



Extracellular Mitochondria Exacerbate Retinal Pigment Epithelium Degeneration in Diabetic Retinopathy

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Advancements in fundus imaging are revealing disruptions in the neurovascular unit in diabetic retinopathy (DR). In the era of anti-vascular endothelial growth factor treatment, a thorough characterization of neurodegeneration is imperative until patients with DR are sufficiently treated. Here, we demonstrate that extracellular mitochondria exacerbate retinal pigment epithelium (RPE) degeneration and inflammation in DR. Extracellular mitochondria increased in the vitreous of patients with DR and were associated with visual impairment but not with proliferative diabetic retinopathy or diabetic macular edema. Animal experiments demonstrated detrimental effects of extracellular mitochondria on RPE and photoreceptors. Lysosomal cell death induced by extracellular mitochondria in RPE cells required mitochondrial DNA but not its pattern recognition receptors. Furthermore, biochemical screening identified candidates for DNA receptors. Among them, DNA-dependent protein kinase was necessary for extracellular mitochondria-induced cell death in both in vitro and in vivo experiments. Extracellular mitochondria further induced interleukin-1 β and tumor necrosis factor- α expression in RPE cells in a Toll-like receptor 9-dependent manner. RNA sequencing suggested that extracellular mitochondria exacerbate inflammation by promoting the proliferation and migration of macrophages, at least in part. In summary, extracellular mitochondria are designated as a novel exacerbating factor of RPE degeneration in DR.

Diabetic retinopathy (DR) is now recognized as a diabetes complication with neurovascular unit impairment (1,2). This insight has led researchers to propose the concept of diabetic retinal disease, in which visual, neural, and vascular functions deteriorate in an integrated manner (3),

ARTICLE HIGHLIGHTS

- A therapeutic strategy for retinal pigment epithelium degeneration should be developed in diabetic retinopathy.
- We investigated the molecular mechanisms underpinning retinal pigment epithelium degeneration by extracellular mitochondria in diabetic retinopathy.
- Extracellular mitochondria were found in the vitreous humor of patients with diabetic retinopathy and exacerbated retinal pigment epithelium degeneration through DNA-dependent protein kinase, cytokine expression via Toll-like receptor 9, and macrophage activation.
- Extracellular mitochondria are designated as an aggravating factor of neurodegeneration and inflammation in diabetic retinopathy.

although the precedence of vascular lesions or neuronal impairments remains controversial (4). Spectral domain optical coherence tomography (SD-OCT) delineates biomarkers of neurodegeneration, including the disruption of the ellipsoid zone of photoreceptors and the disorganization of the retinal inner layers in diabetic macular edema (DME) (5,6). Loss of neurons and activated glia in retinas of animals (7) and humans (8) with diabetes have been documented in experiments. In particular, the retinal pigment epithelium (RPE) contributes to the pathogenesis in outer retinas, although the underlying mechanisms remain poorly understood (9,10).

Mitochondrial function is perturbed by hyperglycemia, resulting in oxidative stress and alterations in three biochemical pathways, which contributes to neurovascular complications (11). Surprisingly, mitochondria are released into extracellular

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spaces in various pathologic states, integrating into ischemic cardiomyocytes or neurons, thereby rescuing them (12). Conversely, extracellular mitochondria (exMT) stimulate microglia, which subsequently induce neuroinflammation (13). Despite the pathologic effects of exMT, the presence of exMT in DR remains to be elucidated.

Mitochondria contain several damage-associated molecular patterns (DAMPs) that elicit inflammation and immune responses. Unmethylated DNA particularly activates a few pattern recognition receptors, which induce cell death and inflammation (14,15). Another group of DNA-binding proteins is involved in DNA damage responses, leading to DNA repair and cell-cycle arrest. Among them, PRKDC interacts with DNA-binding proteins XRCC5 and XRCC6 and serves as an initiator of nonhomologous end joining and phosphorylation signaling (16,17).

This study, for the first time, found exMT in the vitreous humor of patients with DR and investigated how they induce RPE degeneration.

