Long non-coding RNA associated-competing endogenous RNAs are induced by clusterin in retinal pigment epithelial cells

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Abstract. Age related macular degeneration is one of the most common causes of vision loss in the elderly. Long noncoding RNAs (lncRNAs) serve important roles in regulating gene expression by acting as competing endogenous RNAs (ceRNAs). However, the roles of specific lncRNAs and their associated ceRNA function induced by clusterin in cultured retinal pigment epithelial (RPE) cells remain to be fully elucidated. Based on high throughput sequencing data from RPE cells treated with or without clusterin, the present study identified differentially expressed mRNAs, lncRNAs and microRNAs (miRNAs). A lncRNA-mRNA-microRNA (miRNA) network (ceRNA network) was subsequently constructed based on the bioinformatic database miRanda and miRNA targets database miRTarBase. These results demonstrated the expression pattern of several lncRNAs, and a clear clusterin-associated ceRNA network in RPE cells, which included 75 lncRNAs and 32 miRNAs in RPE cells induced by clusterin. Collectively, the present study uncovered and characterized via bioinformatics the global properties of the ceRNA network in human RPE cells in response to clusterin. These results may aid in the elucidation of the molecular mechanisms of clusterin in age-related macular degeneration.

Introduction

Age-related macular degeneration (AMD) is a leading cause of irreversible blindness worldwide, and affects 6.5% of the US population aged ≥40 years (1). AMD is classified into wet and dry AMD. The latter is the most common phenotype, which is characterized by an increase in the number and diameter of drusen, progressive atrophy of the retinal pigment epithelium (RPE), pigmentary irregularities and a graded loss in visual acuity (2-4). Previous studies have indicated that environmental and genetic factors influence the disease, including smoking, obesity and dietary fat intake (5-8).

It has been observed that oxidative damage and inflammation are involved in the pathogenesis of AMD (9,10). Furthermore, the complement system, a vital component of innate immunity and a complex network that contributes to inflammation, has been identified to be associated with the pathogenesis and progression of AMD (11). Clusterin, also known as apolipoprotein J because it is present in high-density lipoprotein complexes, has been indicated to be a negative regulator of the complement cascade and exhibits increased mRNA expression levels in the RPE cells of AMD patients (12). Clusterin is involved in cholesterol transportation and protein aggregation in human plasma, and a deficiency of this complement regulatory protein may contribute to inflammation and neovascularization (13-15).

Epigenetic mechanisms, including DNA methylation, chromatin remodeling, histone modification and non-coding RNA-mediated regulation, have been demonstrated to influence gene expression and interactions between the genetics and the environment during development (16). Non-coding RNA consists of long non-coding RNAs (lncRNAs) and microRNAs (miRNAs). Recent epigenetic and genetic evidence has indicated that miRNAs, such as miR-146A, miRNA-9, miRNA-155 and miRNA-125b are upregulated to modulate a family of potentially pathogenic genes involved in Alzheimer's disease and cancer (17-20). In 2011, Salmena *et al* (21) proposed a competing endogenous RNA (ceRNA) hypothesis, which is now supported by further evidence (22-24). The hypothesis suggests that a regulatory network exists whereby protein-coding and non-coding RNAs compete for binding to miRNAs by sharing miRNA-binding sites. Nevertheless, there is a lack of data on expression patterns of specific lncRNAs in AMD patients. In addition, it is unknown whether lncRNAs participate in the regulation of clusterin-related inflammation, or whether some lncRNAs are induced to aberrantly express or to participate in an altered ceRNA network after RPE cells are exposed to clusterin.

The aim of the present study was to elucidate the effects of clusterin on the expression of coding and non-coding RNA in RPE cells. The expressed mRNAs, miRNAs and lncRNAs were profiled in RPE cells treated with or without clusterin, and the ceRNA network was characterized. To the best of our knowledge, this was the first study to investigate the disease-specific lncRNA expression patterns and the associated ceRNA network in RPE cells.

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Materials and methods

Cell culture. The adult human RPE cell line D407 (Procell Life Science and Technology Co., Ltd., Wuhan, China) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 μg/ml streptomycin, 100 U/ml penicillin and 2.5 μg/ml amphotericin B at 37˚C and 95% humidity with a 5% CO_2 atmosphere.

MTT viability assay. D407 cells were seeded in 96-well plates at a density of $1x10^4$ cells/well and allowed to reach 90% confluence. The growth medium was subsequently replaced with medium supplemented with clusterin at 0 (control), 0.1, 1.0 or 10 μg/μl. Cells were incubated for 48 h and analyzed using an MTT assay. Briefly, the medium was removed and replaced with DMEM supplemented with 5 mg/ml MTT reagent. Following incubation for 4 h, cells were solubilized with 95% dimethyl sulfoxide and the absorbance of the solution was measured at 590 nm using an ELISA plate reader. Each sample well was assessed in triplicate, and all assays were run in triplicate. Statistical analysis was performed using one-way analysis of variance and Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference. Data were presented as the mean ± standard error of the mean.

