

All primate experiments were approved by the Animal Ethics Committee of the Tsukuba Primate Research Center and were conducted in accordance with the The Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Eight eyes from eight normal female Cynomolgus monkeys (*Macaca fascicularis*) whose ages ranged between 13- to 19 years were studied. Eyes were removed approximately one hour after death and treated with RNAlater (Applied Biosystems, Tokyo, Japan). Then a 3 mm diameter macular and peripheral retina containing the RPE and choroidal layer were punched out and frozen until use. The proteins extracted from the tissues of eight eyes were pooled for analyses.

#### *Protein extraction and 2D-gel electrophoresis*

The proteins from the macula and peripheral retina were extracted after homogenization and sonication in sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris, 0.2% Bio-Lyte 3/10 (Bio-Rad Hercules, CA)). After centrifugation for 15 min x 3 at 14,000 rpm (20,800 x g), the lysate was precipitated using Ready Prep 2D cleanup kit (Bio-Rad) and redissolved in sample buffer. The supernatant was collected. The protein concentrations were determined with the RC-DC protein assay kit (Bio-Rad) according to the manufacturer's instruction. Protein samples (300  $\mu$ g) were separated by IEF using 17

cm IPG strips. After 12 to 16 hours of rehydration at 20° C, IEF was used for the first dimension with an initial voltage of 250 V for 15 min and then increased to 10,000 V for 3 hours and held until 60,000 volt-hours was reached. Immediately after the IEF, the IPG strips were stored at -20° C until the equilibration step was carried out. The IPG strips were equilibrated for 20 min in buffer containing 6 M urea, 2% SDS, 0.375 M Tris (pH 8.8), and 20% glycerol under reduced conditions with 2% DTT, followed by another incubation for 10 min in the same buffer under alkylating conditions with 2.5% iodoacetamide. Equilibrated IPG strips were electrophoresed for the second-dimension using 12% acrylamide gel. 2D-gel electrophoresis was performed at four different pH ranges, viz., pH 3-10, pH 4-7, pH 5-8 and pH7-10. After 2D-gel electrophoresis, the proteins were stained with SYPRO Ruby (Bio-Rad). The images of the macular and peripheral retina were compared with ImageMaster 2D Platinum ver.5.0 (GE Healthcare Bio-Sciences, Piscataway NJ) followed by a visual inspection. Then, the gel spots, as shown in Figure 1-4 (arrows) were excised. Butt et al. have described the difficulties of IEF separation by RNAlater because of contaminations (28). Our samples were desalted prior to the IEF separation by Ready Prep 2D cleanup kit (Bio-Rad) using TCA acetone precipitation which resulted in successful separations.

### *In gel digestion and LC-MS/MS analyses*

Each gel piece was cut to approximately one cubic millimeter and washed twice with 50 mM ammonium bicarbonate/50% acetonitrile. After destaining, the gel pieces were rinsed with distilled water, and incubated with acetonitrile for 20 min. The supernatant was discarded and the gel pieces were completely dried before incubation with 10 mM DTT in 100 mM ammonium bicarbonate for 45 min at 56° C. The supernatant was discarded and the pieces were incubated in the dark with 55 mM iodoacetamide in 100 mM ammonium bicarbonate (30 min, at room temperature). The supernatant was discarded, and the gels were washed three times. Finally, the gel pieces were completely dried before tryptic digestion in a solution of sequencing grade modified trypsin (12.5 ng/ul; Promega, Madison, WI) in 50 mM ammonium bicarbonate. The digestion was performed at 37° C overnight, and the extraction step was performed once with 25 mM ammonium bicarbonate, twice with 5% formic acid, and finally with distilled water. The extracted peptides were pooled and dried. After re-suspending in 40 µl of aqueous 0.01% trifluoroacetic acid/2% acetonitrile, the samples were analyzed by LC-MS/MS.