RESEARCH DESIGN AND METHODS

Details on design/methods are provided in the Supplementary Material.

Participants and Biosampling

Participants were recruited for the collection of sera, vitreous humor, and peripheral blood mononuclear cells at the Department of Ophthalmology of Kyoto University Hospital from 2015 to 2023, from 2020 to 2023, and in 2023, respectively.

Data and Resource Availability

Data are available on request.

RESULTS

exMT in the Vitreous Humor of Patients With DR

We first identified exMT in the vitreous humor of patients with DR using transmission electron microscopy (TEM) and immunoblotting (Fig. 1A, Supplementary Fig. 1A, and Supplementary Tables 1 and 2). The vitreous of patients with DR had a higher copy number of mtDNA than that of patients with diabetes without DR (Fig. 1B and Supplementary Table 3). The copy number was associated with poor vision but not with proliferative DR or DME (Fig. 1C–E and Supplementary Table 4). To investigate the effects of exMT on retinas, we performed intravitreal and subretinal administrations of exMT from DR vitreous to C57BL/6J mice. Sectional SD-OCT images showed no significant changes after intravitreal injection (data not shown). In contrast, the subretinal injection reduced intact RPE cells and the outer retinal thickness on SD-OCT images (Fig. 1F–I and Supplementary Table 5).

exMT From Cultured Cells

We next investigated whether exMT were released from 661W cells, a photoreceptor cell line. TEM, flow cytometry,

and Western blot demonstrated that pooled DME sera increased the release of exMT into the supernatants, with no significant effects on cell death (Supplementary Fig. 1B–E and Supplementary Table 6). DME sera also induced DNA fragmentation (Supplementary Fig. 1F). Sera from patients with DME and proliferative DR increased exMT release from 661W cells, compared with sera from patients with diabetes with no apparent retinopathy (Supplementary Fig. 1G and Supplementary Table 7). Notably, inhibition of the complement system reduced exMT release by DME sera (Supplementary Fig. 2). Further investigation showed that interleukin-1 β (IL-1 β) also increased exMT amounts mildly, and high glucose had no effects (Supplementary Fig. 3A and B).

Additionally, we measured the amounts of exMT from human retinal endothelial cells (HRECs) and porcine retinal pericytes. Pooled DME sera did not affect exMT release. Among cytokines, IL-8 and IL-1 β increased the copy number of mtDNA in HRECs and pericytes, respectively (Supplementary Fig. 3C–F).

exMT Induce Lysosomal Cell Death in RPE Cells

To assess the biologic effects of exMT, ARPE-19 cells were incubated with supernatants (conditioned media) from 661W cells. Conditioned media increased cell death, which was reversed by 0.22- μ m filtration. Enriched exMT induced cell death in a dose-dependent manner (Supplementary Fig. 4A–C). TEM showed cytosolic vacuoles but not nuclear deformation (Supplementary Fig. 4D). In untreated cells, acridine orange accumulated in lysosomes, emitting dot-like red signals, whereas exMT treatment caused blurred staining (Supplementary Fig. 4E). Western blot analyses revealed the leakage of cathepsin D, a lysosomal enzyme, into the cytosol and JNK phosphorylation (Supplementary Fig. 4F and G). These characteristics suggest lysosomal cell death, at least in part (18). Furthermore, exMT also induced cell death in HRECs but not in porcine retinal pericytes (Supplementary Fig. 5).

Mitochondrial DNA Is Necessary for RPE Degeneration Induced by exMT

Because DR is inflammatory and neurodegenerative, we investigated the effects of unmethylated DNA among various mitochondrial DAMPs (4,8,15,19,20). Competitive inhibition using single-strand oligoDNAs A151 and C151 mitigated exMT-induced cell death in ARPE-19 cells (Fig. 2A). After exMT from 661W cells were administered into subretinal spaces in C57BL/6J mice, RPE flatmounts and TEM revealed that exMT-induced RPE cell damage was reversed by these oligoDNAs (Fig. 2B–D). SD-OCT demonstrated reduced thickness of the outer retinal layers, and TEM showed nuclear deformation and the accumulation of extracellular fluids in the outer nuclear layer in eyes treated with exMT (Fig. 2E–G). Such detrimental effects were reversed by C151.