RNA extraction. Total RNA was isolated from D407 cells treated without clusterin (0 μg/μl; control) or 1.0 μg/μl clusterin using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The extracted RNA sample was treated with DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) to remove the DNA. The RNA quality was analyzed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.) and 10% gel electrophoresis. RNA samples were stored at -80˚C for sequencing library construction.

Construction of sequencing library and RNA-sequencing (Seq). An mRNA library was constructed according to the manufacturer's instructions using TruSeq Stranded mRNA Library Prep kit (Illumina, Inc., San Diego, CA, USA). Briefly, mRNA was enriched using oligo (dT) magnetic beads and then broken to ~200 bp fragments. The first-strand cDNA was synthesized using random hexamer primers, and then the second strand was synthesized. The purified DNA fragments were then sequenced using an Illumina Hiseq 2000 (Illumina, Inc.) by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The small RNA library was constructed according to Illumina's recommended protocols and sequenced on a Hiseq 2000 platform at RiboBio Co., Ltd. All sequences were deposited onto the GEO database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE87167).

Data analysis. Sequence reads were aligned to the hg19 human genome using Bowtie (25) with Tophat (26). Only reads that had ≤2 mismatches and 2 gaps to the hg19 genome were accepted. To assess the transcript level in the sample (slug-ml-3) and control (NC-3), the reads per kilobase of transcript per million mapped reads value was calculated. The number of mapped reads was calculated for each miRNA and the read count of each unique sequence was normalized using the reads per million (RPM) normalization, according to the formula: RPM = (number of reads mapping to miRNA reference / total number of mapped reads) x 10^6. Differential expression analysis was performed using Cuffdiff (26). Protein-coding transcripts and lncRNAs with fold change (FC)>1.5 or FC<0.67 and $P<0.05$ were considered to be differentially expressed in D407 cells treated with clusterin, whereas miRNAs with FC>2 or FC<0.5 ($P<0.05$) were considered aberrantly expressed.

Each sequence was annotated by aligning to known RNA sequences downloaded from Rfam11.0 (http://rfam.xfam.org/; rRNA, snRNA and snoRNA), miRBase (http://www.mirbase.org/ release version 21.0; miRNA), UCSC (http://genome.ucsc.edu; tRNA) and pirnabank (http://pirnabank.ibab.ac.in/; piRNA). Novel miRNAs were predicted using the miREAP algorithm (27) according to the manual. RNA secondary structures were predicted using RNAfold (28).

Functional annotation of differentially expressed mRNAs. Gene Ontology (GO) (29) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (30) pathway analysis of differentially expressed mRNAs was performed using the database for annotation, visualization and integrated discovery (DAVID, version 6.7) (31). The obtained GO terms and KEGG categories were filtered at a threshold significance value of $P<0.05$.

Construction of ceRNA network. The ceRNA network was constructed and visualized using Cytoscape software as preciously described (32). The process included prediction of lncRNA-miRNA interactions by PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html) (33), RNAhybrid (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (34) and miRanda (http://www.microrna.org) (35), targeting of mRNAs by miRNAs based on experimental data using miRTarBase (http://www.microrna.gr/tarbase) (36), miRwalk (http://mirwalk.uni-hd.de/) (37) and miRanda, and filtration of specific miRNA-lncRNA and miRNA-mRNA pairs, where the interaction pairs containing differentially expressed miRNA and lncRNA or mRNA were retained. A network map was constructed and visualized using Cytoscape software v3.5.1 (32).

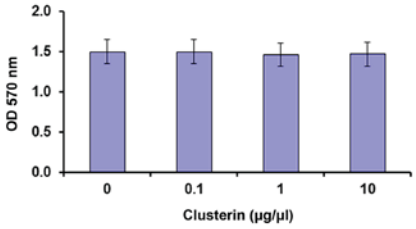

Figure 1. Viability of clusterin-treated D407 cells determined by MTT assay. Data are presented as the mean of three independent experiments. The bars represent standard deviation. OD, optical density.

Results

Effect of clusterin on cell viability. In consideration of the abnormal secretion of clusterin in RPE cells from AMD donors (38), clusterin activity on the viability of D407 cell viability was investigated using MTT assay. The viability of D407 cells was not affected by clusterin at tested concentrations (Fig. 1).

Table I. The distribution of mapped reads in experimental and control groups.

Region	Slug-ml-3 mapped reads	NC-3 mapped reads
All	12,378,042	12,385,191
Upstream/ downstream	171,120	160,934
Exonic	66,229	80,603
Intergenic	3,191,258	2,709,256
Intronic	1,116,005	1,116,007
NcRNA_exonic	7,642,921	8,092,812
NcRNA_intronic	110,292	134,888
Splicing	2,720	2,856
UTR	77,497	87,835

Nc RNA, non-coding RNA; UTR, untranslated region.

