Altered Long Non-coding RNAs Involved in Immunological Regulation and Associated with Choroidal Neovascularization in Mice

Liwei Zhang\textsuperscript{1,2}, Huilan Zeng\textsuperscript{1,2}, Jiang-Hui Wang\textsuperscript{3,4}, Han Zhao\textsuperscript{1,2}, Boxiang Zhang\textsuperscript{1,2}, Jingling Zou\textsuperscript{1,2}, Shigeo Yoshida\textsuperscript{5}, Yedi Zhou\textsuperscript{1,2}* 

1. Department of Ophthalmology, The Second Xiangya Hospital, Central South University, Changsha, Hunan, China
2. Hunan Clinical Research Center of Ophthalmic Disease, Changsha, Hunan, China
3. Centre for Eye Research Australia, Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria, Australia
4. Ophthalmology, Department of Surgery, University of Melbourne, East Melbourne, Victoria, Australia
5. Department of Ophthalmology, Kurume University School of Medicine, Kurume, Fukuoka, Japan

Short title: LncRNAs in CNV

Correspondence: Yedi Zhou, MD, PhD
Department of Ophthalmology, The Second Xiangya Hospital, Central South University, Changsha, Hunan 410011, China
Telephone: +86-731-85292175
E-mail: zhouyedi@csu.edu.cn

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Abstract

Choroidal neovascularization (CNV) is a severe complication of the late-stage form of wet age-related macular degeneration (AMD). Long non-coding RNAs (lncRNAs) have been implicated in the pathogenesis of different ocular neovascular diseases. To identify the function and therapeutic potential of lncRNAs in CNV, we assessed lncRNAs and messenger RNAs (mRNAs) expression profile in a mouse model of laser-induced CNV. Microarray analyses were performed to identify altered lncRNAs and mRNAs in CNV mice. The results were validated by qRT-PCR. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses were conducted to clarify the biological functions and signaling pathways with which altered genes are most closely related. Moreover, a coding-non-coding gene co-expression (CNC) network was performed to identify the interaction of lncRNAs and mRNAs. By microarray analysis, we identified 716 altered lncRNAs and 821 altered mRNAs in CNV mice compared to controls. A CNC network profile based on 7 validated altered lncRNAs (uc009ewo.1, AK148935, uc029sdr.1, ENSMUST00000132340, AK030988, uc007mds.1, ENSMUST0000180519) as well as 282 interacted and altered mRNAs was composed of 713 connections. GO and KEGG analyses suggested that altered mRNAs as well as those lncRNA-interacted mRNAs were enriched in immune system process and chemokine signaling pathway. Thus, lncRNAs are significantly altered in this mouse model of CNV and are involved in immunological regulation, suggesting that lncRNAs may play a critical role in the pathogenesis of CNV. Thus, dysregulated lncRNAs and their target genes might be promising therapeutic targets to suppress CNV in AMD.
Introduction

Age-related macular degeneration (AMD) is one of the leading causes of visual impairments and blindness in developed regions [1]. Choroidal neovascularization (CNV) is a severe complication of the late-stage form of neovascular or wet AMD [2]. It is well known that vascular endothelial growth factor (VEGF) plays key roles in the pathogenesis of neovascular AMD [3, 4]. Numerous studies have demonstrated that anti-VEGF therapy is effective and safe to patients with CNV due to AMD for which it has been widely used in clinics [5, 6]. However, recent studies suggest that some VEGF neutralizing proteins are not effective in some patients [7, 8]. There is increasing evidence that a number of factors may be responsible for non-responses including tolerance or tachyphylaxis (a term refers to the sudden decrease in response to a drug after its administration), roles for proangiogenic factors other than VEGF alone [9, 10]. Therefore, there is an urgent need for the mechanistic understanding of CNV, to explore novel therapeutic targets for early intervention of CNV, which would provide potential alternatives to anti-VEGF therapies.

Long non-coding RNAs (lncRNAs) is a subtype of non-coding RNAs with the transcripts of more than 200 nucleotides [11]. Although lncRNAs have no protein-coding capacity, they may regulate physiological functions as well as pathological processes in many diseases [12-15]. In particular, the activation of lncRNAs might regulate expression of protein-coding genes through sophisticated mechanisms in ocular disorders [16-19]. It has been reported that lncRNAs have differential expression profiles in a mouse model of ischemia-induced retinal neovascularization [20]. The role of lncRNAs in AMD has also been investigated in a number of studies. A study showed that lncRNAs are differentially expressed in RPE/choroid samples in patients with early AMD compared to controls and might be involved in important regulative functions [21]. Among altered lncRNAs in patients with early AMD, RP11-234O6.2 has been demonstrated of having protective effects in aging RPE model [21]. A few in vitro studies
investigated roles of several certain lncRNAs (such as ZNF503-AS1 and BANCR) in retinal pigment epithelium (RPE) cells [22, 23]. Nevertheless, it still remains unclear what the expression profiles, targets and effects of lncRNAs and their contribution to pathogenesis of CNV as well as neovascular AMD.

Laser-induced CNV is a well-established mouse model to investigate the pathogenesis of CNV and neovascular AMD [24]. Previous studies using CNV mouse model to examine the role of VEGF and many other molecules [25-27]. We recently identified altered microRNAs and tRNA-derived small RNAs in the laser-induced CNV model[28].