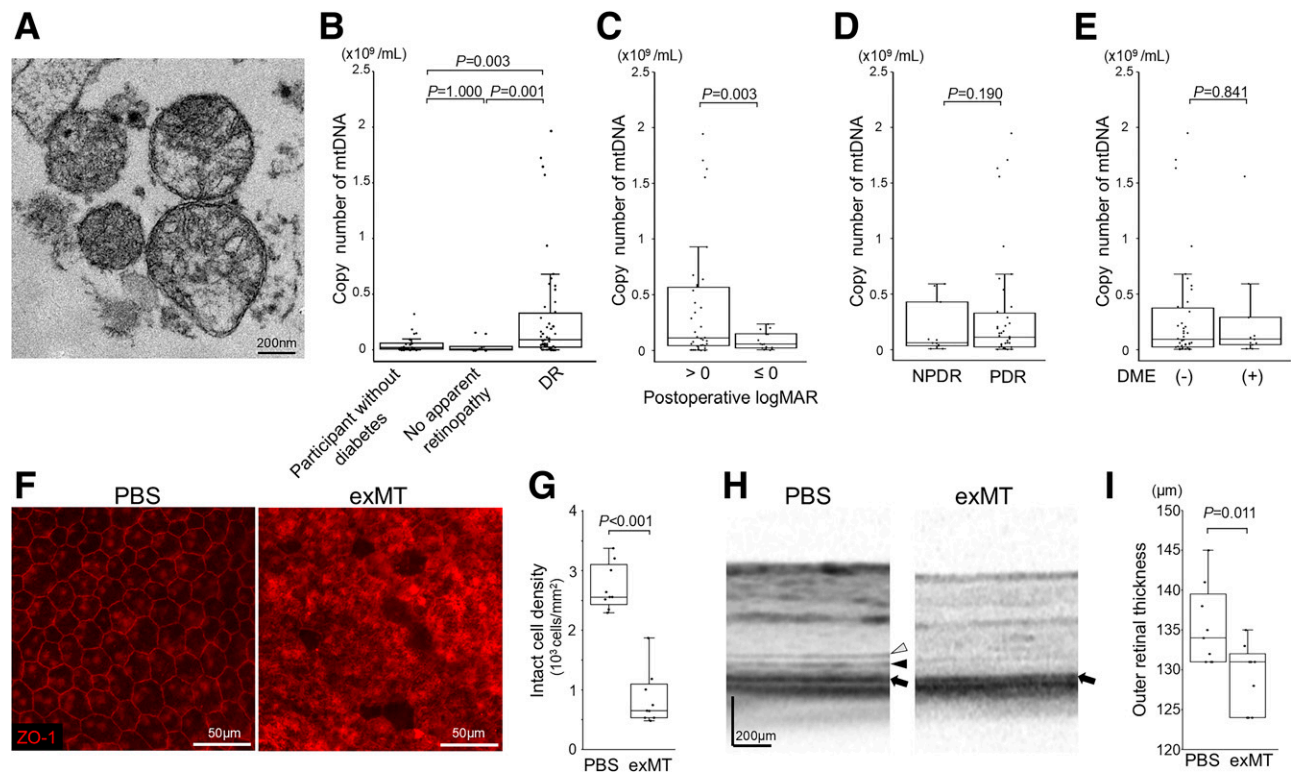


Figure 1—exMT in vitreous humor of patients with DR. **A**: TEM shows swollen exMT from vitreous humor of patient with proliferative DR (PDR). **B**: Increased copy number of mtDNA in vitreous humor of patients with DR compared with participants without diabetes. **C**: Eyes with good vision (logarithm of minimum angle of resolution visual acuity [logMAR VA] ≤ 0) after vitrectomy surgery have lower copy number than eyes with poor vision (logMAR VA > 0). **D**: No significant differences in copy number between eyes with PDR and those with nonproliferative DR (NPDR). **E**: Copy number in eyes with DME was not different from that in eyes without DME. **F** and **G**: Representative immunofluorescent images demonstrate catastrophic damage (e.g., absence, deformation, and loss of intercellular junction [ZO-1 staining]) in RPE cells of C57BL/6J mice injected with pooled exMT derived from nine patients with DR. Quantification reveals significant reduction in intact RPE cell density under exMT treatment. **H** and **I**: Representative SD-OCT images demonstrate ellipsoid zone (EZ) loss in retinas treated with exMT. Gray arrowhead indicates external limiting membrane. Black arrowhead indicates EZ. Arrow indicates RPE. Measurements demonstrate thinning of outer retinal layers in retinas injected with exMT.

Additionally, the intracellular administration of DNA (purified from 661W cells) using lipofectamine induced cell death, compared with no effects of DNA by itself (Supplementary Fig. 6A). Particularly, mtDNA increased cell death more than nuclear DNA (Supplementary Fig. 6B). Inhibitors of three major pathways in DNA-stimulated cell death had no discernible effects on exMT-induced cell death, suggesting the involvement of other DNA receptors (Supplementary Fig. 6C) (15).

