlcNac) to N-acetyl-D-mannosamine (ManNAc) and consecutive phosphorylation reactions, forming ManNAc 6-phosphate. Enzyme activity is a limiting factor in sialic acid synthesis. It is known that a missense mutation of *GNE* gene can result in hereditary skeletal muscle atrophy or distal myopathy with rimmed vacuoles (DMRV) [4–6].

However, *GNE* mutations can affect various cell types as well as muscle cells, since *GNE* is ubiquitously expressed. In order to confirm the significance of sialic acids control in RPE, we utilized DMRV patient-derived iPS cells, which we have established from the peripheral blood mononuclear cells, and examined whether sialic acids are essential for normal development and autophagy progression of RPE.

MATERIALS AND METHODS

Generation of distal myopathy with rimmed vacuoles (DMRV)- patient-derived iPS cells

This study was approved by the ethics board of Tokai University Hospital in Japan (13I-36, 19I-02). DMRV patient-derived iPS cells were established as previously described [1], in which mononuclear cells

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obtained from sialidosis patient and healthy volunteers were induced to iPS cells. In brief, mononuclear cells obtained from the peripheral blood of a DMRV patient, in which GNE gene missense mutation was detected in both alleles (C529T; R177C and G1714C; V572L), were incubated with anti-CD3/CD28 coated beads (DB11131, Thermo Fisher Scientific, Waltham, MA, USA), and bead-attached cells were collected and cultured in the presence of rIL-2 (Novartis, Basel, Switzerland) for 2 days. The cells were transduced with reprogramming factors (Oct4, Sox2, Klf4, and c-myc) via retrovirus vector, which were provided by Dr. Shin Kaneko (The Institute of Medical Science, Tokyo University, Tokyo, Japan) and cultured on mitomycin C-treated mouse embryonic fibroblasts (MEF) in DMEM F12 (D6421, Sigma-Aldrich, St. Louis, MO, USA) containing 20% KnockOut Serum Replacement (KSR; 10828-028, GIBCO, Thermo Fisher Scientific), 1% non-essential amino acids (11140, GIBCO, Thermo Fisher Scientific), 1 × L-glutamine-penicillin-streptomycin (G1146, Sigma-Aldrich), and 5 ng/mL bFGF (060-04543, Wako Chemicals, Neuss, Germany). The generated iPSC colonies were harvested and expanded.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed with PBS (phosphate buffer saline). The fixed cells were incubated in PBS containing 5% goat serum with 0.5% Triton X-100. The cells were then incubated with the primary antibody in PBS containing 1% goat serum and 0.5% Triton X-100 overnight at 4˚C. After washing with PBS 3 times, the cells were incubated with Alexa Fluor 488-labeled or Alexa Fluor 594-labeled secondary antibodies for 2 h at room temperature. Thereafter, the cells were washed with PBS 3 times and counter-stained with DAPI (1 μg/mL) for 15 min. Photomicrographs were taken using a microscope (Carl Zeiss Axio Vert A1). The following primary antibodies were used: anti-LC3 pAb (PM036, MBL International, Woburn, MA, USA), anti-SSEA-4 (#MAB4304, Millipore, Billerica, MA, USA), anti-TRA-1-60 (#MAB4360, Millipore), and anti-TRA-1-81 (#MAB4381, Millipore). Alexa Fluor 488- / Alexa Fluor 594-labeled anti-mouse IgG (A-11017 and A-11020, respectively, Thermo Fisher Scientific) and anti-rabbit IgG (A-11070 and A-11072, respectively, Thermo Fisher Scientific) were used as secondary antibodies. Alkaline phosphatase was detected using an alkaline phosphatase substrate kit II (#SK-5200, Vector Labs).