In the present study, microarray analyses were applied to clarify the expression profiles of lncRNAs and mRNAs in CNV mouse model. In addition, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were also conducted to explore the biological functions and potential signaling pathways of altered genes. Moreover, a coding-non-coding gene co-expression (CNC) network analysis was performed to investigate the correlation of differentially expressed lncRNAs and mRNAs, and to further predict the potential roles of altered lncRNAs in CNVs.

**Materials and Methods**

**Animals**

Male C57BL/6J mice (aged 7-8 weeks old) (SJA Laboratory Animal Co., Ltd., Hunan, China) were used in the present study. All protocols of the experiments were approved by the Institutional Animal Care and Use Committee of Central South University, China. The animal experiments are according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Laser-induced CNV mouse model**
As previously described, CNV mouse model was induced by laser photocoagulation [27, 29] and has been modified in this study. Briefly, the photocoagulation was performed around the optic disc using a 532-nm diode laser (100 mW, 0.1 s duration, 50 μm). Twenty-five spots were burned on each eye, and the eyes were enucleated 7 days after laser photocoagulation. Age-matched mice without laser photocoagulation treatment were used as controls.

**LncRNA and mRNA microarray**

Total RNA was isolated from the RPE-choroid-sclera complexes following instructions of the manufacturer using Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA). RPE-choroid-sclera complexes from 4 eyes were pooled as one sample. By a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA), isolated RNA was quantified, and standard denaturing agarose gel electrophoresis was used to assess RNA integrity. The Mouse LncRNA Microarray (Arraystar, Rockville, MD, USA) (V3.0, consisting of 24881 coding transcripts and 35923 lncRNAs) were used to detect the expression of mRNAs and lncRNAs in a total of 6 samples (3 CNV vs 3 control samples).

**RNA labeling, array hybridization and microarray analysis**

RNA labeling and array hybridization were conducted based on the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technologies, Santa Clara, CA, USA) as described with some modifications [30]. In brief, mRNA was purified by utilizing the mRNA-ONLY™ Eukaryotic mRNA Isolation Kit (Epicentre Biotechnologies, Madison, WI, USA). Each sample was then amplified and transcribed into fluorescent cRNA using a Flash RNA Labeling Kit (Arraystar). Labeled cRNAs were purified by an RNeasy Mini Kit (Qiagen, Hilden, German) and quantified by NanoDrop ND-1000. The labeled cRNAs were then hybridized into the lncRNA expression microarray slide and were incubated in an Agilent
Hybridization Oven (Agilent Technologies) at 65°C for 17 hours. After washing and fixing, the hybridized arrays were scanned by the Agilent G2505C DNA Microarray Scanner (Agilent Technologies). Acquired array images were analyzed by Feature Extraction version 11.0.1.1 software (Agilent Technologies). Subsequent data normalization and processing were performed by using the GeneSpring GX v12.1 software package (Agilent Technologies). The raw data of microarray has been uploaded to the database of Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) for public access (accession number GSE129743). Altered lncRNAs and mRNAs expression were identified by a Volcano Plot filtering [fold change (FC) $\geq 1.5$] and $P<0.05$.

**Validation by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)**

The validation of lncRNA and mRNA microarray results was further processed by qRT-PCR [31]. RNA was transcribed into cDNA utilizing SuperScript III Reverse Transcriptase (Invitrogen) following instructions of the manufacturer by Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). qRT-PCR was performed by the ViiA 7 RT PCR System (Applied Biosystems) with the 2 $\times$ PCR Master Mix (Arraystar). The reaction conditions were as follows: incubating at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 60 s. LncRNAs' relative expression levels were calculated by 2$^{-\Delta\Delta Ct}$ method [32] and normalized with GAPDH [33, 34]. Each gene's primers are listed in Table 1. The data are representing three experiments' means.

**GO and KEGG pathway analyses**

The Gene Ontology (GO) (http://www.geneontology.org/) provided a way to describe CNC-associated mRNA attributes in the target organism. Pathway analysis was applied to investigate
the significant pathways of the altered mRNAs or CNC related mRNA on the basis of KEGG database (http://www.genome.jp/kegg/). The more overlapping between the GO/KEGG annotation list and the gene list was analyzed by Fisher’s exact test. A threshold of P<0.05 was applied to denote the significant enrichment of GO terms/pathway.

Construction of IncRNA-mRNA co-expression network

According to validated altered IncRNAs and their related mRNAs, we constructed a CNC network profiles to explore the relationship between IncRNAs and mRNAs. We selected Pearson correlation coefficients (PCCs) $\geq 0.97$ to construct the network utilizing Cytoscape version 2.8.1 software (The Cytoscape Consortium, San Diego, CA, USA) based on the PCCs of correlation analysis of IncRNA and mRNA.

Statistical analyses

All data are shown in the present study as the mean ± SEM. The statistical difference between two groups was assessed using Student’s t-test. P<0.05 was considered as statistically significant differences.

Results

Expression profiles of IncRNA and mRNA in CNV mice

To assess the expressions of IncRNAs in CNV compared to control mice, microarray analysis was conducted in the collected RPE-choroid-sclera complexes. Our results showed that 716 IncRNAs were significantly altered in CNV mice compared to control mice: 442 were upregulated and 274 were downregulated (FC $\geq 1.5$, P<0.05). The top 20 most significantly upregulated and downregulated IncRNAs are shown in Table 2-3. Among the differentially expressed IncRNA transcripts, AK036888 tops among upregulated IncRNAs with an FC of
13.38, whereas ENSMUST00000135495 tops among downregulated lncRNAs with an FC of 7.47. The clustering analysis demonstrated the relevance of lncRNA expression patterns in CNV and control samples by showing top 20 up- and downregulated lncRNAs (Fig. 1A). The variation of lncRNA expression between CNV and control mice is shown in a volcano plot (Fig. 1C) and a scatter plot (Fig. 1E).