Identification of DNA-PK as a Regulator of Cell Death

Biochemical screening identified several proteins involved in DNA damage responses as candidates for DNA-binding proteins (Fig. 3A, Supplementary Fig. 7A, and Supplementary Table 8). Notably, we focused on XRCC5 and XRCC6, which collaboratively bind DNA, recruit PRKDC, and form DNA-dependent protein kinase (DNA-PK) (Fig. 3B) (16,17). exMT induced PRKDC phosphorylation (Fig. 3C and Supplementary Fig. 7B). PRKDC inhibitors Nu7441 and Nedisertib and knockdown of DNA-PK components partially mitigated exMT-induced cell death in ARPE-19 cells (Fig. 3D and E

and Supplementary Fig. 7C–E). Furthermore, such inhibition also reduced the cathepsin D leakage into the cytosol and JNK phosphorylation (Fig. 3F and G and Supplementary Fig. 8). These findings suggest that lysosomal cell death partially depends on PRKDC in ARPE-19 cells (18).

Animal experiments confirmed that simultaneous injection of exMT from 661W cells and Nu7441 restored intact cell density in RPE flatmounts and reversed lysosomal cell death in C57BL/6J mice (Supplementary Fig. 9A–C). OCT measurements demonstrated that Nu7441 reversed exMT-induced thinning in the outer retinal layers (Supplementary Fig. 9D–F). PRKDC gene ablation in mice prevented the reduction in intact RPE cell density and outer retinal thicknesses caused by exMT (Supplementary Fig. 9G–J). Translational studies affirmed that C151 and Nu7441 inhibited this damage by exMT from the DR vitreous (Supplementary Fig. 10).

Additionally, we explored the gene expression levels in ARPE-19 cells and found that exMT increased mRNA levels of IL-1 β and tumor necrosis factor- α but not interferon- β 1. This gene expression was reduced by an inhibitor and knockdown of Toll-like receptor 9 (TLR9) (Supplementary Fig. 11).