Retinal pigment epithelial (RPE) cell induction through embryoid body-like 3D culture of iPS cells

Differentiation into RPE cells was induced as described by Takahashi's group [7] with a slight modification [1]. Dissociated clumps of iPS colonies of DMRV-patient and healthy volunteer [1] were cultured in non-adherent dishes (Prime Surface dish #MS-9035X, Sumitomo Bakelite, Tokyo, Japan) with DMEM F12 containing 20% KSR, 0.1 mM 2-ME, 1 × non-essential amino acids (11140-050, GIBCO) and 2 mM LGlutamate for 3 days, then cultured in ES differentiation medium (GMEM, 0.1 mM non-essential amino acids, 1 mM pyruvate, and 0.1 mM 2-Mercaptoethanol) containing 20% KSR for 3 days, and then in GMEM containing 15% KSR for 9 days. During this period, cells were cultured in non-adherent dishes and formed embryoid body-like 3D structures. Then, the aggregates were transferred to adherent dishes and cultured in GMEM containing 10% KSR for 6 days. Finally, CKI-7 (final 5 mM) and SB431542 (final 5 mM) were added to the medium, and the medium was changed every 2 days. RPE cells emerged approximately after three weeks of culturing.

Estimation of autophagy progression

Control and DMRV patient-derived RPE cells were cultured under nutrient-rich (10% FCS containing DMEM medium) and starving conditions (serum and amino acid-free EBSS medium) with or without CQ for 3 h. Cells were fixed and stained with anti-LC3 Ab (red) and DAPI (blue). Putative cell border was calculated using Cellomics Scan/View program. The numbers and areas of LC3 puncta were measured and calculated using the Cellomics ArrayScan VTI cell image analyzer (Thermo Fisher Scientific) and Cellomics Scan/View program (Thermo Fisher Scientific).

Statistical analysis

Data were analyzed using R software (v.3.4.3), R-studio, and additional R-packages. Student's t-test was used to analyze immunofluorescence data. Results are presented as mean ± SD. The level of statistical significance was set at $P \leq 0.05$.

Transmission electron microscopy

The cells were fixed in 2.5% 0.05 M phosphate buffer for 2 h, post-fixed in 1% osmium tetroxide/0.05 M phosphate buffer for 1 h, and then stained with uranyl acetate. After dehydration with a graded ethanol series, the specimens were embedded in Quetol 812. Semithin sections were stained with toluidine blue, and suitable areas for ultrastructural studies were chosen under a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined using a transmission electron microscope (JEM 1400, JEOL, Tokyo, Japan).

RESULTS

Mononuclear cells were separated from the blood sample of a Japanese 34-year old female patient with distal myopathy with rimmed vacuoles (DMRV), with which the patient was diagnosed by missense mutation of GNE gene of both alleles, when she was 25 years old. The cells were then activated with anti-CD3 plus anti-CD28 antibody-coated beads in the presence of rIL-2. After two days of culture, the activated cells were transduced with reprogramming factors (Oct4, Sox2, Klf4, and c-myc) via retrovirus vector. The cells were then plated on mitomycin C (MMC)-treated mouse embryonic fibroblasts (MEF) in RPMI 1640 medium containing 10% FBS, which was gradually replaced with iPS medium. Emerging iPS colonies were picked up and expanded. Established patient-derived iPS cells expressed iPS cell markers such as SSEA-4, TRA1-60, TRA1-81, and alkaline phosphatase (Fig. 1).

To induce iPS cells to differentiate into RPE cells, small clumps of iPS cells were cultured in non-adhesive culture dishes with a series of differentiation