Table II. Annotation of noncoding RNAs.

	Slug-ml-3		NC-3	
Type	Total sRNA	%	Total sRNA	%
All	13,393,799	100.0	13,233,217	100.0
miRNA	5,547,804	41.4	6,436,571	48.7
tRNA	472,065	3.5	452,319	3.4
rRNA	1,648,269	12.3	1,417,235	10.7
snRNA	621,981	4.6	435,469	3.3
snoRNA	423,883	3.2	440,867	3.3
piRNA	504,767	3.8	451,597	3.4
Y_RNA	126,656	1.0	126,592	1.0
Others	4,048,374	30.2	3,472,567	26.2

sRNA, small non-coding RNA; miRNA, micro RNA; tRNA, transfer RNA; rRNA, ribosomal RNA; snRNA, small nuclear RNA; snoRNA, small nucleolar RNA; piRNA, Piwi-interacting RNA; slug-ml-3, experimental group; NC-3, control group.

RNA profiling in RPE cells. Total RNA isolated from D407 cells treated or untreated with clusterin was sequenced. After filtering out reads without a 3', 5' adaptor sequence and poly-A reads, the slug-ml-s and NC-3 mRNA libraries had 12,378,042 and 12,385,191 mapped reads, respectively (Table I). Notably, the distribution of the mapped reads in the experimental and control groups were similar between control and clusterin-treated RPE cells. The small RNA sequences were annotated according to their overlap with the sequences of known RNAs. The annotated results suggested that small RNA libraries from slug-ml-3 and NC-3 contained the same classes of RNAs (Table II). Furthermore, the abundance of each class was similar between the two libraries.

Identification of differentially expressed coding and non-coding genes. The expression levels of differentially expressed mRNA, miRNA and lncRNA were further analyzed between slug-ml-3 and NC-3 cells. A total of 321 genes were significantly differentially expressed, including 71 up- and 250 downregulated genes. Among them, 67 important genes are presented in Fig. 2A.

Aberrantly expressed miRNAs were detected based on the threshold of FC>2 or FC<0.5, and P<0.05. When miRNA expression was compared between D407 cells treated with and without clusterin, 36 miRNAs were observed as aberrantly expressed (Fig. 2B). To identify novel miRNAs from the reads mapped to unannotated genomic regions, the described miRNA discovery algorithm, miREAP, which is a tool for discovering novel miRNAs from small RNA deep sequencing data, was employed. A total of 71 and 76 candidate miRNAs were identified and considered to be novel in slug-ml-3 and NC-3, respectively.

Differentially expressed lncRNAs were also detected between slug-ml-3 and NC-3 cells. In total, 296 previously annotated lncRNAs and 90 novel lncRNAs were aberrantly regulated in cells treated with clusterin.

ceRNA network generation. miRNAs interact with lncRNAs via miRNA response elements within the ceRNA network. Therefore, potential miRNA response elements in lncRNAs were investigated. The results indicated that 22 of 36 specific miRNAs may interact with 75 of 386 lncRNAs (data not shown). To establish a lncRNA-miRNA-mRNA network (ceRNA network), mRNAs that were targeted by miRNAs were investigated. Using the list of miRNAs that may interact with lncRNAs, miRNA targeting mRNAs were searched for using miRTarBase, miRwalk and miRanda. From this analysis, 23 miRNAs were identified (data not shown). Most of the targets of these miRNAs were AMD-associated genes such as engrailed homeobox (EN)1, EN2, endothelin 2, myosin regulatory light chain interacting protein and complement component 5a receptor 2 (C5AR2). Based on these data, a ceRNA network (Fig. 3) was established; 75 lncRNAs and 32 miRNAs were involved in the proposed ceRNA network.

Functional analysis of differentially expressed mRNAs. In order to elucidate the molecular model of action of clusterin in AMD; biological processes and pathways regulated by clusterin were analyzed using DAVID. Enriched GO terms including Biological Process, Cellular Component and Molecular Function annotations were assessed. The results indicated that differentially expressed mRNAs were predominantly associated with the regulation of lipid transport across the blood brain barrier, receptor catabolic process, postsynaptic membrane organization and phospholipid transport (Fig. 4A and B). To understand the signaling pathways involved in the ceRNA network, the mRNAs were analyzed using the DAVID algorithm. The principally enriched pathways were environmental information processing, genetic information processing, cellular processing, organismal systems, drug development, human diseases and metabolism (Fig. 4C and D). The results indicated that differentially expressed mRNAs were enriched in the adherens junction and cell adhesion molecules in addition to extracellular matrix-receptor interactions. Furthermore, these mRNAs were largely involved in diseases including small cell lung cancer, arrhythmogenic right ventricular cardiomyopathy and hypertrophic cardiomyopathy.