We also identified that 821 mRNAs were significantly altered in CNV mice compared to control mice: 588 were upregulated and 233 were downregulated (FC ≥ 1.5, P < 0.05). The top 20 significantly altered mRNA are shown in Table 4-5. Among those upregulated mRNAs, Aplnr (NM_011784) tops with an FC of 11.11, while expression of Prpmp5 (NM_001024705) tops among downregulated genes with FC of 3.61. The heatmap plot revealed the clustering analysis among mRNA expression patterns by presenting top 20 up- and downregulated mRNAs in the collected samples (Fig. 1B). A volcano plot (Fig. 1D) and a scatter plot (Fig. 1F) showed the variation of mRNA expression between CNV and control mice.

Validation of the microarray data of lncRNAs by qRT-PCR
To validate the accuracy and reliability of the microarray profiling data, seven lncRNAs (uc009ewo.1, AK148935, uc029sdr.1, ENSMUST00000132340, AK030988, uc007mds.1, ENSMUST00000180519) were randomly selected for qRT-PCR. The qRT-PCR results are consistent with the microarray analyses where expression of lncRNAs AK148935, ENSMUST00000132340, uc009ewo.1 and uc029sdr.1 were significantly upregulated and expression of lncRNAs AK030988, ENSMUST00000180519 and uc007mds.1 were significantly downregulated in CNV mice compared to control mice (Fig. 1G).

GO and KEGG pathway analyses of differentially expressed genes
GO analyses of the 821 altered mRNAs showed that upregulated genes were enriched in
immune system process (ontology: biological process, GO: 0002376), extracellular region (ontology: cellular component, GO: 0005576) and protein binding (ontology: molecular function, GO: 0005515) (Fig. 2A); while downregulated genes were enriched in anion transport (ontology: biological process, GO: 0006820), plasma membrane region (ontology: cellular component, GO: 0098590) and secondary active transmembrane transporter activity (ontology: molecular function, GO: 0015291) (Fig. 2B).

KEGG pathway analysis of those differentially expressed mRNAs found that the upregulated genes were enriched in chemokine signal pathway, cytokine-cytokine receptor interaction and Staphylococcus aureus infection (Fig. 2C), while the downregulated genes were enriched in GABAergic synapse, Hippo signaling pathway and phototransduction (Fig. 2D).

**CNC networks with GO and KEGG analyses**

LncRNA and mRNA co-expression network profiles were constructed based on 7 validated altered lncRNAs as well as 282 interacted mRNAs which were differentially expressed. This CNC network was composed of 289 nodes and 713 connections. There were 512 positive and 201 negative interactions within the network (Fig. 3). The lncRNA uc009ewo.1 is correlated with 138 mRNAs, ENSMUST00000132340 is correlated with 132 mRNAs, uc029sdr.1 is correlated with 152 mRNAs, ENSMUST00000180519 is correlated with 127 mRNAs, AK148935 is correlated with 76 mRNAs, while uc007mds.1 and AK030988 are correlated with 49 and 39 mRNAs respectively. According to the networks, the most relevant mRNAs are B430306N03Rik, C-type lectin receptor 4e (Clec4e) and paired immunoglobulin-like receptor A6 (Pira6), all correlated with 6 lncRNAs. To predict the functions of the lncRNAs, GO analyses and KEGG pathway analyses of those interacted mRNAs which were differentially expressed were conducted based on the results of the co-expression network. GO analysis showed that the most enriched GO terms of the targeted genes were immune system process
(ontology: biological process, GO: 0002376), plasma membrane (ontology: cellular component, GO: 0005886) and protein binding (ontology: molecular function, GO: 0005515) (Fig. 4A). KEGG pathway analysis showed those targeted mRNAs were enriched in chemokine signal pathway, osteoclast differentiation and cytokine-cytokine receptor interaction (Fig. 4B).

**Discussion**

Despite several studies reported important roles of particular lncRNAs such as ZNF503-AS1 and BANCR in RPE cells through *in vitro* studies [22, 23], none of them have investigated the role of lncRNAs in CNV. The present study profiled lncRNA and mRNA expression in the mouse model of laser-induced CNV by integrated microarray analysis. We identified 821 significantly altered mRNAs (588 upregulated; 233 downregulated) and 716 differentially expressed lncRNAs (442 upregulated; 274 downregulated) in the RPE-choroid-sclera complexes from CNV mice compared to control mice. Validation of seven randomly chosen lncRNAs by qRT-PCR further confirmed the reliability of microarray analysis. The profiles of the lncRNAs and mRNAs in CNV mice provide novel insights into our understanding of the pathogenesis of CNV.