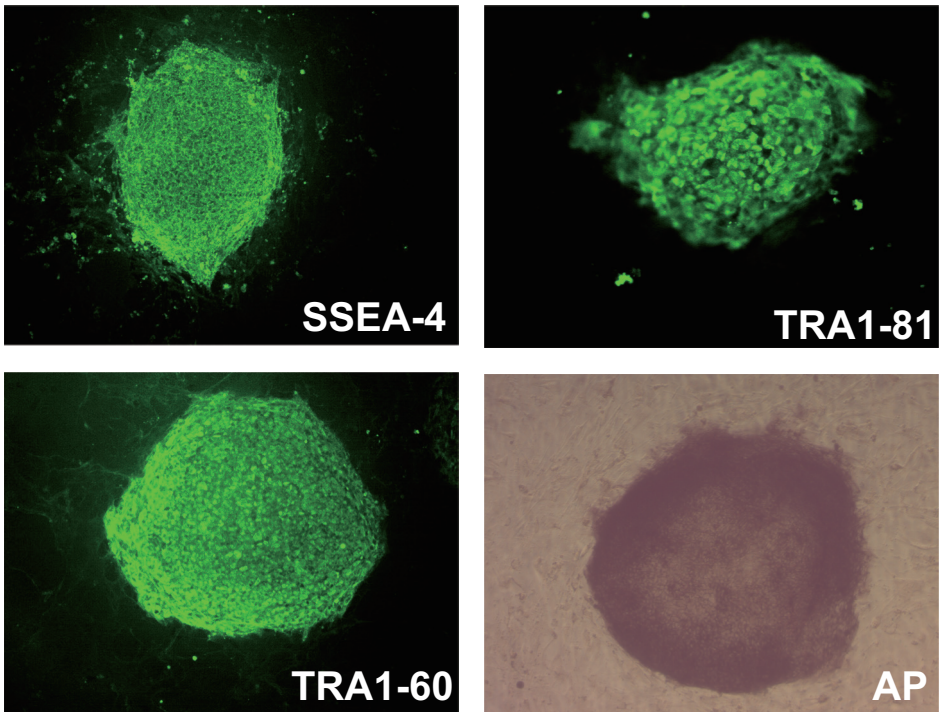

Fig. 1 Characterization of induced pluripotent stem (iPS) cells derived from a distal myopathy with rimmed vacuoles (DMRV) patient.
Patient-derived iPS cell line expressed pluripotency markers: (A) SSEA-4, (B) TRA1−81, (C) TRA1−60, and (D) alkaline phosphatase (AP) activity.

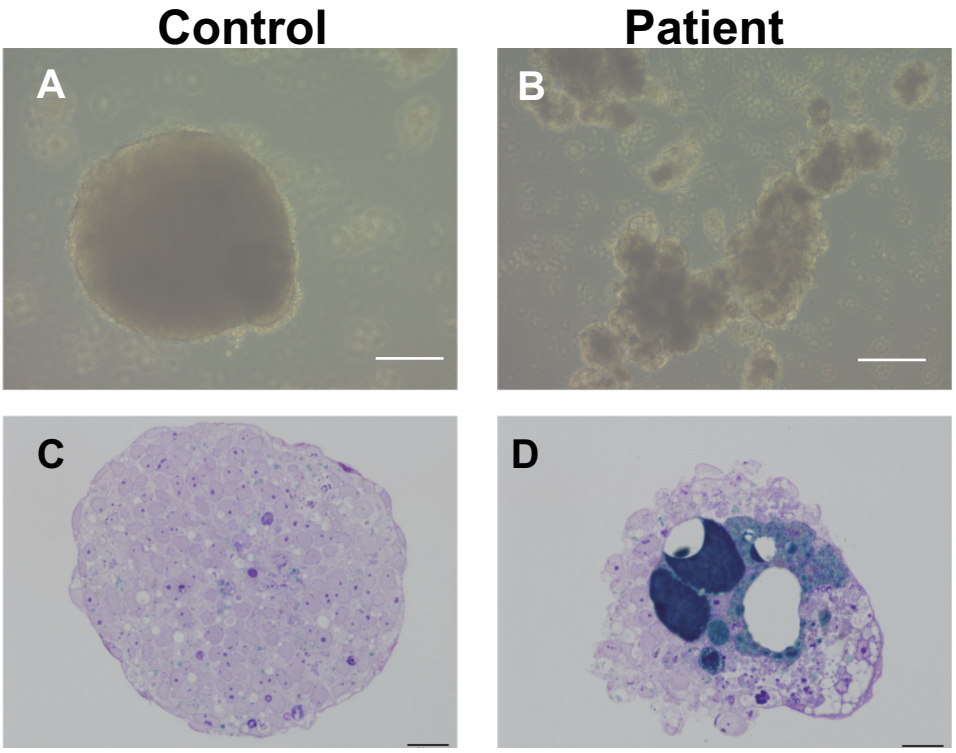

Fig. 2 Aberrant morphology of embryoid body (EB)-like structures induced from DMRV patient derived iPS cells.
Small clumps of iPS cells were cultured in non-adhesive dishes in differentiation medium for 21 days. While control iPS cells formed a tightly packed EB-like structure (A), DMRV patient-derived cells formed irregularly shaped EBs (B). Toluidine blue-stained sections showed some strongly stained portions in patient-derived EB-like cell aggregates (D), but not in control cell aggregates (C). Scale bars, 50 μm (A, B) and 20 μ m (C, D).