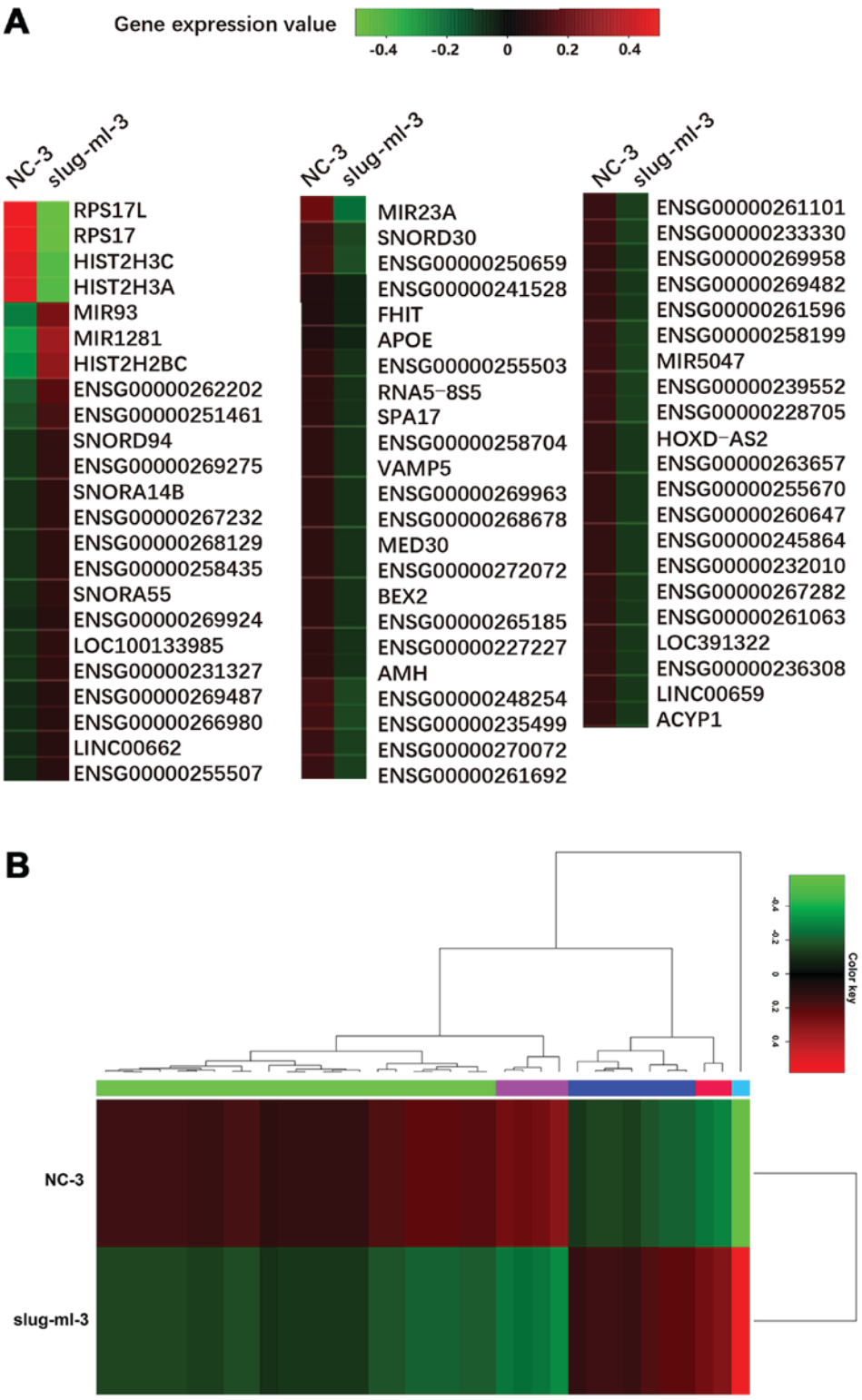

Figure 2. Hierarchical clustering analysis using Genesis clustering software of differentially expressed genes and miRNAs. Differentially expressed (A) genes and (B) miRNAs identified from human retinal pigment epithelial cell line D407 treated with or without clusterin. Slug-ml-3, experimental group; NC-3, control group; miRNA, microRNA.

Discussion

lncRNAs are considered as important regulators of biological processes in human diseases (39,40). However, few studies have reported the lncRNAs expression profile in retinal disorder-associated diseases including AMD, using a high-throughput sequencing strategy (41). Increasing evidence has demonstrated that there are interactions between mRNA and lncRNA (42) or lncRNA and miRNA (43,44) in cancer development, suggesting that lncRNAs may function as a part of ceRNA network. However, ceRNA networks in AMD are still poorly explored. The present study served to identify

