Original Paper

Long Noncoding RNA Profiling from *Fasciola Gigantica* Excretory/Secretory Product-Induced M2 to M1 Macrophage Polarization

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Key Words

Long non-coding RNA • *Fasciola gigantica* • Macrophage polarization • Excretory /secretory products (ESP) • CeRNA

Abstract

Background/Aims: Long noncoding RNAs (lncRNAs) are well known regulators of gene expression that play essential roles in macrophage activation and polarization. However, the role of lncRNA in *Fasciola gigantica* excretory/secretory products (ESP)-induced M2 polarization into M1 macrophages is unclear. Herein, we performed lncRNA profiling of lncRNAs and mRNAs during the ESP-induced macrophage polarization process. **Methods:** *F. gigantica* ESP was used to induce peritoneal cavity M2 macrophages in BALB/c mice (5–6 weeks old) *in vivo*, and these cells were subsequently isolated and stimulated with IFN- γ + LPS to induce M1 cells *in vitro*. lncRNA and mRNA profiling was performed via microarray at the end of both polarization stages. **Results:** In total, 2,844 lncRNAs (1,579 upregulated and 1,265 downregulated) and 1,782 mRNAs (789 upregulated and 993 downregulated) were differentially expressed in M2 macrophages compared to M1 macrophages, and six lncRNAs were identified during polarization. We selected 34 differentially expressed lncRNAs and mRNAs to validate the results of microarray analysis using quantitative real-time PCR (qPCR). Pathway and Gene Ontology (GO) analyses demonstrated that these altered transcripts were involved in multiple biological processes, particularly peptidase activity and carbohydrate metabolism.

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Furthermore, coding and non-coding gene (CNC) and mRNA-related ceRNA network analyses were conducted to predict lncRNA expression trends and the potential target genes of these lncRNAs and mRNAs. Moreover, we determined that four lncRNAs and four mRNAs might participate in *F. gigantica* ESP-induced M2 polarization into M1 macrophages. **Conclusions:** This study illustrates the basic profiling of lncRNAs and mRNAs during *F. gigantica* ESP-induced M2 polarization into M1 macrophages and deepens our understanding of the mechanism underlying this process.

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Introduction

Macrophages play a central role in inflammation and host defense. In response to tissue microenvironmental signals, macrophages activate and differentiate into diverse phenotypes as required. Classically activated macrophages (M1 cells) induced by IFN- γ /LPS secrete high levels of IL-12, IL-23, IL-6, TNF- α , and iNOS, but low levels of IL-10, thereby promoting Th1-type inflammatory responses [1, 2]. In contrast, alternatively activated macrophages (M2 cells) induced by IL-4/IL-13 express high levels of IL-10, YM-1, arginase-1, and RELM α , but low levels of IL-12 to promote Th2-type responses, wound healing, and inflammation resolution [3, 4]. The helminth can survive within its hosts due to its ability to manipulate host immunity, in particular the suppression of Th1 immune responses which is correlated with immune protection in the host. Helminth infection induces a polarized Th2 response characterized by an increase of IL-4, IL-5, and IL-13, but a decrease in IFN γ [5]. Like other helminths, *F. hepatica* infection and its excretory/secretory products (ESP) inhibit the development of M1 which facilitates the switch to the M2 phenotype, and modulates the host immune response by inducing potent polarized M2 (Th2) and by downregulating the production of M1 (Th1) macrophages [6, 7]. Fascioliasis, which is caused by *F. hepatica* or *F. gigantica* afflicts at least 2.6 million people around the world [8, 9], causes hepatic pathologies such as liver fibrosis, cirrhosis, and even cancer [10]. Moreover, fascioliasis outbreaks caused by *F. gigantica* in Yunnan, China further suggested that this pathogen might be zoonotic [11]. Therefore, decoding the biological and immunological feature of *F. gigantica* is imperative for public health. Though few reports have shown that *F. gigantica* could induce M1 or M2 phenotype, our preliminary experimental results suggested that *F. gigantica* was capable of inducing strong M2 and weak M1 phenotype during infection of water buffalo and mice (unpublished). This observation suggested that *F. gigantica* infection can induce M1/M2 mixed macrophages skewed towards an M2 phenotype, and might induce macrophage phenotypes similar to *F. hepatica*. Since M1 and M2 represent two different major immune responses (Th1 and Th2 response, respectively), we hypothesized that different immunomodulatory molecules play varying roles following *F. gigantica* infection. Specifically in *F. gigantica* ESP proteins, we identified 297 proteins, including 118 cytosol proteins, 22 nuclear proteins, 59 mitochondria proteins, 3 endoplasmic reticulum, 1 peroxisome, 3 cytosol and mitochondria proteins, 9 cytoskeleton proteins, 52 extracellular proteins, 7 plasma membrane proteins, and 23 cytosol and nuclear proteins (unpublished). We also found that heat shock protein 70, cathepsin B-like peptidase and thioredoxin-glutathione reductase might play key roles on the immune cell differentiation. Thus, the objective of our present study was to identify potential lncRNAs and mRNAs that might be novel targets for preventive, diagnostic or therapeutic strategies against fasciola infections. We posit that the identification of molecular targets that can regulate the M1 and M2 switch will spur strategies that can lead to early diagnosis, prevention or control *F. gigantica* infections. Moreover, elucidating the mechanism underlying macrophage polarization from the M2 to M1 subtypes during *F. gigantica* infection may provide insights into illuminating the mechanisms underlying helminth ESP-induced macrophage polarization.