media. After 3 weeks of culture, aggregates of healthy volunteer-derived control iPS cells showed well-packed, round embryoid body (EB)-like structures (Fig. 2A and C). However, DMRV-patient-derived cell aggregates had less tightly packed EB-like structures of irregular shape (Fig. 2B and D). Aggregates of patient-derived

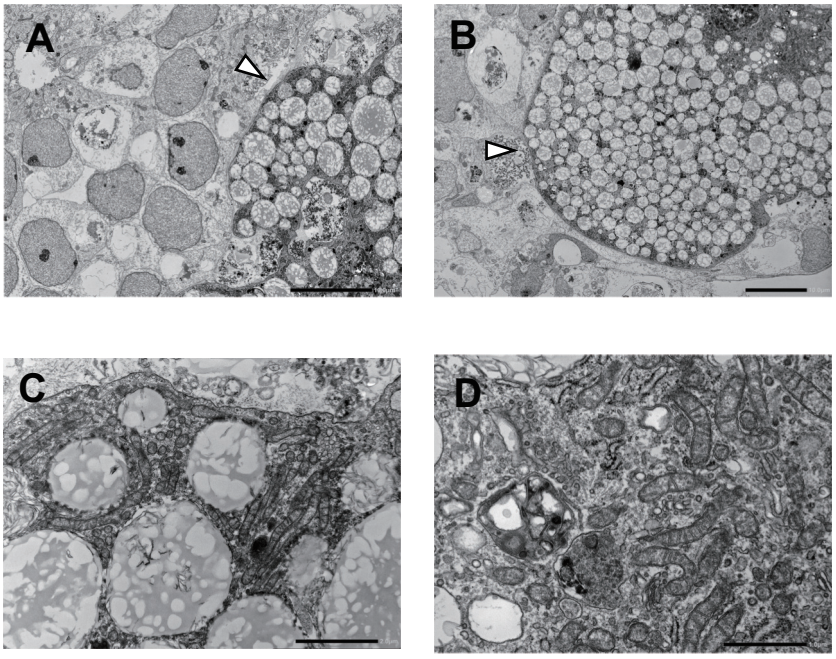

Fig. 3 Transmission electron microscopy (TEM) of DMRV patient-derived EB-like structures.
Structures strongly stained with toluidine blue (A and B, white arrowhead) included lipid droplets and mitochondria (C and D, respectively). Scale bars: 10 μm (A, B) and 2 μm (C, D).

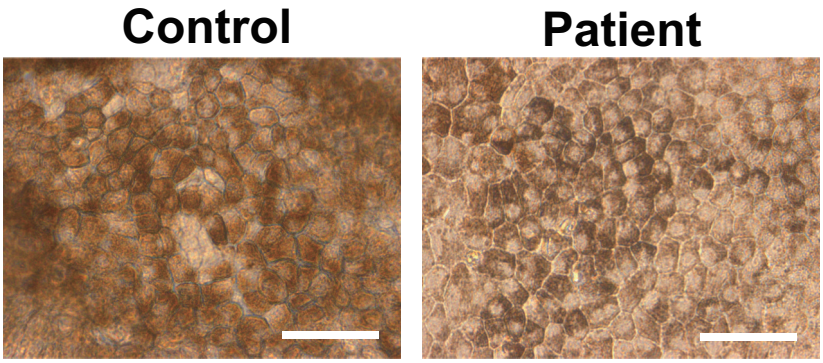

Fig. 4 Retinal pigment epithelial (RPE)-like cells induced from iPS cells.
Phase-contrast microscopy showed tightly packed monolayer cells with pigment. Scale bars 20 μm.

cells were easily dissociated by gentle pipetting due to reduced cell adhesion. This may be consistent with the notion that sialic acids are involved in cell adhesion.