A number of studies have reported that different cytokines, such as IL-10, IL-12, IFN-γ, IL-17, IL-18 and IL-33, may play a role in the pathogenesis of CNV [35-39]. The pro- and anti-angiogenic effects of the cytokines relevant to Th1, Th2 and Th17 cells, participate in a complicated immunological network [40]. It has been reported that the number of macrophages increased significantly after laser photocoagulation [24], and M1-M2 polarization of macrophages have diverse distributions and functions in laser-induced CNV as well as in wet AMD [41-43]. Pro-inflammatory M1 macrophages increased in the site of CNVs [41], suggesting that inflammation might play a key role in the pathogenesis of CNV and AMD. Our GO analyses showed that the most upregulated genes participate in immune system process,
whereas the downregulated genes participate in plasma membrane region. Moreover, the KEGG pathway analysis revealed that the functions of altered genes are enriched in the chemokine signal pathway, cytokine-cytokine receptor interaction and the GABAergic synapse. Similarly, GO and KEGG pathway analyses of the lncRNAs-interacted mRNAs showed that the most enriched GO terms are associated with immune system process and immune response, and the most enriched KEGG pathway is also chemokine signaling pathway. These analyses demonstrated that CNV might be mainly immune-regulated by cytokines and chemokines.

Macrophage inducible Ca2+-dependent lectin receptor (Mincle) is a member of the C-type lectin family of immune receptors, which encoded by the gene of Clec4e, and Clec4e is also a type 2 transmembrane receptor [44]. As the CNC network analysis showed, Clec4e is a significantly altered gene correlated with 6 validated lncRNAs. Lv et al. reported that clec4e is induced specifically on M1 macrophages, and it plays an essential role in maintaining the phenotype of M1 macrophage in acute renal inflammation [45]. Another study showed that Clec4e enhanced proinflammatory phenotype of macrophages through activation of the unfolded protein response [46]. Our previous study demonstrated that M1 macrophages mainly distribute around the site of CNVs, and inflammatory M1 macrophage-associated cytokines increased in RPE and choroid in laser-induced CNV mouse model [41]. Thus Clec4e, as well as macrophage polarization are possibly involved in mRNA-lncRNA network.

Nevertheless, the relevance of lncRNAs and cytokines or macrophages in intraocular neovascularization is still unclear. Our GO analysis suggested that several altered lncRNAs are involved in the regulation of cytokines and immunological network in the pathogenesis of CNV, however, further studies should be guaranteed to investigate the exact functions and mechanisms of the dysregulated lncRNAs. Chemokines are a group of small chemoattractant cytokines, which play great roles in inflammation and regulating angiogenesis as well as macrophage polarization [47, 48]. Studies have shown that chemokine (C-X-C motif) ligand 8
(CXCL8) and monocyte chemoattractant protein-1 (MCP-1), and chemokine receptors such as CXC chemokine receptor 3 (CXCR3) have been reported to be involved in CNV and AMD pathogenesis [49, 50]. A study showed that miR-539-5p attenuates experimental CNV through targeting CXC chemokine receptor 7 (CXCL7) [51], indicating that non-coding RNAs are involved in the functional effect of chemokines and their receptors that contribute to the formation of CNV. In the present study, GO and KEGG analyses showed that altered genes enriched in immune system process, immune response, chemokine signaling pathway and cytokine-cytokine receptor interaction, indicating that lncRNAs may regulate chemokines, cytokines and their receptors in the pathogenesis of CNV formation through their target genes. Thus, it is worth to further investigate the mechanisms of involvement of lncRNAs in chemokines and chemokine receptors in CNV and AMD.

Zhu et al. presented identified lncRNAs which involved in early AMD [21]. In that study, GO and KEGG analyses showed that lncRNA-related genes enriched different functions, such as visual perception, sensory perception of light stimuli, and phototransduction pathway, but not immunological regulations. It might because that study focused on early stage AMD patients without geographic atrophy and CNV, and our present study investigated the pathogenesis of CNV, which demonstrated different roles of lncRNAs in AMD.

We recently demonstrated that both mRNAs and lncRNAs have differential expression profiles in a mouse model of oxygen-induced retinopathy (OIR) [20], which is used for investigation of retinal neovascular diseases. We recognized 539 altered mRNAs and 373 altered lncRNAs in OIR retinas (FC≥2.0, P<0.05) [20]. And we tried to check the intersection of altered RNAs in both models, and identified 161 mRNAs and 53 lncRNAs which altered in OIR as well as CNV models, and the majority of the RNAs have same trend of alteration. For instance, ENSMUST00000165968 and CD68 were up-regulated in both models, while ENSMUST00000144657 and cpna2 were down-regulated in both models. These double-
dysregulated mRNAs and lncRNAs might play more essential roles in angiogenesis, and this suggested that lncRNAs could have potential roles in the pathogenesis of ocular neovascular diseases.

In conclusion, the present study reveals that several lncRNAs and mRNAs are significantly altered in CNV mice through lncRNA and mRNA microarray analyses. Further, GO and pathway analyses indicate that altered lncRNAs are not only involved in biological processes of immune mechanisms and inflammation but also are involved in the related pathways which might contribute in CNV and AMD pathogenesis. In particular, immunological network, including cytokines, chemokines and their receptors are mainly involved and relevant to the effect of lncRNAs in CNV. The limitation of this study is the lack of functional assessment of the identified lncRNA and mRNAs. Therefore, further studies are required to investigate the potential roles and exact mechanisms of the altered lncRNAs and mRNAs in CNV.