Long non-coding RNAs (lncRNAs) are key regulators with diverse roles in various biological processes [12, 13]. Studies have shown that lncRNAs play essential roles in macrophage polarization and activation. For example, lnc-MC regulates polarization of

human mononuclear macrophages *via* absorption of miR-199a-5p [14], and overexpression of lncRNA E330013P06 induces inflammatory gene expression and enhances inflammatory signaling pathways [15]. In addition, TCONS_00019715 promotes macrophage polarization to the M1 phenotype [16]. However, whether lncRNAs play regulatory roles in helminth ESP-induced macrophage polarization remains unclear. To address this hypothesis, an lncRNA microarray-based assay was employed to identify the differentially expressed lncRNAs between *F. gigantica* ESP induced-M2 phenotype and IFN- γ + LPS-induced M1 phenotype. Our data showed that the expression of various lncRNAs and mRNAs consistently changed, and important biological processes and essential signaling pathways, were identified during polarization. These findings suggest that lncRNAs are important regulators of helminth ESP-induced macrophage polarization.

Materials and Methods

Ethics statement

This study was performed in accordance with the Guide for Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Ethics and Animal Welfare Committee of the Guangxi Institute of Fishery Sciences.

*Preparation of *F. gigantica* ESP*

All animal experimental procedures were performed according to the International Guiding Principles for Biomedical Research Involving Animals as documented by the International Organizations of Medical Sciences. The preparation of *F. gigantica* ESP was performed as described by El Ridi et al [39], with the exception that the *F. gigantica* adults were obtained from infected water buffalo instead of sheep. Concentrations of ESPs were tested and stored -80°C.

**F. gigantica* ESP-induced M2 cell purification and culture*

BALB/c mice (5-6 weeks old) were intraperitoneally injected with 0.1 mg *F. gigantica* ESP per mice at two days interval. The peritoneal cavity macrophages were isolated and collected at 1, 2, and 3 weeks after the first injection by density gradient centrifugation on Ficoll-Paque (Sigma-Aldrich, USA). Mice injected with PBS were used as controls. The purified macrophages were cultured in RPMI1640 medium containing 10% FBS and 0.05% glutamine (Sigma) for 48 h at 5% CO₂ and 37 °C [40], then the cells were collected for RNA isolation. M2 macrophage phenotype was identified by qPCR by using M2 macrophage molecule marker YM-1, arginase-1, and RELM α specific primers (Table 3).

M1 macrophage polarization

To induce M1 macrophage polarization, the identified *F. gigantica* ESP-induced M2 macrophages were treated with 10 ng/mL of IFN- γ (R&D Systems, USA) and 50 ng/mL of LPS (Sigma). After 24 h of polarization, the cells were collected for RNA isolation and M1 macrophage phenotype was identified by qPCR by using M1 macrophage molecule marker iNOS-specific primer (Table 3).

qPCR

Purified total RNA was reverse-transcribed by using the PrimeScript RT reagent (Takara, Japan). The expression pattern of selected lncRNAs and mRNA correlating with ESP-induced macrophage polarization were measured. qPCR was performed on ABI 7500 PCR instrument using SYBR green reagents (Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. The specific primer sequences are listed in Table 3. All qPCR experiments were performed in triplicate. The expression levels of RNA were normalized to internal control β -actin, and calculated with Δ CT method.