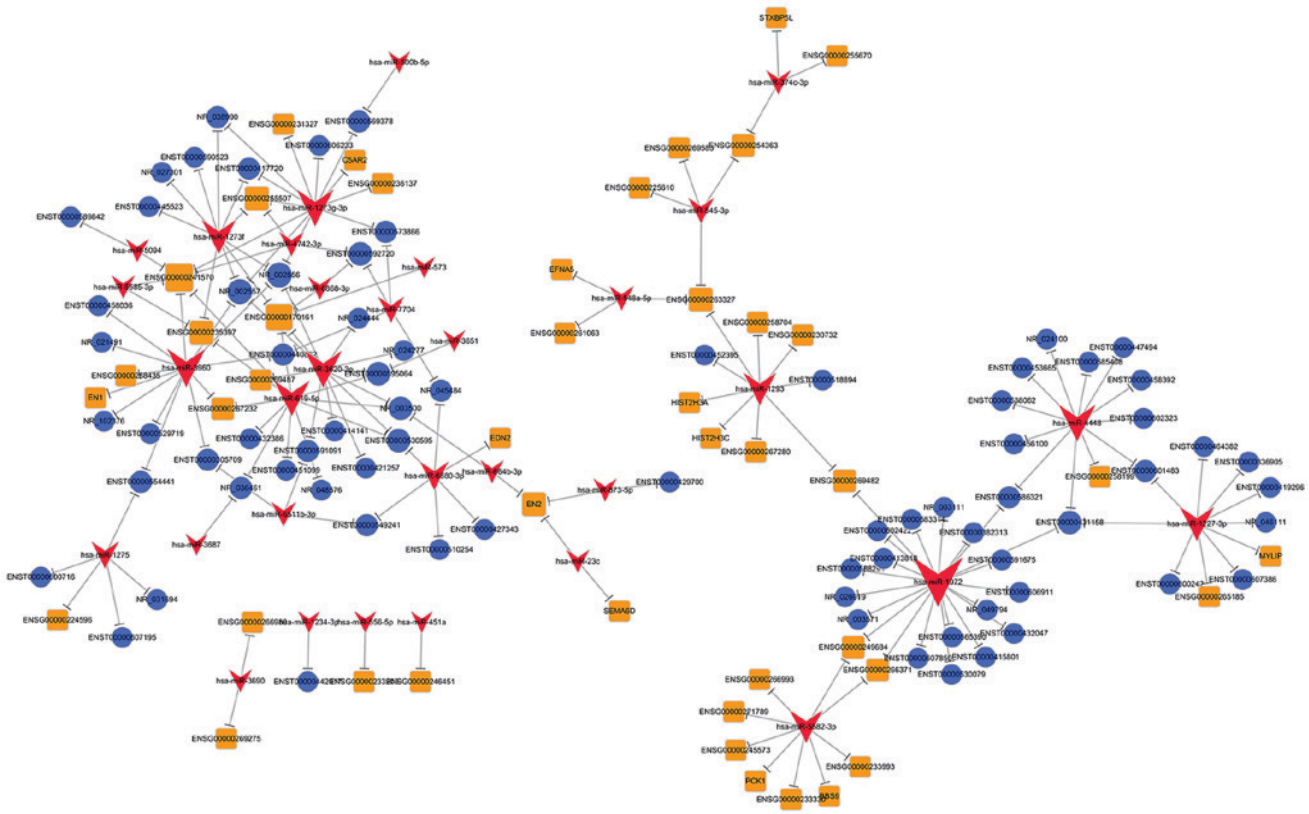

Figure 3. ceRNA network in retinal pigment epithelial D407 cells. Blue circular nodes represent long-noncoding RNAs, orange squares represent mRNA and red inverted triangles represent miRNA. ceRNA, competing endogenous RNA.