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Figure Legends

Figure 1. Altered expressions of lncRNAs and mRNAs between CNV group and control group by microarray.
Heat map and hierarchical clustering analysis of the top 20 most significantly upregulated and downregulated lncRNAs(A) and mRNA(B) between CNV and control samples. The top column represents lncRNA and mRNA relative expression varies according to the color scale. The volcano plot presents all identified lncRNA (C) and mRNA (D) expression variation between the CNV and control samples. The horizontal green line shows the default 1.5-fold change and the vertical green line represents a P value of 0.05. The red and green plots represent differentially expressed genes with both fold change $\geq 1.5$ and P<0.05. The scatter plot presents all identified lncRNA (E) and mRNA (F) expression variation between the CNV and control samples. The x-axis and y-axis values are each sample's normalized values (log2 transformed). The gray line shows the default 1.5-fold changes. The red and green plots represent differentially expressed genes with fold change $\geq 1.5$. Red indicates a high relative expression level and green indicates a low relative expression level. The qRT-PCR validation of seven randomly selected lncRNAs: AK148935, ENSMUST00000132340, uc009ewo.1, uc029sdr.1, AK030988, ENSMUST00000180519, uc007mds.1 (G).

Figure 2. The GO and KEGG analyses of altered mRNAs.
The GO analyses of up- (A) and downregulated (B) mRNAs. KEGG pathway analysis of altered mRNAs indicated: the top 10 significant pathways which were correlated with the upregulated genes (C), and the top 5 significant pathways which were correlated with the downregulated genes (D).

Figure 3. CNC networks by validated lncRNAs.
The co-expression network profiles of lncRNAs and mRNAs based on 7 validated lncRNAs and correlated mRNAs which were differentially expressed. This co-expression network composed of 713 edges and 289 nodes. The diamond nodes represent lncRNAs, in which yellow denotes upregulated lncRNAs and blue denotes downregulated lncRNAs. The round nodes represent mRNAs, in which green denotes upregulated mRNAs and red denotes
downregulated mRNAs. Continuous lines indicate positive interactions and dotted lines indicate negative interactions between lncRNAs and mRNAs.

**Figure 4. The GO and KEGG analyses of interacted and altered mRNAs in CNC networks.**
The GO analyses of CNC associated mRNA attributes in the target organism (A). KEGG pathway analysis of significant pathways of CNC associated mRNA (B).
### Table 1. Sequence of the primers for lncRNAs.

| Gene name     | Forward and reverse primer | Tm (℃) | Product length (bp) |
|---------------|---------------------------|--------|---------------------|
| GAPDH(MOUSE)  | F:5' CACTGAGCAAGAGAGGCCCTAT3' R:5' GCAGCGAACTTTATGGATGGATT3' | 60     | 144                 |
| uc009ewo.1    | F:5' GGTAAGTCCCCCCTATCATTCTC 3' R:5' AAACACCCCCCTCCTC 3' | 60     | 184                 |
| AK148935      | F:5' TTTGTGGGCTGCTTCTCTTC 3' R:5' TGGTACCTGTGAGTGTTCCCTA 3' | 60     | 72                  |
| uc029sdr.1    | F:5' CTCTTGATGTATCCGGTG 3' R:5' CAGGAACACACAGCTCTCTC 3' | 60     | 210                 |
| ENSMUST00000132340 | F:5' CGGAATGTTACTGCCCATAG 3' R:5' TGACTATTAGGATAAGTCTG 3' | 60     | 254                 |
| AK030988      | F:5' TGGGACCTAAGGATGGAAGA 3' R:5' AGACCAGACCTGCTGAGC 3' | 60     | 167                 |
| uc007mds.1    | F:5' TTCAGGCCGACGACGCAC 3' R:5' GAAAGGTGGTTCTGCTGCTAC 3' | 60     | 187                 |
| ENSMUST00000180519 | F:5' TCTTGTTCTGCGCTGATGTC 3' R:5' AAACACGACACAAACAGG 3' | 60     | 129                 |