Western blotting assay

Samples were mixed with loading mix (Sample Reducing Agent; NuPAGE), denatured and resolved by SDS-PAGE, using pre-cast 4-12% NuPAGE gel followed by transfer onto nitrocellulose filter (BioRad Trans Blot) for Western blotting by electrophoresis at 30 volts for 1 h. Membranes were washed with 1xTBS and

Table 1. Top 15 differentially expressed lncRNAs and corresponding coding RNA

No	Sequence	Coding Gene	Fold Change	RNA length	Relationship	P-value
1	uc02evd1	Ptk6a5	1.8282361	1074	exon sense-overlapping	0.0296717
2	AK089651	AK089651	5.8302576	2688	intronic antisense	0.0437345
3	AK134149	AK134149	5.4947724	1461	natural antisense	0.0260267
4	uc007gc1	AK083606	5.1287667	3520	intergenic	0.0169856
5	ENSMUST00000133801	Aqpfh	4.8448616	889	exon sense-overlapping	0.0361825
6	ENSMUST00000154800	Gm13069	4.4691894	317	intronic antisense	0.0277560
7	ENSMUST00000131267	Kcub1	4.2878054	702	exon sense-overlapping	0.0408023
8	uc008uch.1	AK148461	4.0100251	2064	bidirectional	0.0351751
9	uc007fa.1	AK016100	3.8638325	793	intergenic	0.0468449
10	ENSMUST00000142699	Fam167b	3.8142052	527	exon sense-overlapping	0.0160623
11	AK005751	AK005751	3.5970826	692	intronic antisense	0.0312759
12	ENSMUST00000117368	Gm13916	3.3586363	628	intergenic	0.0265817
13	Human lincRNA1799+	Hs- lincRNA1799	2.9765229	8248	intergenic	0.0184289
14	ENSMUST00000142279	Gm11494	2.8129311	1738	intergenic	0.0296844
15	ENSMUST00000163450	Tno	2.6993409	888	exon sense-overlapping	0.0438254
16	AK089651	AK089651	5.8302576	2688	intronic antisense	0.0437345
17	AK134149	AK134149	5.4947724	1461	natural antisense	0.0260267
18	uc007gc1	AK083606	5.1287667	3520	intergenic	0.0169856
19	ENSMUST00000133801	Aqpfh	4.8448616	889	exon sense-overlapping	0.0361825
20	ENSMUST00000154800	Gm13069	4.4691894	317	intronic antisense	0.0277560
21	ENSMUST00000131267	Kcub1	4.2878054	702	exon sense-overlapping	0.0408023
22	uc008uch.1	AK148461	4.0100251	2064	bidirectional	0.0351751
23	uc007fa.1	AK016100	3.8638325	793	intergenic	0.0468449
24	ENSMUST00000142699	Fam167b	3.8142052	527	exon sense-overlapping	0.0160623
25	AK005751	AK005751	3.5970826	692	intronic antisense	0.0312759
26	ENSMUST00000117368	Gm13916	3.3586363	628	intergenic	0.0265817
27	humanlincRNA1799+	Hs- lincRNA1799	2.9765229	8248	intergenic	0.0184289
28	ENSMUST00000142279	Gm11494	2.8129311	1738	intergenic	0.0296844
29	ENSMUST00000163450	Tno	2.6993409	888	exon sense-overlapping	0.0438254
30	TC005_00025154	XL0C_018794	2.6404995	225	intergenic	0.0170768

then incubated for 1 h in 5% skim milk in TBS. Primary antibodies Anti-YM1 (1/1000) and Anti-iNOS (1/500) were then incubated with the blots overnight at 4°C. β -actin (1:200, Abcam, UK) was used as control. Incubation with Goat-anti-Rabbit IgG- HRP: 1/1000 for 1 h was followed with detection by enhanced chemiluminescence method according to the manufacturer's protocol (ECL kit; Amersham).

ELISA assay

The expression of cytokines IL-12(p70), and IL-10 in the culture supernatants was measured with ELISA according to the manufacturer's instructions (Invitrogen, UK). Briefly, 96 wells plates were coated at 50 μ l/well with capture antibody in carbonate buffer and incubated at 4°C overnight, followed by incubation in PBS + 4%BSA (200 μ l/well) 2 h at room temperature in dark. Then 50 μ l/well 2-folds diluted standard (Top concentration: IL10 at 10 ng/ml, IL-12(p70) at 50 ng/mg diluted with PBS-BSA 1%) was added, followed by the addition of 50 μ l/well samples and incubation at 4°C overnight. Plates were then incubated in biotinylated antibody (final solution: IL10 at 2 μ g/ml, IL-12(p70) at 1 μ g/ml) 1 h followed by 30 min incubation in AMDEX streptavidin-peroxidase (Sigma, UK) with dilution 1: 6000 in PBS-1%BSA. Finally, 50 μ l/well TMB (Invitrogen UK) was added and plates were incubated at room temperature. When blue color was intense only in upper standard wells, the reaction was blocked by addition of 20 μ l H₂SO₄ at 1mM. Plates were read at 450nm.