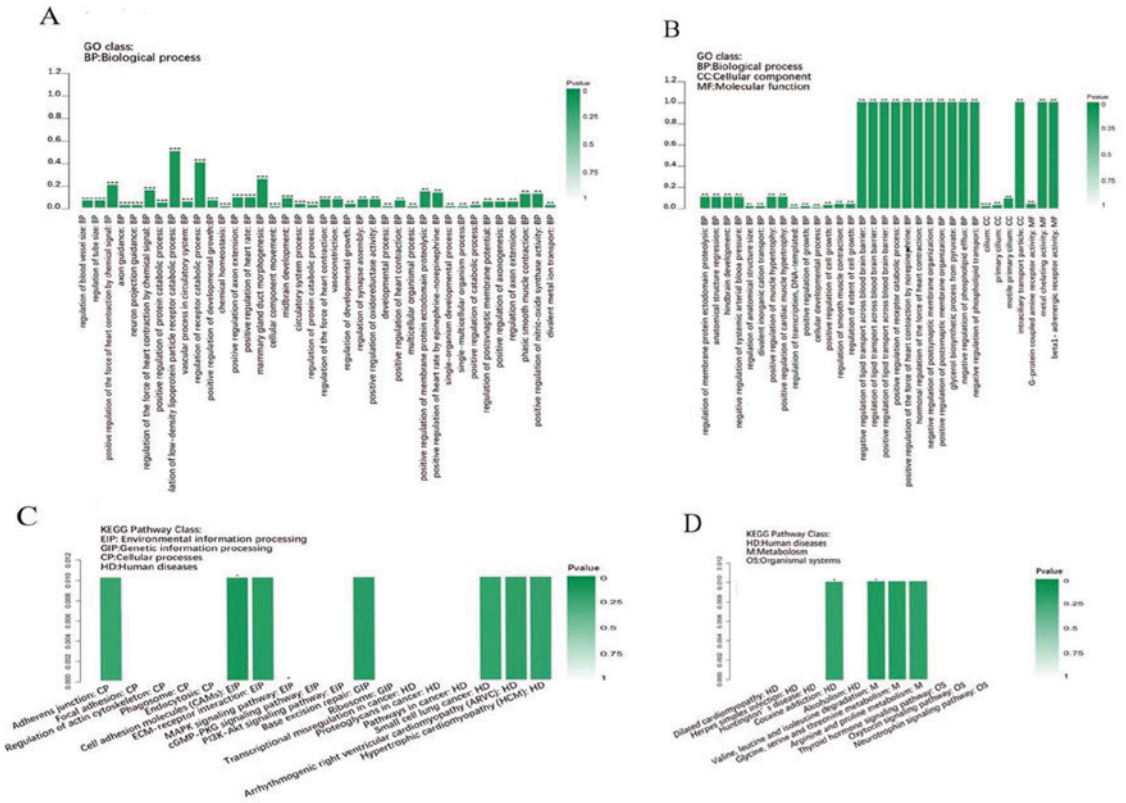

Figure 4. GO enrichment analysis of differentially expressed genes. (A and B) GO analysis and (C and D) KEGG pathway enrichment analysis of differentially expressed genes identified from human retinal pigment epithelial cell line D407 treated with or without clusterin. GO, gene ontology; BP, biological process; CC, cellular component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genomes; EIP, environmental information processing; GIP, genetic information processing; CP, cellular processes; HD, human diseases; M, metabolism; OS, organismal systems. ***P<0.001; **P<0.01; *P<0.05.

miRNA and lncRNA expression and distribution in RPE cells following treatment with clusterin, based on genome-wide expression profiling. Furthermore, a ceRNA network was constructed among mRNAs, miRNAs and lncRNAs, to provide a systematic view of lncRNA-miRNA-mRNA interactions.

Based on next generation RNA sequence data, 386 lncRNAs were observed to be differentially expressed between RPE cells treated with or without clusterin. Abnormally expressed miRNAs and mRNAs were also identified, and 75 lncRNAs were identified to be involved in the ceRNA network, suggesting they may serve role in AMD development. Among the lncRNAs involved in the ceRNA network, NR_036461 (HIST2H2BC) was reported to promote cell proliferation and invasion in triple-negative breast cancer (45). Furthermore, NR_102376 (small integral membrane protein 27) was identified to be a potential biomarker for human breast cancer (46). From these results, it was concluded that there was a differential expression pattern of certain lncRNAs between RPE cells with and without clusterin treatment. Further studies are required to elucidate the role of these differentially expressed lncRNAs.

To improve the predicted network, three databases including PITA, RNAhybrid and miRanda were applied to filter the pair-wised relationships based on lncRNA-miRNA expression correlations. This strategy served to identify additional lncRNAs, compared with using a single database. To enhance the reliability of the data, validated miRNA target genes were acquired from the miRTarBase database that had associated experimental supporting data.

Several genes involved in inflammation and cell adhesion were identified to participate in the ceRNA network after treating RPE cells with clusterin. For instance, engrailed (EN)1, EN2, endothelin 2, myosin regulatory light chain interacting protein, complement component 5a receptor 2 (C5AR2), lncRNA NR_036461 (HIST2H2BC) and NR_102376 (TOPORS-AS1) miRNA hsa-miR-1273g-3p, hsa-miR-5582-3p and hsa-miR-1293 were identified to be associated with AMD. Moreover, we found that hsa-miR-1273g-3p was closely associated with inflammation. C5AR2, which is mediated by hsa-miR-1273g-3p, has been indicated to be involved in C5a-mediated human mast cell adhesion and proinflammatory mediator production (47). Inflammation is well recognized as a molecular mechanism of AMD (48). Therefore, differentially expressed lncRNAs may serve important roles in AMD development.

In conclusion, the present study identified lncRNAs specifically induced by clusterin in RPE cells and identified an abnormal expression pattern of these lncRNAs. A ceRNA network was constructed that may provide a novel method to identify AMD-related lncRNA alterations in AMD. These results suggest that lncRNAs specifically identified in RPE cells may participate in a complex ceRNA network and in unknown regulatory pathways in the network, and may contribute to our understanding of the biological mechanism underlying AMD.