Tm: temperature. bp: base pair
| Sequence Name | P-value     | FDR         | Fold Change | Regulation | Strand | Relationship | CNV 1 | CNV 2 | CNV 3 | Control 1 | Control 2 | Control 3 |
|---------------|-------------|-------------|-------------|------------|--------|--------------|-------|-------|-------|-----------|-----------|-----------|
| AK036888      | 0.000171    | 0.150561    | 13.378940   | up         | -      | intergenic   | 6.585418 | 6.480785 | 6.071276 | 3.077727  | 2.512265  | 2.321811  |
| uc007pgi.1    | 0.014847    | 0.390976    | 9.578848    | up         | -      | intergenic   | 5.211190 | 7.107776 | 4.430212 | 2.325983  | 2.321827  | 2.321811  |
| AK078702      | 0.000536    | 0.150561    | 7.117427    | up         | -      | intergenic   | 6.359641 | 6.132188 | 6.161450 | 3.862077  | 3.371296  | 2.925838  |
| AK035526      | 0.005083    | 0.298875    | 6.657825    | up         | +      | natural antisense | 5.724644 | 7.089529 | 7.076339 | 4.231499  | 3.878298  | 3.575563  |
| uc009ewo.1    | 0.000984    | 0.182037    | 5.719433    | up         | -      | intron sense-overlapping | 8.163315 | 7.829017 | 7.371524 | 5.300458  | 5.567779  | 4.949003  |
| AK036625      | 0.005126    | 0.298875    | 5.353162    | up         | -      | intergenic   | 5.726749 | 5.680111 | 5.143081 | 2.325983  | 3.345529  | 3.617254  |
| ENSMUST00000148005 | 0.001561    | 0.218932    | 5.264145    | up         | +      | exon sense-overlapping | 6.509248 | 6.302205 | 5.601678 | 3.998450  | 3.479648  | 3.746437  |
| ENSMUST00000153541 | 0.000164    | 0.150561    | 4.957192    | up         | +      | exon sense-overlapping | 11.456490 | 11.349358 | 10.999685 | 9.005207  | 9.098501  | 8.773256  |
| AK008836      | 0.004393    | 0.293747    | 4.888444    | up         | -      | intron antisense | 7.371524 | 6.772026 | 6.602773 | 4.573347  | 5.202004  | 4.102845  |
| ENSMUST00000146254 | 0.000028    | 0.106609    | 4.640225    | up         | -      | exon sense-overlapping | 7.526886 | 7.218386 | 7.363228 | 5.129217  | 5.254380  | 5.082319  |
| NR_026843     | 0.001265    | 0.203615    | 4.591574    | up         | -      | natural antisense | 6.264922 | 6.398417 | 5.991045 | 4.478630  | 3.930072  | 3.648715  |
| ENSMUST00000122340 | 0.001105    | 0.191468    | 4.442445    | up         | -      | intron antisense | 8.984519 | 8.526921 | 8.527271 | 6.376287  | 6.936308  | 6.272055  |
| AK082144      | 0.000016    | 0.106609    | 4.424734    | up         | -      | intergenic   | 6.554079 | 6.639836 | 6.748626 | 4.380675  | 4.513834  | 4.609461  |
| AK048117      | 0.001609    | 0.221747    | 4.382340    | up         | -      | intergenic   | 9.343423 | 9.431755 | 9.431267 | 7.627238  | 7.620520  | 7.463583  |
| ENSMUST00000143423 | 0.000941    | 0.181475    | 4.307678    | up         | +      | exon sense-overlapping | 4.561347 | 5.009892 | 4.564078 | 2.963872  | 2.321827  | 2.528887  |
| AK148935      | 0.000638    | 0.156222    | 4.263167    | up         | -      | intergenic   | 8.259856 | 8.525405 | 8.330367 | 6.882964  | 6.086507  | 6.073080  |
| AK047865      | 0.001065    | 0.191468    | 4.256438    | up         | -      | intergenic   | 9.039629 | 8.963352 | 9.098866 | 7.275191  | 6.469285  | 7.088430  |
| ENSMUST00000141975 | 0.000283    | 0.150561    | 4.232972    | up         | -      | exon sense-overlapping | 6.736901 | 6.384502 | 6.153523 | 4.416513  | 4.338800  | 4.274602  |
| uc028sdr.1    | 0.000052    | 0.120829    | 4.185189    | up         | +      | intergenic   | 10.768113 | 10.641598 | 10.589025 | 8.507404  | 8.798939  | 8.496515  |
| NR_045384     | 0.010196    | 0.362008    | 4.172997    | up         | +      | intergenic   | 5.724329 | 5.884554 | 5.893274 | 4.532355  | 3.801238  | 2.985313  |

P values were calculated using unpaired t-test. FDR: false discovery rate. Fold change: the absolute ratio (no log scale) of average normalized intensities between two groups (CNV vs Control). CNV 1 to 3 and Control 1 to 3: each sample's normalized intensity (log2 scale). Hereinafter the same.
### Table 3. Top 20 significantly down-regulated lncRNAs.