RNA isolation

Total RNA of the M1 and M2 macrophages was extracted by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA concentration and integrity was evaluated by NanoDrop ND-1000 spectrophotometer and denaturing electrophoresis, respectively.

Table 2. Top 15 differentially expressed mRNA

No.	Seq name	Gene Symbol	Fold Change	RNA length	P-value
1	NM_001159497	Ppm1b	6.6124735	1609	0.031609609
2	NM_134173	Vmn1r24	3.6058195	891	0.022019815
3	NM_009063	Rgs5	3.4941021	1809	0.024945756
4	NM_021307	Zfp112	3.2544098	3507	0.024820576
5	NM_001272045	Ces2h	3.057114	1677	0.009364195
6	NM_175263	Notum	2.928983	2020	0.01027892
7	NM_147079	Olfr547	2.7983575	940	0.004879411
8	NM_001113487	Sept9	2.6263821	3796	0.042643463
9	NM_001177778	Dlg3	2.5646848	4958	0.024382693
10	NM_027721	Katnal2	2.4535415	1673	0.049252194
11	NM_198962	Hcrtr2	2.3592752	3728	0.023600725
12	NM_001135991	Krtap10-4	2.3089433	902	0.011179904
13	NM_011997	Casp8ap2	2.2716324	6805	0.049740276
14	NM_029002	Nkx6-3	2.2297996	2236	0.0231563
15	NM_172873	Cdkp2	2.1194175	2611	0.033573453
16	NM_009690	Cd5l	47.1045255	2037	0.033771358
17	NM_001025574	Bpifb9b	23.9093383	2083	1.56801E-05
18	NM_174990	Gimap4	20.0433599	1814	0.031241985
19	NM_018781	Egr3	7.517049	1416	0.011625404
20	NM_001199224	Agbl1	6.5710109	3369	0.000778652
21	NM_009638	Crisp1	6.1722717	1416	0.048338258
22	NM_152839	Igj	5.4467555	2111	0.030702143
23	NM_001039223	Gm14137	5.4000889	3067	0.02536975
24	NM_016696	Gpc1	4.5873121	3552	0.003945357
25	NM_009994	Cyp1b1	4.5443241	5128	0.048170482
26	NM_021985	Tnfrsf18	4.2782858	817	0.04512882
27	NM_024245	Kir23	4.0384839	3439	0.043661422
28	NM_011346	Sell	3.8263997	2341	0.017107466
29	NM_016707	Bcl11a	3.516451	3425	0.012461752
30	NM_001077508	Tnfrsf9	3.5154845	1999	0.030462784

<http://www.kegg.jp/>) was utilized to confirm pathway enrichment. The *p*-value was used to indicate the significance of the pathway, and the lower of the *p*-value, the higher the significance of the pathway.

CNC analysis

CNC analysis was performed by calculating the Pearson correlation coefficient (PCC) between the expression levels of coding and noncoding genes. We initially selected the DE lncRNAs and mRNAs from microarray data, and the expressions of these molecules in ESP-induced macrophages and M1 macrophages were respectively confirmed by qPCR. Based on the qPCR results and the PCC value ($PCC \geq 0.995$ and $P < 0.05$) between the lncRNA and mRNA, the co-expression network was built and illustrated using Cytoscape (v3.4.0). In the network, the value of interaction represented the likelihood that the two genes were co-expressed. Higher values indicated stronger interaction, and the degree value represented the number of interacting genes. Furthermore, higher degrees suggested higher likelihood of centrality location of the mRNA or lncRNA in the network. Analyses were performed by KangChen Bio-tech, Shanghai, China.