Furthermore, some "balloon-like" structures frequently protruded from the surface of the aggregates of patient cells. In addition, patient-derived cell aggregates appeared spongy, containing several vacuoles. With toluidine blue dye staining, some strongly stained blue colored large bodies were detected in patient-derived cell aggregates, while only nuclei were stained purple in control cell aggregates (Fig. 2C, D). Transmission electron microscopy revealed that the body contained many lipid droplets (Fig. 3A, B) and mitochondria (Fig. 3C, D). These features of cell aggregates of the DMRV-patient-derived iPS cells were quite similar to those of sialidosis-patient-derived iPS cells, as previously reported [1]. This strongly suggests that lysosomal degradation is impaired in DMRV-patient-derived cells as well as in sialidosis-patient-derived cells.

Floating cell aggregates of control and patient-derived cells were collected and transferred to adhesive culture dishes in order to induce differentiation toward retinal pigment epithelial (RPE) cells [1, 7]. After 3 to 8 weeks of culture, squamous and polygonal cells with pigment appeared among various cell types. The cells were then excised and planted onto another culture plate. The isolated cells grew well and were maintained by serial passaging (Fig. 4). The cells exhibited epithelial sheet-like morphology and had tight junctions with pigment, very similar to RPE cells. Efficiency of RPE cell differentiation after the transfer to adhesive culture was not evidently different between control and patient-derived cells.

Considering the deformed embryoid body (EB)-like structures and electron-dense bodies filled with mitochondria and lipid droplets of DMRV patient-derived cells, which were also observed in sialidosis patient-derived cells [1], it is quite plausible that autophagy is also impaired in DMRV patient-derived RPE cells. Next, we examined the autophagy progression of DMRV patient-derived RPE cells in response to amino acid starvation (Fig. 5).

LC3 is known to be involved in autophagosome formation and has been widely used as an autophagy