| Sequence Name         | P-value  | FDR      | Fold Change | Regulation | Strand                  | Relationship               | CNV1     | CNV2     | CNV3     | Control1 | Control2 | Control3 |
|-----------------------|----------|----------|-------------|------------|--------------------------|----------------------------|----------|----------|----------|-----------|-----------|-----------|
| ENSMUST00000135495   | 0.044869 | 0.516189 | 7.465422    | down       | -                        | exon sense-overlapping      | 4.264994 | 6.259270 | 3.847529 | 8.927113  | 6.595256  | 7.550095  |
| ENSMUST00000151832   | 0.042431 | 0.506906 | 3.682991    | down       | +                        | exon sense-overlapping      | 3.973213 | 5.239059 | 4.472576 | 5.519285  | 4.674242  | 7.331772  |
| AK079094             | 0.000472 | 0.150561 | 2.875171    | down       | -                        | natural antisense           | 4.097720 | 3.991172 | 3.967711 | 5.716774  | 5.645347  | 5.265424  |
| ENSMUST00000171338   | 0.003991 | 0.284707 | 2.831007    | down       | -                        | exon sense-overlapping      | 4.347292 | 4.591844 | 4.052868 | 6.017756  | 5.435850  | 6.042344  |
| ENSMUST00000147716   | 0.002243 | 0.244886 | 2.805529    | down       | -                        | intergenic                  | 7.029440 | 6.772935 | 7.329016 | 8.414517  | 8.813207  | 8.368486  |
| AK030988             | 0.004724 | 0.293747 | 2.406930    | down       | -                        | bidirectional               | 2.530040 | 3.314212 | 2.545198 | 4.152919  | 4.397770  | 3.778787  |
| ENSMUST00000155217   | 0.000964 | 0.182037 | 2.460863    | down       | -                        | intergenic                  | 7.949919 | 8.236430 | 8.067902 | 9.477743  | 9.536148  | 9.137853  |
| NR_037995            | 0.000706 | 0.335485 | 2.453643    | down       | -                        | bidirectional               | 7.028992 | 6.600705 | 6.301580 | 8.136388  | 8.017734  | 7.661932  |
| ENSMUST00000155625   | 0.004510 | 0.293747 | 2.389210    | down       | +                        | natural antisense           | 3.883898 | 2.440709 | 2.323922 | 3.600419  | 4.496326  | 3.688754  |
| uc007ilt.1           | 0.039689 | 0.498925 | 2.389210    | down       | +                        | exon sense-overlapping      | 2.323979 | 2.189701 | 2.172476 | 4.039022  | 4.044215  | 3.541541  |
| ENSMUST00000141135   | 0.027081 | 0.383884 | 2.485166    | down       | -                        | natural antisense           | 5.561865 | 5.562532 | 4.940521 | 6.790447  | 6.549662  | 6.353641  |
| NR_033584            | 0.001977 | 0.240414 | 2.331771    | down       | +                        | exon sense-overlapping      | 3.421041 | 2.750947 | 2.558318 | 3.748812  | 3.811546  | 4.667192  |
| TCONS_00016118       | 0.007575 | 0.340233 | 2.312752    | down       | -                        | intronic antisense          | 5.561865 | 5.562532 | 4.940521 | 6.790447  | 6.549662  | 6.353641  |
| AK029573             | 0.013265 | 0.380110 | 2.305416    | down       | +                        | natural antisense           | 4.500548 | 3.852448 | 3.562495 | 5.301735  | 5.106782  | 5.122617  |
| ENSMUST00000144044   | 0.040861 | 0.503207 | 2.225878    | down       | +                        | exon sense-overlapping      | 4.548734 | 5.325275 | 4.296475 | 6.335644  | 5.575793  | 5.720369  |
| NR_033469            | 0.005124 | 0.298875 | 2.225231    | down       | +                        | intergenic                  | 6.057274 | 6.533446 | 6.704603 | 7.941327  | 7.926619  | 7.339692  |
| AK013439             | 0.011666 | 0.378564 | 2.208164    | down       | +                        | intergenic                  | 6.454914 | 6.874332 | 6.432593 | 7.343084  | 8.090536  | 7.756705  |
Table 4. Top 20 significantly up-regulated mRNAs.

| Sequence Name | Gene Symbol | P-value | Fold Change | Regulation | Chrom | CNV 1 | CNV 2 | CNV 3 | Control 1 | Control 2 | Control 3 |
|---------------|-------------|---------|-------------|------------|-------|-------|-------|-------|-----------|-----------|-----------|
| NM_011784     | Aplnr       | 0.000012| 11.110603   | up         | chr2  | 7.033988| 6.843315| 7.167412| 3.528313  | 3.702581  | 3.392226  |
| NM_011646     | Try4        | 0.001518| 10.794919   | up         | chr6  | 5.868813| 6.207469| 6.427937| 3.563739  | 2.321827  | 2.321811  |
| NM_008372     | Il7r        | 0.000099| 9.886701    | up         | chr15 | 6.023879| 5.297020| 5.565189| 2.325983  | 2.321827  | 2.321811  |
| NM_177843     | Gm14461     | 0.001185| 8.779374    | up         | chr2  | 7.331272| 7.067940| 6.677774| 3.347988  | 4.486817  | 3.839826  |
| NM_001003405  | Try5        | 0.000012| 8.310638    | up         | chr6  | 5.221975| 5.431142| 5.596919| 2.325983  | 2.321827  | 2.321811  |
| NM_206535     | Cd200r2     | 0.000381| 7.767570    | up         | chr16 | 6.312686| 6.182743| 5.670891| 2.734021  | 3.294071  | 3.265839  |
| NM_175406     | Atp6v0d2    | 0.001067| 7.672187    | up         | chr4  | 10.914325| 10.474780| 10.348221| 7.376840  | 8.241582  | 7.299991  |
| NM_011089     | Pira2       | 0.000091| 7.671351    | up         | chr7  | 6.094800| 5.933179| 5.760588| 2.792479  | 3.300848  | 2.876798  |
| NM_207244     | Cd200r4     | 0.006075| 7.548943    | up         | chr16 | 6.464434| 6.282340| 5.594127| 2.325983  | 3.989506  | 3.276587  |
| NM_008311     | Hut2b       | 0.000706| 7.192641    | up         | chr1  | 10.557041| 10.345577| 9.884406| 7.819662  | 7.395292  | 7.032505  |
| NM_011088     | Pira11      | 0.000196| 6.976422    | up         | chr7  | 10.466558| 10.217773| 10.150017| 7.762837  | 7.555972  | 7.113078  |
| NM_011094     | Pira7       | 0.000161| 6.963833    | up         | chr7  | 10.961688| 10.969189| 10.845609| 8.455120  | 8.155355  | 7.766367  |
| NM_028973     | Lrc15       | 0.012774| 6.827810    | up         | chr16 | 8.322155| 8.931940| 10.139378| 6.901063  | 6.511550  | 5.666592  |
| NM_178241     | Cxcr1       | 0.003685| 6.805119    | up         | chr1  | 6.005635| 5.869263| 5.788451| 3.879585  | 3.231827  | 3.162076  |
| NM_001166672  | Gm14548     | 0.000097| 6.673624    | up         | chr7  | 11.580840| 11.296632| 11.179657| 8.847641  | 8.877864  | 8.406313  |
| NM_019948     | Clec4e      | 0.000563| 6.596644    | up         | chr6  | 5.670682| 5.027602| 5.075513| 2.887439  | 2.399350  | 2.321811  |
| NM_011087     | Pira1       | 0.003576| 6.187779    | up         | chr7  | 5.488713| 5.426346| 5.204069| 2.325983  | 3.583068  | 2.321811  |
| NM_001267695  | Cts5        | 0.000957| 5.934620    | up         | chr3  | 9.404564| 8.806512| 8.751737| 6.192754  | 6.833984  | 6.228608  |
| NM_008848     | Pira6       | 0.002555| 4.851621    | up         | chr7  | 4.801415| 4.635361| 4.962917| 2.325983  | 2.839596  | 2.399350  |
| NM_008404     | Itgb2       | 0.001909| 4.841209    | up         | chr10 | 9.670052| 8.907650| 8.869934| 6.768681  | 7.213496  | 6.639358  |
Table 5. Top 20 significantly down-regulated mRNAs.