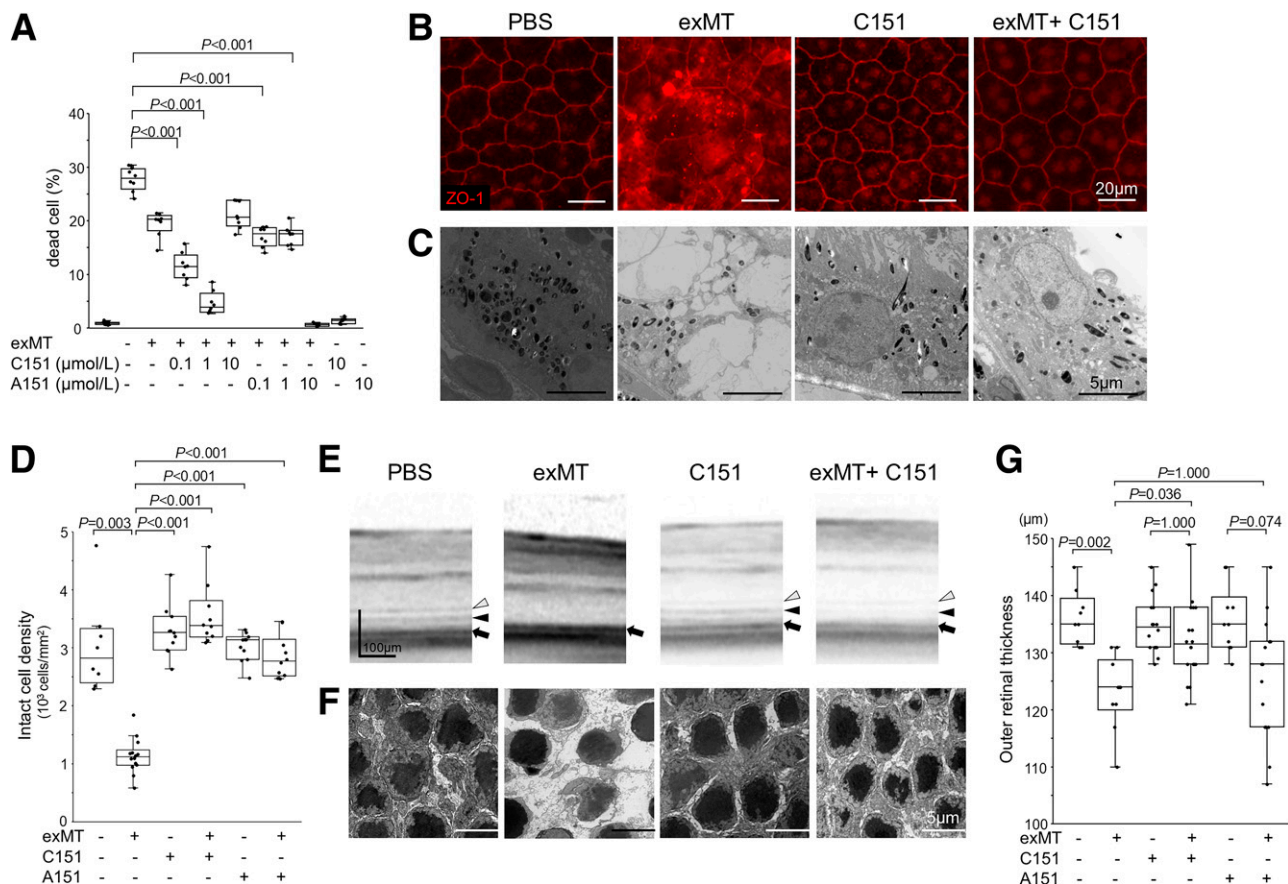


Figure 2—RPE cell death is mediated via DNA. **A**: exMT are collected from 661W cells treated with pooled sera from 56 patients with DME. LIVE/DEAD assay reveals that single-strand oligoDNA fragments, C151 and A151, reduce exMT-induced cell death in ARPE-19 cells. **B** and **D**: exMT are administered into subretinal spaces of C57BL/6 J mice, followed by immunostaining with ZO-1. **B**: Representative images. Intact RPE cell density in C57BL/6J mice decreases under exMT treatment, whereas simultaneous administration with exMT and C151 (or A151) reverses this effect. **C**: TEM confirms that RPE cells have large vacuoles within cytosol in eyes treated with exMT, whereas C151 decreases them. **E** and **G**: SD-OCT shows that outer retinal thicknesses decrease, and ellipsoid zone (EZ) line disappears in mice administered with exMT from 661W cells. These changes are reversed under simultaneous injection with exMT and C151. **E**: Representative SD-OCT images. Gray arrowhead indicates external limiting membrane. Black arrowhead indicates EZ. Arrow indicates RPE. **F**: TEM shows that extracellular fluid accumulates, and nuclei in some photoreceptor cells are fragmented in outer nuclear layer of mice treated with exMT. These changes do not occur in mice treated with exMT and C151.