LC-MS/MS was performed with a combined system of Paradigm system (Michrom BioResources, Auburn, CA) and an ESI mass spectrometer (LCQ Deca XP plus or Finnigan LTQ; Thermo Fisher Scientific, Yokohama, Japan; assembled by AMR Inc.,

Tokyo, Japan). For the LCQ analysis, sample peptides were separated on nano column (AMR Inc.) with solvent A (2% acetonitrile/0.1% formic acid) and B (90% acetonitrile/0.1 % formic acid) at a flow rate of 0.6  $\mu$ l/min, gradient of 5 to 95% solvent B over 40 min. For the LTQ analysis, peptides were separated on Magic C18 (Michrom BioResources) with solvent A and B, a flow rate 1.5  $\mu$ l/min, gradient of 5 to 95% solvent B over 30 min. The identification of the proteins from the MS/MS spectra was performed using protein identification software (Bioworks ver.3.1, Thermo Fisher Scientific) and UniProtKB/Swiss-Prot database (Release 48.8) which was preliminarily extracted by the species "human" (13361 entries). Peak list generation and database search were performed with the following parameters; mass tolerance for precursor ions, 2.5 amu; mass tolerance for fragment ions, 0.00 amu; enzymatic cleavage position before lysine or arginine; number of missed cleavage sites permitted 2; fixed modification, carbamide-methylation (+57.02Da) for cysteine; variable modification, oxidation (+16Da) for methionine. The peptide sequences were filtered by peptide Cross Correlation (XC) score and delta Cn score. The threshold level of delta Cn score were >0.1 for peptide sequences from both measurement devices. The threshold level of XC score for each charge (+1/+2/+3) were >1.5/2.0/3.7 for LCQ and >1.9/2.2/3.7 for LTQ. Then correlation between observed in gel images and theoretical molecular weight and pl were

considered. When peptides matched multiple members of the protein family, the protein which had the most number of peptides matched to the amino acid sequence was selected. In case of equal numbers, both proteins were listed.

#### *Western blotting and immunohistochemical analysis of macula*

Five to fifteen micrograms of macula or peripheral retina homogenates were diluted in double volume of SDS buffer and separated by either 1D or 2D-PAGE followed by PVDF membrane transfer. Membranes were blocked with a Blocking Solution Concentrate (KPL, Gaithersburg, MD) and probed with one of the following primary antibodies (Abs): chicken Ab to human arrestin-C (GenWay Biotech, San Diego, CA), rabbit Ab to human Mn- superoxide dismutase (Mn-SOD) (upstate, Temecula, CA), rabbit Ab to human synuclein gamma (Novus Biologicals, Littleton, CO), rat Ab to human epidermal fatty acid binding protein (E-FABP) (R&D Systems, Inc. Minneapolis, MN), rabbit Ab to tropomyosin Br-1, Br-3 (CHEMICON International, Temecula, CA), mouse Ab to chicken tropomyosin TM311 (Abcam, Cambridge, UK), goat Ab to human heterogeneous nuclear ribonucleoproteins (hnRNPs) A2/B1 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse Ab to human hnRNPs C1/C2 (abcam). The specific signals were detected with one of the following secondary antibodies: goat Ab to mouse IgG horseradish peroxidase

(HRP) conjugated (Jackson ImmunoResearch Laboratories, West Grove, PA), goat Ab to rabbit IgG HRP conjugated (Pierce, Rockford, IL), rabbit Ab to chicken/turkey IgG HRP conjugated (Zymed Laboratories, South San Francisco, CA), donkey Ab to goat IgG HRP conjugated (Jackson ImmunoResearch Laboratories), goat Ab to rat IgG HRP conjugated (Zymed Laboratories). The signals were made visible by chemiluminescence reactions and examined with a chemiluminescence imager (Lumi-Imager F1; Roche Diagnostics, Tokyo, Japan). The detection of Mn-SOD by 2D western blotting of macular or peripheral retina samples was made by 2D gel electrophoresis. Thirty micrograms of protein from each sample were separated using 7cm IPG strip at pH 3 to 10. The second dimensional separation was made on 12.5% polyacrylamide gel and then transferred to PVDF membranes. For quantitative comparisons of the band intensities of the western blots, the blots were scanned and measured by Lumi Analyst ver.3.1 (Roche Diagnostics).