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References

1. Klein R, Chou CF, Klein BE, Zhang X, Meuer SM and Saaddine JB: Prevalence of age-related macular degeneration in the US population. Arch Ophthalmol 129: 75-80, 2011.
2. Gehrs KM, Anderson DH, Johnson LV and Hageman GS: Age-related macular degeneration-emerging pathogenetic and therapeutic concepts. Ann Med 38: 450-471, 2006.
3. Gehrs KM, Jackson JR, Brown EN, Allikmets R and Hageman GS: Complement, age-related macular degeneration and a vision of the future. Arch Ophthalmol 128: 349-358, 2010.
4. Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, Hancox LS, Hu J, Ebright JN, Malek G, *et al*: The pivotal role of the complement system in aging and age-related macular degeneration: Hypothesis re-visited. Prog Retin Eye Res 29: 95-112, 2010.
5. Chakravarthy U, Wong TY, Fletcher A, Piault E, Evans C, Zlateva G, Buggage R, Pleil A and Mitchell P: Clinical risk factors for age-related macular degeneration: A systematic review and meta-analysis. BMC Ophthalmol 10: 31, 2010.
6. Chong EW, Robman LD, Simpson JA, Hodge AM, Aung KZ, Dolphin TK, English DR, Giles GG and Guymer RH: Fat consumption and its association with age-related macular degeneration. Arch Ophthalmol 127: 674-680, 2009.
7. Seddon JM, George S and Rosner B: Cigarette smoking, fish consumption, omega-3 fatty acid intake, and associations with age-related macular degeneration: The US twin study of age-related macular degeneration. Arch Ophthalmol 124: 995-1001, 2006.
8. Age-Related Eye Disease Study Research Group, SanGiovanni JP, Chew EY, Clemons TE, Ferris FL III, Gensler G, Lindblad AS, Milton RC, Seddon JM and Sperduto RD: The relationship of dietary carotenoid and vitamin A, E, and C intake with age-related macular degeneration in a case-control study: AREDS Report No. 22. Arch Ophthalmol 125: 1225-1232, 2007.
9. Donoso LA, Kim D, Frost A, Callahan A and Hageman G: The role of inflammation in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 51: 137-152, 2006.
10. Beatty S, Koh H, Phil M, Henson D and Boulton M: The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 45: 115-134, 2000.
11. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B and Seddon JM: Plasma complement components and activation fragments: Associations with age-related macular degeneration genotypes and phenotypes. Invest Ophthalmol Vis Sci 50: 5818-5827, 2009.
12. Suuronen T, Nuutinen T, Ryhänen T, Kaarniranta K and Salminen A: Epigenetic regulation of clusterin/apolipoprotein J expression in retinal pigment epithelial cells. Biochem Biophys Res Commun 357: 397-401, 2007.
13. Anderson DH, Mullins RF, Hageman GS and Johnson LV: A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol 134: 411-431, 2002.
14. Jenne DE, Lowin B, Peitsch M, Böttcher A, Schmitz G and Tschopp J: Clusterin (complement lysis inhibitor) forms a high density lipoprotein complex with apolipoprotein AI in human plasma. J Biol Chem 266: 11030-11036, 1991.
15. Gemenetzi M and Lotery AJ: The role of epigenetics in age-related macular degeneration. Eye (Lond) 28: 1407-1417, 2014.
16. Bell JT and Spector TD: A twin approach to unraveling epigenetics. Trends Genet 27: 116-125, 2011.
17. Li YY, Cui JG, Hill JM, Bhattacharjee S, Zhao Y and Lukiw WJ: Increased expression of miRNA-146a in Alzheimer's disease transgenic mouse models. Neurosci Lett 487: 94-98, 2011.
18. Li YY, Cui JG, Dua P, Pogue AI, Bhattacharjee S and Lukiw WJ: Differential expression of miRNA-146a-regulated inflammatory genes in human primary neural, astroglial and microglial cells. Neurosci Lett 499: 109-113, 2011.
19. Pogue AI, Percy ME, Cui JG, Li YY, Bhattacharjee S, Hill JM, Kruck TP, Zhao Y and Lukiw WJ: Up-regulation of NF-kB-sensitive miRNA-125b and miRNA-146a in metal sulfate-stressed human astroglial (HAG) primary cell cultures. J Inorg Biochem 105: 1434-1437, 2011.
20. Kociok N and Joussen AM: Enhanced expression of the complement factor H mRNA in proliferating human RPE cells. Graefes Arch Clin Exp Ophthalmol 248: 1145-1153, 2010.
21. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP: A ceRNA hypothesis: The rosetta stone of a hidden RNA language? Cell 146: 353-358, 2011.