Macrophage Stimulation by exMT

Because phagocytes play a pivotal role in DR pathogenesis, we conducted RNA sequencing in macrophages stimulated with exMT from the DR vitreous (19,20). Hierarchic clustering of 1,000 genes with high variation showed that gene expression patterns were primarily influenced by the source of the macrophages (Fig. 4A and Supplementary Fig. 12). MA plots identified 26 upregulated and 25 downregulated genes, visualized in heat maps (Fig. 4B and C). Pathway analysis and Kyoto Encyclopedia of Genes and Genomes graph revealed upregulated expression levels in genes associated with cellular proliferation (Fig. 4D and Supplementary Fig. 13). Overrepresentation analysis indicated increased expression in genes related to immunity, inflammatory responses, and migration (Fig. 4E). These results suggest that exMT promote the proliferation and migration of macrophages and inflammation.

Hyperreflective foci in SD-OCT images generally correspond to lipid-laden macrophages (20). Clinical investigation

showed that eyes with hyperreflective foci in central 1-mm areas had higher exMT amounts than those without such foci (Supplementary Fig. 14A). Similarly, mtDNA copy number was significantly higher in eyes with foci in outer retinal layers, which are associated with vision (Supplementary Fig. 14B) (21). Conditioned media from exMT-treated macrophages promoted cell death in ARPE-19 cells (Supplementary Fig. 14C). These results suggest that exMT induce RPE degeneration partially mediated by macrophage stimulation.

DISCUSSION

In this study, we demonstrated for the first time the presence of exMT in the vitreous humor of patients with DR. exMT induced RPE cell death both directly and indirectly through macrophage stimulation. In vitro and in vivo experiments revealed that exMT-induced cell death and cytokine expression in RPE depended on DNA-PK and TLR9, respectively. exMT also induced cell death in HRECs. RNA