*Protein detection followed 1D western blotting*

Enucleated eye from normal female cynomolgus monkey (age 13 yrs) were fixed in 10% neutralized and buffered formaldehyde solution at 4° C overnight and then dehydrated. The specimens were embedded in paraffin and serial sectioned at 4  $\mu$ m thickness. The

specimens were treated for antigen retrieval by autoclaving in Target Retrieval Solution (Dako, Carpinteria, CA) for 20 min at 121° C. The sections were then blocked with Dako protein block (Dako) or skim milk or BSA in PBS. The primary antibodies were the same as used for the western blotting. For signal detection after rinsing off the primary antibodies, the sections were incubated with one of following secondary antibodies: Alexa 488 conjugated goat Ab mouse IgG (invitrogen, Tokyo, Japan), Alexa 568 conjugated goat anti-rabbit IgG (invitrogen), Alexa 488 conjugated donkey anti-goat IgG (invitrogen), Alexa 568 conjugated goat anti-chicken IgG (invitrogen), Alexa 488 conjugated goat anti-rat IgG (invitrogen). After rinsing off the antibodies, the sections were examined by confocal laser scanning microscope (Radiance 2100, Bio-Rad). The cell nuclei were stained with DAPI (4',6-diamino-2-phenylindole). To determine the location of the signals, one of sections was stained with hematoxylin and eosin.

## **Results**

### *Identification of macula enriched proteins*

Approximately 700 spots were detected in the macular and peripheral retinal tissues by 2D-gel (pH range 3-10; Fig. 1). Sixty percent of these spots were found in both

samples. The gel spots indicating high expression of proteins are listed in Table 1. Forty eight known proteins from earlier proteomics studies of the retina were identified (12-14, 29, 30). Fifteen spots were specific to the macula. The neurofilament triplet L protein identified in this study has not been previously reported by proteomic approach but by previous SAGE analysis (6). To obtain a further separation, the pH range of the 2D-gel electrophoresis was narrowed to three ranges; 4-7, 5-8, and 7-10 (Fig. 2-4). Under these conditions, 40 spots specific to macula were further detected and 26 proteins were identified (Table 2). To obtain the relative expression levels of the identified proteins, western blotting analysis was performed.

#### *Quantification of macula enriched proteins*

Western blotting was performed on the following seven proteins identified by mass spectrometry; arrestin-C, Mn-SOD,  $\gamma$ -synuclein, E-FABP, tropomyosin1a chain, hnRNPs A2/B1, and hnRNPs C1/C. Arrestin-C showed a 1.5-fold higher expression in the macula than the peripheral retina (Fig. 5-1). Arrestin-C has been previously identified as a cone photoreceptor specific protein (31), and thus served as a positive control for this study. Mn-SOD, an anti oxidative enzyme located in mitochondria (32), showed equal expression in the macular and peripheral retina (Fig. 5-2). For Mn-SOD, 2D-gel western



blotting was performed to observe the distribution of spots. Figure 6 showed an additional spot in the acidic position (arrow) on the macula membrane compared with peripheral retina.  $\gamma$ -synuclein, a protein known to be up-regulated in cancer cells (33), showed 2.9-fold higher expression in the macula. E-FABP, a reactive lipid scavenger (34), showed 1.9-fold higher expression in the macula. Tropomyosin1a chain Br-1 and Br-3 showed 1.8-fold higher expression in the macula than in the peripheral retina, while tropomyosin1a chain TM311 showed 8-fold higher expression in the macular (Fig. 5-5 & 5-6). Tropomyosin1a chain has been reported in many isoforms by alternative splicing (35). MS/MS data from spot M4 (Fig.1) identified peptide sequence CAELEELK (Table 2), which corresponded to isoform 1 (skeletal muscle type) or isoform 5 (brain type, TMBR-3) of tropomyosin1a chain in the UniProtKB/Swiss-Prot database.