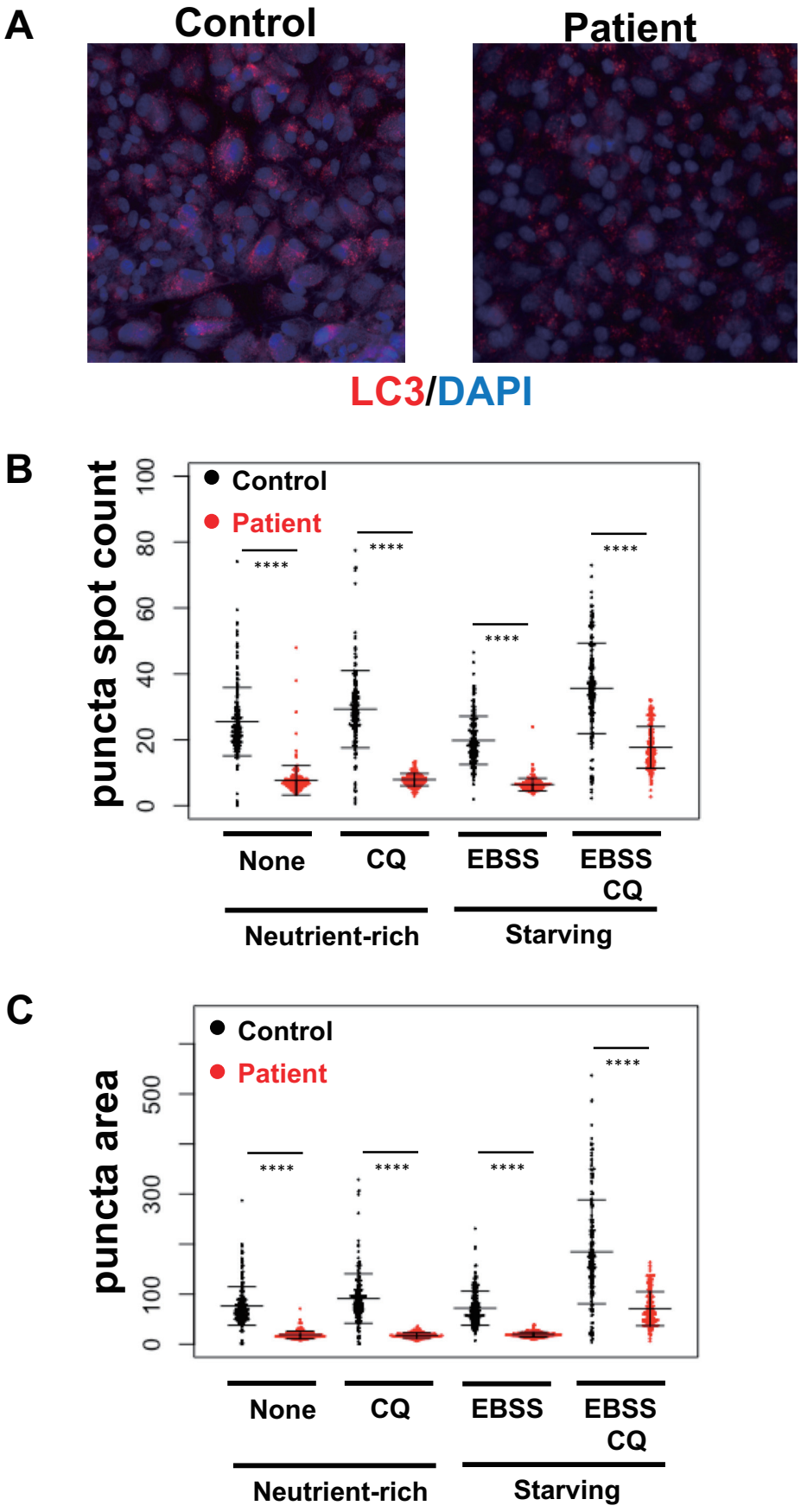

Fig. 5 Impaired autophagy in DMRV patient-derived RPE cells.
(A) Control and DMRV patient-derived RPE cells were cultured under nutrient-rich (10% FCS containing DMEM) and starving conditions (serum and amino acid-free EBSS medium) with or without CQ for 3 h. Cells were fixed and stained with anti-LC3 Ab (red) and DAPI (blue). Putative cell border was calculated using Cellomics Scan/View program, and the numbers and areas of LC3 puncta were measured by a cell image analyzer. The values of puncta spot count (B) and area (C) per cell in each visual field were calculated and plotted (n = 200). Average and standard error values are indicated as horizontal bars and error bars, respectively. Asterisks indicate a significant difference compared to control cells (****; $P < 0.0001$).

marker. At the initiation step of autophagy, LC3 is lipidated and associated with the membrane of autophagosome, resulting in "puncta" formation. Thereafter, LC3 is degraded by autophagy itself through the fusion of autophagosomes with lysosomes. Since chloroquine (CQ) inhibits lysosomal degradation by raising lysosomal pH, CQ treatment aids visualization of autophagosomes, which would be degraded during the incubation period. Thus, we can estimate autophagy progression by detecting LC3 puncta using specific antibodies. When control and DMRV-RPE cells were subjected to amino acid starvation in Earle's balanced solution (EBSS) in the presence of chloroquine (CQ), LC3 puncta were less prominent in DMRV-RPE cells than in control RPE cells (Fig. 5A), suggesting that autophagy was impaired in DMRV patient cells.

To confirm this, autophagosome formation was examined by measuring LC3 puncta using a cell imaging analyzer. The number and area of LC3 puncta per cell in each observed field was calculated, and the values from 200 fields are shown (Fig. 5B, C). Under nutrient-rich conditions, basal autophagy can be estimated. The average numbers of LC3 puncta in the presence of CQ were 28.7 and 7.7 in control and patient RPE, respectively. Under starved conditions, induced (presumably maximal) autophagy can be estimated. The average numbers of LC3 puncta in the presence of CQ were 32.6 and 16.9, in control and patient RPE, respectively (Fig. 5B). Values for the puncta area showed a similar tendency (Fig. 5C). This clearly indicates that autophagy in RPE cells induced by DMRV was impaired.

DISCUSSION

In this study, we cobserved impaired autophagy in DMRV patient-derived cells; deformed EB-like structure of iPS cell aggregates with internal bodies filled with lipid droplets and mitochondria and also iPS-derived RPE cells with reduced LC3 puncta formation. These observations seem to be consistent with the notion that myopathy is closely associated with compromised autophagy [8–10]. It is noteworthy that the phenomena were also observed in sialidosis patient-derived cells, suggesting that impairment of both sialic acid synthesis (DMRV) and cleavage (sialidosis) results in compromised autophagy. Future research should focus on how the control of sialic acid modification affects cellular homeostasis, especially autophagy.

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