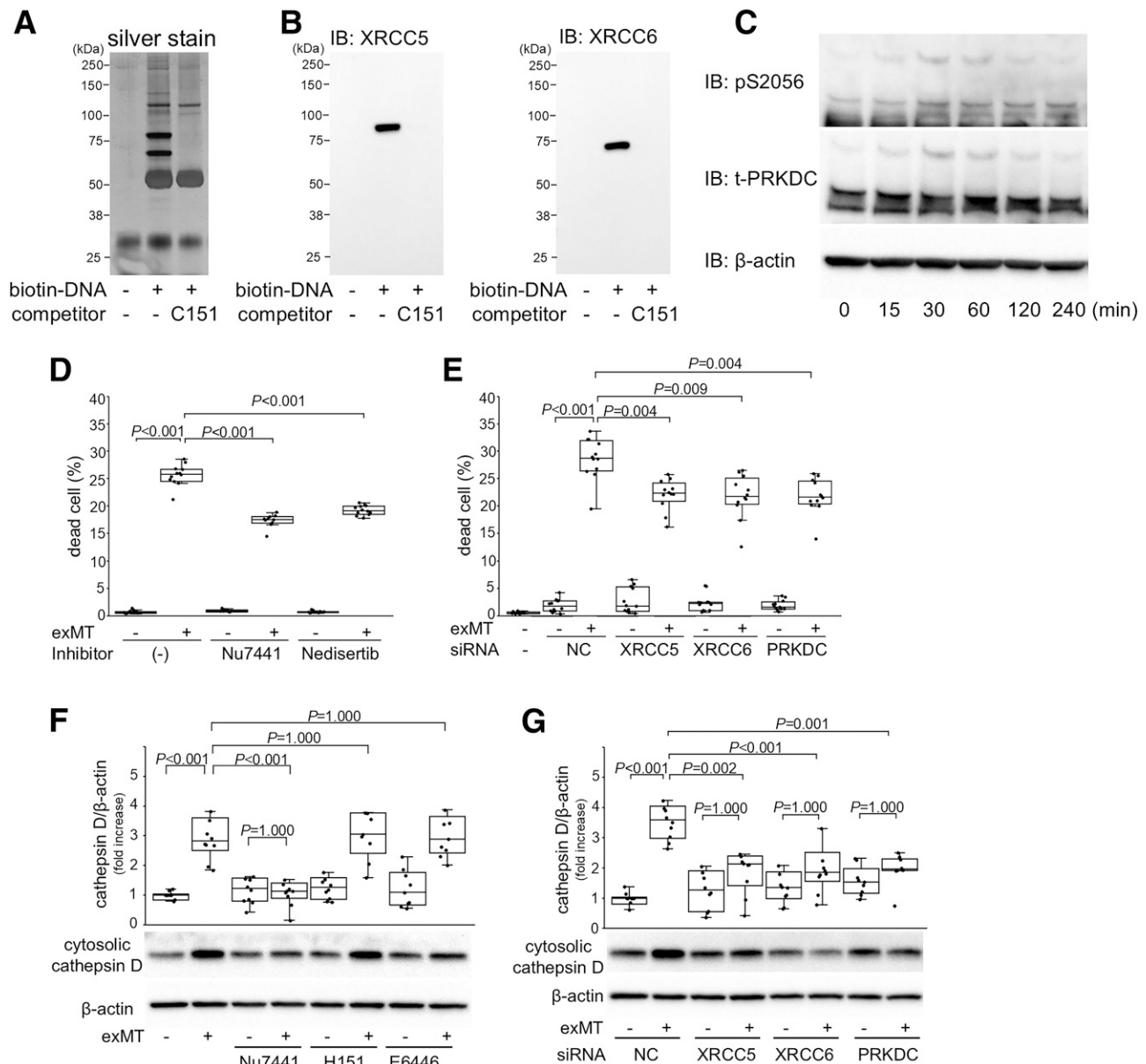


Figure 3—DNA-PK is necessary for RPE cell death at least in part. **A**: After pulling down DNA-binding proteins using biotin-labeled PCR products, they are separated by SDS-PAGE and visualized by silver staining. **B**: Western blot of DNA-binding proteins with anti-XRCC5 and anti-XRCC6 antibodies. **C**: Representative blot of PRKDC phosphorylation at S2056 (pS2056) by exMT (from 661W cells) in a time-dependent manner. **D**: LIVE/DEAD assay shows that PRKDC inhibitors (Nu7441 and nedisertib) reduce cell death in ARPE-19 cells treated with exMT from 661W cells. **E**: Cell death is reduced by knockdown of XRCC5, XRCC6, and PRKDC. **F**: ARPE-19 cells are treated with exMT from 661W cells. Western blot shows that Nu7441 but not H151 or E6446 decreases cathepsin D leakage into cytosol. **G**: Knockdown of XRCC5, XRCC6, and PRKDC in ARPE-19 cells also reverses increase in cytosolic cathepsin D induced by exMT from 661W cells. NC, negative control (scramble siRNA); t-PRKDC, total PRKDC.

sequencing showed that exMT increased the expression of gene sets regarding cell-cycle progression and migration, potentially promoting inflammatory responses. These results demonstrate just a few pathologic aspects of exMT regarding the neurovascular unit in diabetic retinal disease.

Recently, antimitochondrial antibodies were identified in the sera of patients with DME, and extracellular vesicles were seen to elicit immunologic responses (22,23). We therefore searched for and found exMT in the vitreous of

patients with DR. We hypothesized that exMT are released from photoreceptor cells at least partly, because mitochondria are densely accumulated in the inner segment of photoreceptors. We showed that DME sera-induced exMT depend on complement activation, but the mechanisms underpinning exMT release and mtDNA fragmentation remain to be elucidated (24). Cytokines also modestly increased exMT release from vascular cells, and additional studies should explore the sources of exMT comprehensively.

that secretory factors from RPE cells and macrophages contribute to photoreceptor damage.

This translational research revealed that exMT induce RPE degeneration in DR through the mtDNA/DNA-PK axis and indirect effects by macrophage activation.

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