Based on these data, western blotting was performed using two antibodies for tropomyosin1a chain isoform 1 and 5. The anti-tropomyosin antibody TM311 detects 19 amino acids in exon 1a of tropomyosin gene family in mammalian tissues, viz., alpha-, beta-, gamma-, delta- tropomyosin, including the skeletal muscle type but it does not detect brain type (TMBR-3). The other antibodies used were specific to the brain isoforms TMBR-1 and TMBR-3. hnRNPs A2/B1 had 1.5-fold higher expression in the macula, while hnRNPs C1/C2, had 2.6-fold higher expression in the macula than peripheral retina (Fig.

5-7 & 5-8). hnRNPs A2/B1 is known to be up-regulated in carcinoma cells (36) and hnRNPs C1/C2 for amino acid modification by oxidative stress (37). hnRNPs A2/B1 and hnRNPs C1/C2 products are known as alternative splicing variants. A unique amino acid sequence of hnRNP B1 isoform was identified from spot M37 and M38 but other hnRNPs A2/B1 and all hnRNPs C1/C2 spots contained only common amino acid sequences of each variant.

#### *Tissue localization of macula enriched proteins*

To determine the location of the 7 proteins in the macula, immunohistochemistry was performed using antibody against each protein (Fig. 7). Arrestin-C was detected in photoreceptor outer segments and outer plexiform layer (Fig. 7B) as reported (31).  $\gamma$ -synuclein was located in the retinal ganglion cells and the nerve fiber layer. E-FABP was detected throughout the sensory retina except the photoreceptors and RPE cells (Fig. 7E). Tropomyosin Br-1 and Br-3 were located in the photoreceptor inner segments and outer plexiform layer (Fig. 7F), while TM311 was detected in the choroidal layer (Fig. 7G). hnRNPs A2/B1 and hnRNPs C1/C2 were located in the nucleus of a number of retinal cells including the cells in the retinal ganglion cell layer, the inner nuclear layer, the outer nuclear layer, and the RPE layer. However, a difference in the signal intensity was

observed between hnRNPs A2/B1 and hnRNPs C1/C2.

## Discussion

Comparison of the different protein levels in the macula and peripheral retina was determined by proteomic analyses, and the results showed a number of quantitatively abundant proteins in the primate macula. One of the proteins was arrestin-C known to be specifically expressed in cone photoreceptors, a cell type highly concentrated in the macula (4). The identification of arrestin-C indicated that this proteomic approach was methodologically effective for the identification of macula enriched proteins. Another example is 3'(2'),5'-bisphosphate nucleotidase 1 previously identified by SAGE study was reported to be highly expressed in cone photoreceptors (7), and our results identified it as another macular enriched protein. Guanylate kinase (Table 2, M3), which catalyzes the phosphorylation of GMP(38) and is essential for recycling cGMP in the photoreceptors (39), was also identified as one of the macular enriched protein.

$\gamma$ -synuclein, a member of the synuclein family, was identified as a macular specific spot in the 2D-gel. Previous SAGE study also showed that  $\gamma$ -synuclein was highly expressed in the macula (6). Immunostaining showed that this protein was predominantly

located in the retinal ganglion cells and nerve fiber layer (Fig. 7D), which is also consistent with a previous study (40). These findings are in agreement with the high concentration of retinal ganglion cells in the macular area.  $\gamma$ -synuclein was initially identified as a breast cancer specific gene 1 by high throughput differential cDNA sequencing (33). This protein was expressed in peripheral nervous system, brain (41), and in ocular tissues (40).  $\gamma$ -Synuclein knockout mice are viable, fertile, with no abnormal behavior compared to wild type mice (42) with morphologically normal retina (43). The cellular function of  $\gamma$ -synuclein is still unknown, but an over-expression of synoretin, a bovine ortholog of  $\gamma$ -synuclein, activates the Elk-1 signal transduction pathway (44) and the chaperonic activity (43). Snyder et al. hypothesized that  $\gamma$ -synuclein is the regulator of 20S proteasome degradation and has a role in cellular response to oxidative stress because 20S ubiquitin-independent proteasomal pathway is responsible for the degradation of 70-80% of all mildly oxidized proteins (45). In the macula,  $\gamma$ -synuclein is likely to play an important role in the degradation of oxidized proteins.