22. Ebert MS, Neilson JR and Sharp PA: MicroRNA sponges: Competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4: 721-726, 2007.
23. Poliseno L, Salmena L, Zhang J, Carver B, Haveman WJ and Pandolfi PP: A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature 465: 1033-1038, 2010.
24. Wu H: CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer. Nucleic Acids Res 38: 5366-5383, 2010.
25. Langmead B: Aligning short sequencing reads with Bowtie. Curr Protoc Bioinformatics Chapter 11: Unit 11.7, 2010.
26. Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL and Pachter L: Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. Nat Protoc 7: 562-578, 2012.
27. Chen X, Li Q, Wang J, Guo X, Jiang X, Ren Z, Weng C, Sun G, Wang X, Liu Y, *et al*: Identification and characterization of novel amphioxus microRNAs by Solexa sequencing. Genome Biol 10: R78, 2009.
28. Hofacker IL, Fontana W, Stadler PF, Bonhoeffer LS, Tacker M and Schuster P: Fast folding and comparison of RNA secondary structures. Monatshefte für Chemie/Chemical Monthly 125: 167-188, 1994.
29. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT, *et al*: Gene ontology: Tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25-29, 2000.
30. Kanehisa M and Goto S: KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28: 27-30, 2000.
31. Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC and Lempicki RA: DAVID: Database for annotation, visualization, and integrated discovery. Genome Biol 4: P3, 2003.
32. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B and Ideker T: Cytoscape: A software environment for integrated models of biomolecular interaction networks. Genome Res 13: 2498-2504, 2003.
33. Kertesz M, Iovino N, Unnerstall U, Gaul U and Segal E: The role of site accessibility in microRNA target recognition. Nat Genet 39: 1278-1284, 2007.
34. Rehmsmeier M, Steffen P, Hochsmann M and Giegerich R: Fast and effective prediction of microRNA/target duplexes. RNA 10: 1507-1517, 2004.
35. John B, Enright AJ, Aravin A, Tuschl T, Sander C and Marks DS: Human MicroRNA targets. PLoS Biol 2: e363, 2004.
36. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, Anastasopoulos IL, Maniou S, Karathanou K, Kalfakakou D, *et al*: DIANA-TarBase v7.0: Indexing more than half a million experimentally supported miRNA:mRNA interactions. Nucleic Acids Res 43 (Database issue): D153-D159, 2015.
37. Dweep H and Gretz N: miRWalk2.0: A comprehensive atlas of microRNA-target interactions. Nat Methods 12: 697, 2015.
38. An E, Lu X, Flippin J, Devaney JM, Halligan B, Hoffman EP, Strunnikova N, Csaky K and Hathout Y: Secreted proteome profiling in human RPE cell cultures derived from donors with age related macular degeneration and age matched healthy donors. J Proteome Res 5: 2599-2610, 2006.
39. Wapinski O and Chang HY: Long noncoding RNAs and human disease. Trends Cell Biol 21: 354-361, 2011.
40. Batista PJ and Chang HY: Long noncoding RNAs: Cellular address codes in development and disease. Cell 152: 1298-1307, 2013.
41. Guduric-Fuchs J, O'Connor A, Cullen A, Harwood L, Medina RJ, O'Neill CL, Stitt AW, Curtis TM and Simpson DA: Deep sequencing reveals predominant expression of miR-21 amongst the small non-coding RNAs in retinal microvascular endothelial cells. J Cell Biochem 113: 2098-2111, 2012.
42. Yuan JH, Yang F, Wang F, Ma JZ, Guo YJ, Tao QF, Liu F, Pan W, Wang TT, Zhou CC, *et al*: A long noncoding RNA activated by TGF-β promotes the invasion-metastasis cascade in hepatocellular carcinoma. Cancer Cell 25: 666-681, 2014.
43. Augoff K, McCue B, Plow EF and Sossey-Alaoui K: miR-31 and its host gene lncRNA LOC554202 are regulated by promoter hypermethylation in triple-negative breast cancer. Mol Cancer 11: 5, 2012.
44. Tsang FH, Au SL, Wei L, Fan DN, Lee JM, Wong CC, Ng IO and Wong CM: Long non-coding RNA HOTTIP is frequently up-regulated in hepatocellular carcinoma and is targeted by tumour suppressive miR-125b. Liver Int 35: 1597-1606, 2015.
45. Liu YR, Jiang YZ, Xu XE, Hu X, Yu KD and Shao ZM: Comprehensive transcriptome profiling reveals multigene signatures in triple-negative breast cancer. Clin Cancer Res 22: 1653-1662, 2016.
46. Su X, Malouf GG, Chen Y, Zhang J, Yao H, Valero V, Weinstein JN, Spano JP, Meric-Bernstam F, Khayat D and Esteva FJ: Comprehensive analysis of long non-coding RNAs in human breast cancer clinical subtypes. Oncotarget 5: 9864-9876, 2014.
47. Pundir P, MacDonald CA and Kulka M: The novel receptor C5aR2 is required for C5a-mediated human mast cell adhesion, migration, and proinflammatory mediator production. J Immunol 195: 2774-2787, 2015.
48. Donoso LA, Kim D, Frost A, Callahan A and Hageman G: The role of inflammation in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 51: 137-152, 2006.