| Sequence Name | Gene Symbol | P-value  | Fold Change | Regulation | Chrom | CNV 1 | CNV 2 | CNV 3 | Control 1 | Control 2 | Control 3 |
|---------------|-------------|----------|-------------|------------|--------|--------|--------|--------|-----------|-----------|-----------|
| NM_001024705  | Prpmp5      | 0.014868 | 3.605863    | down       | chr6   | 9.067530 | 8.986222 | 9.954781 | 11.192267 | 11.751723 | 10.615577 |
| NM_019840     | Pde4b       | 0.001704 | 2.927001    | down       | chr4   | 3.256077 | 3.624609 | 3.076331 | 5.079052  | 4.632161  | 4.894074  |
| NM_175296     | Mael        | 0.013959 | 2.785800    | down       | chr1   | 5.162247 | 5.565056 | 5.125866 | 7.062294  | 7.111513  | 6.113637  |
| NM_008623     | Mpz         | 0.008683 | 2.769509    | down       | chr1   | 12.174806| 13.137897| 12.638795| 13.881432 | 14.154913 | 14.324043 |
| NM_183160     | Tmem252     | 0.006698 | 2.677545    | down       | chr19  | 3.883402 | 4.178289 | 4.299822 | 5.111757  | 5.964037  | 5.548451  |
| NM_207280     | Ccdc121     | 0.014313 | 2.450424    | down       | chr1   | 2.805933 | 2.324675 | 2.323922 | 3.639443  | 3.399682  | 4.294497  |
| NM_001025255  | Mbp         | 0.00151  | 2.427744    | down       | chr18  | 2.949733 | 3.182399 | 3.054139 | 4.463824  | 4.263468  | 4.297829  |
| NM_026925     | Pnlp        | 0.028181 | 2.426672    | down       | chr19  | 3.116513 | 2.324675 | 2.323922 | 3.895614  | 3.380287  | 4.326145  |
| NM_001113373  | Shank2      | 0.023401 | 2.379186    | down       | chr7   | 3.648482 | 3.438832 | 2.672217 | 4.131874  | 4.666463  | 4.712598  |
| NM_001081153  | Unc13c      | 0.034934 | 2.372052    | down       | chr9   | 11.000760 | 10.198641| 11.528724| 12.222275 | 12.268342 | 11.975915 |
| NM_001122594  | Phlp2       | 0.001196 | 2.356591    | down       | chr8   | 3.131635 | 2.660591 | 2.931842 | 4.163803  | 4.026565  | 4.243805  |
| NM_001161722  | Tfeb        | 0.003061 | 2.335554    | down       | chr17  | 2.690098 | 3.230295 | 2.904800 | 4.046248  | 4.066880  | 4.383359  |
| NM_028807     | Exoc314     | 0.026008 | 2.331869    | down       | chr12  | 4.272006 | 3.720309 | 3.146549 | 5.178742  | 4.693262  | 4.931320  |
| NM_001127685  | BC048943    | 0.013403 | 2.324064    | down       | chr12  | 9.447387 | 8.766418 | 8.507593 | 10.125555 | 10.236406 | 10.009386 |
| NM_001204253  | Cle1c1b     | 0.008321 | 2.321635    | down       | chr6   | 4.644223 | 5.290836 | 4.979474 | 5.859919  | 6.408708  | 6.291329  |
| NM_007545     | Hrk         | 0.003712 | 2.290870    | down       | chr5   | 7.678339 | 8.078760 | 7.425854 | 8.965889  | 8.983374  | 8.821377  |
| NM_010220     | Fkbp5       | 0.029949 | 2.281321    | down       | chr17  | 6.708265 | 6.429901 | 7.553089 | 8.247108  | 8.176554  | 7.837201  |
| NM_020599     | Rlbp1       | 0.007127 | 2.235286    | down       | chr7   | 8.194874 | 8.097004 | 7.941646 | 9.260371  | 9.602041  | 8.852490  |
| NM_147110     | Olfr570     | 0.003044 | 2.223418    | down       | chr7   | 5.491363 | 5.307785 | 5.178571 | 6.613100  | 6.653939  | 6.169017  |
| NM_027790     | Dhrs2       | 0.003418 | 2.203722    | down       | chr14  | 5.776111 | 6.199949 | 5.806094 | 6.968227  | 7.311111  | 6.922644  |
Figure 1
Figure 2