Six macula specific mitochondria proteins were identified in this study. Mitochondria have been recognized as one of the major cellular generators of reactive oxygen species through electron transport chain reactions (55). Oxidative stress is believed to be important in the aging process and pathogenesis of diseases including AMD (56).

Photoreceptors densely localized in the macula have higher concentration of mitochondria with 2 to 3-fold greater oxygen consumption compared to the inner retina (46) and the distribution and morphological differences of mitochondria in the different regions of the retina have been reported (47). The macula may due to the higher concentration of cones exposed to more oxidative stress than peripheral retina.

Retinal abnormality of the Cu, Zn-SOD knockout mice (57) and Mn-SOD knockdown mice (58) mimic to patients with AMD. A higher demand of ATP production in cones than in rods has been suggested by the analysis of mitochondrial crista structure (59). An accumulation of 4977 bp deleted mitochondrial DNA (mtDNA<sup>4977</sup>), which is mainly caused by free radicals generated from mitochondria in the inner segments, has been shown to increase with age, and at higher concentration in the macula than in the peripheral retina (60). In our results, additional horizontal spots for Mn-SOD were observed in the 2D-western blot (Fig. 6, arrow), indicating a posttranslational modification specific to the macula. The phosphorylation of Mn-SOD by calcium ion has been demonstrated to inactivate enzyme activity (61). The activity of Mn-SOD is essential for the survival (62) and protection of the retina from oxidative stress (63). The presence of numerous phosphorylated forms of Mn-SOD in different region of the retina may explain the preferentially impairment of the macula under high oxidative stress levels.

Addition to these observation, the identified voltage-dependent anion-selective channel protein 1 (VDAC1) is located in the outer membrane of mitochondria (48, 49) and involved in cell volume regulation and apoptosis (X). Age-related maculopathy susceptibility 2 (*ARMS2/LOC387715*), a functionally unknown susceptibility gene for AMD (50-52), encodes a protein which is also localized to the outer membrane of the mitochondria (53, 54). Further functional study of VDAC1 and ARMS2 in correlation with AMD is notable.

E-FABP also plays a role as an antioxidant. 4-hydroxynonenal (4-HNE) is one of the aldehydes produced from lipid peroxidation. E-FABP is a molecular target for 4-HNE modification and functions as an antioxidant protein by scavenging reactive lipids through covalent modification of Cys-120 (34). 4-HNE modified E-FABP has been detected in the rat retina (64) and even higher expression levels in the primate macula to cope with the higher oxidative stress than the peripheral retina. In a transcriptional comparison study between macular and peripheral RPE, the expression of E-FABP was higher in the peripheral than macular RPE (9). Our immunostaining showed that E-FABP was predominantly located in the neural retina except the photoreceptors (Fig. 7E) which is consistent with the results of Kingma et al. (65). Our observation of a higher E-FABP expression in the macula is probably from the neural retina and not the RPE.

Four macula enriched hnRNPs were identified. hnRNPs are RNA-binding proteins predominantly located in the nucleus and function in splicing, transporting, and stabilizing mRNAs (66, 67). hnRNPs A2/B1 and hnRNPs C1/C2 were confirmed to be expressed at significantly higher levels in the macula by western blotting (Fig. 5G and 5H). These proteins were identified from multiple horizontal spots in the same gel indicating the possibility of phosphorylation. hnRNPs C1/C2 has been observed in several posttranslational phosphorylated forms and these forms are dependent on the duration of the oxidative stress (37). It also modulates the affinity of the mRNA binding to hnRNPs C1/C2 (68). The regional specificity of phosphorylation in this protein may affect the pathological process of macula-related diseases. For example, it has been reported that this protein stabilizes the mRNA of amyloid precursor protein (APP) and enhances translation of APP (69). APP and Amyloid beta (A $\beta$ ) are found in drusen from AMD patients (X). Enrichment and posttranslational modification of hnRNPs C1/C2 may associate with A $\beta$  accumulation. In addition to the hnRNPs C1/C2 phosphorylation, hnRNPs A2/B1 phosphorylation is involved in modifying its molecular function. In the central nerve system, phosphorylation of hnRNPs A2/B1 has been considered to be essential for myelination at the axon-glia contact site by mRNA transportation (70).

Tropomyosin 1 a chain belongs to a multi-gene family of actin-binding proteins and 5

isoforms are listed in UniProtKB/Swiss-Prot database. In mammals, tropomyosin can give rise to many isoforms by splicing variants and specific isoforms are present in each tissue (35). Although the tropomyosin1a chain and other tropomyosins have been identified in photoreceptors (72), this is the first report to observe distribution of tropomyosin isoforms in macula and to identify brain isoform from photoreceptors. Anti-tropomyosin1a chain antibody TM311 was used for western blotting and immunohistochemistry. TM311 is a monoclonal antibody which detects broad range of alpha-, beta-, gamma- and delta- tropomyosin expressed in both muscle and non-muscle tissue except TMBR-3. By immunostaining, tropomyosin isoforms detected by TM311 were located around the choroidal vessel (Fig. 7G). MacDonald et al. demonstrated that endostatin, a cleavage fragment of collagen XVIII, functions as an anti-angiogenic factor (73) but it requires binding to tropomyosin to gain activity (74). Higher expression of tropomyosin1a chain in the macula has the potential to act as an anti-angiogenic factor for AMD by binding to endostatin.

Phosphorylation of the tropomyosin1a chain has also been described. Using human umbilical vascular endothelial cells, Houle et al. reported tropomyosin phosphorylation by oxidative stress through activation of the ERK pathway. Inhibition of the ERK pathway in the presence of oxidative stress resulted in membrane blebbing which then led to



damage of the vascular endothelium (71).

Spot number M4 corresponded to TMBr-3. TMBr-3 is expressed in all regions of the brain (75, 76) and was detected in the outer plexiform layer and photoreceptor inner segments (Fig. 7F) by double immunostaining with arrestin-C (Fig. 7B). The function of TMBr-3 in the photoreceptor inner segments is still unknown. Tropomyosin has a role in stabilizing actin filaments and regulates actin filaments in competition with other actin binding protein in non-muscle cells (77). Actin filaments located in the photoreceptor connecting cilia are considered the cytoskeletal core necessary for initiating new disk formation (78). Disk turnover is essential for maintenance of normal vision and consequently accumulation of lipofuscin in RPE cells by phagocytosis of disks has been linked to AMD (79). The function of TMBr-3 might involve disk formation by actin binding and rate of lipofuscin accumulation. Furthermore one of peptide fragment, which contains nitro tyrosine, in tropomyosin 1 alpha chain was identified from skeletal muscle (X). Nitration has been considered the potential for pathogenesis of many diseases (X). Brain type isoform also contains same amino acid sequence. In the retina, nitration of tropomyosin might be occurred and be associated with pathogenesis of AMD. Further studies are needed to understand the function of TMBr-3 in the photoreceptors.

## **Conclusions**

Proteomic analyses using 2D gel electrophoresis and LC-MS/MS were performed for comparison of regional differences in primate retina. Twenty-six proteins were identified as macula unique 2D-gel spots and 6 of these proteins were confirmed to be macula enriched by western blotting. In this study, brain type isoform of tropomyosin 1 alpha chain was first reported from retina. Furthermore, additional horizontal spots for Mn-SOD were specifically observed in the macula. Although some of these proteins are derived because of difference in major cell types between the two regions, identification of protein from macular enriched cells is important to understand the pathogenesis of AMD.

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