Construction of ceRNA networks

lncRNAs or mRNAs can interact with miRNAs through their MicroRNA Recognition Elements (MREs) within a ceRNA network. The ceRNA network was constructed as follows: we initially selected the DE lncRNAs and mRNAs in microarray data, and the expressions of these molecules in ESP-induced macrophages and M1 macrophages were confirmed by qPCR, respectively. Based on the qPCR results and the screening parameter (fold change of ≥ 2.0 and P value < 0.05), the dysregulated lncRNA or mRNA was used to perform miRNA target prediction using the latest miRBase, which collects abundant miRNAs and is extensively used as miRNA reference [41-43]. The obtained miRNAs were screened by miRanda and TargetScan programs. lncRNAs or mRNAs that possessed the MREs of targeted miRNAs were predicted by RNA22 (<https://cm.jefferson.edu/rna22/Precomputed/>) and PITA (http://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). The ceRNA network was built and illustrated by using Cytoscape (v3.4.0). Analyses were performed by KangChen Bio-tech, Shanghai, China.

Statistical analysis

Numerical data were analyzed by software SPSS Statistics 17.0 (SPSS Inc.) using the two-tailed Student's *t*-test, ANOVA, and the Mann-Whitney test. All data were presented as the mean $\pm$ standard error of the mean (SEM). Spearman correlation was used to determine the relationship between lncRNAs and their target genes. An absolute fold change ≥ 2 , $P < 0.05$ was used to screen differentially expressed lncRNA and mRNA. $P < 0.05$ was considered statistically significant.

Results

Differentially expressed lncRNAs and mRNAs in ESP-induced M2 cells compared to polarized M1 cells

Peritoneal macrophages from *F. gigantica* ESP-injected BALB/c mice were isolated, and the phenotypes were identified by PCR and qPCR using phenotype-specific primers. Fig. 1A shows that all three M2 phenotypic molecular markers resulted in positive amplification (Fig. 1A (a)), thereby suggesting successful induction of ESP-mediated M2 cells. Western blotting and qPCR assay further verified stronger M2 phenotype was induced by *F. gigantica* ESP and the phenotype was successfully switched into M1 macrophage (Fig. 1A (b, c)). Furthermore, ELISA results showed that ESP-stimulated M2 macrophages secreted higher levels of IL10 and low IL12 than M1 cells, and upon IFN γ +LPS-induced M2 to M1 polarization, lower levels of IL10 and increased IL12 secretion were observed (Fig. 1A (d)). To explore the dysregulated lncRNAs and mRNAs during IFN γ +LPS-induced polarization from M2 to M1 macrophages, we determined the lncRNA and mRNA expression profiles using microarray analyses of M2 and M1 macrophages. Heatmaps, scatter plots, and volcano plots were used to assess variations in lncRNA expression between M1 and M2 cells (Figs. 1B-G). All lncRNAs and mRNAs showing a two-fold change in signal intensity (p value < 0.05) were identified as differentially expressed when the M1 subtype was compared to the M2 cells. In total, 35,667

Fig. 1. Differentially expressed lncRNAs in *F. gigantica* ESP-induced M2 macrophages compared with polarized M1 macrophages. (A) Identification of the phenotype of *F. gigantica* ESP-induced macrophages. a. PCR identification of ESP-induced macrophages and the macrophages re-induced by IFN γ +LPS. The molecules YM1, RELM α and Arginase-1 were used as markers for M2 phenotype, while iNOS was as M1 phenotype. b. Western blotting of the *F. gigantica* ESP-induced macrophages and the macrophages re-induced by IFN γ +LPS. c. qPCR assay of the *F. gigantica* ESP-induced macrophages and the macrophages re-induced by IFN γ +LPS. Three duplicated wells of each phenotype of macrophage were collected for qPCR assay (n = 3). d. ELISA

assay of the *F. gigantica* ESP-induced macrophages and the macrophages re-induced by IFN γ +LPS. IL10 represents M2 macrophage cytokine, while IL12 represents M1 macrophage cytokine. (B and C) Differentially expressed lncRNAs (B) and mRNAs (C) between *F. gigantica* ESP-induced M2 macrophages and IFN γ +LPS re-induced M1 macrophages were analyzed using hierarchical clustering; Three parallel samples in each group were analyzed. 'red' indicates high expression, and 'green' indicates low expression. (D and E) A scatter plot is used to assess lncRNA (D) and mRNA (E) expression variations between *F. gigantica* ESP-induced M2 macrophages and polarized M1 macrophages. lncRNAs and mRNAs above the top green line and below the green line exhibited a greater than 2.0-fold changes. (F and G) A volcano plot is used to assess lncRNA (F) and mRNA (G) expression variations between *F. gigantica* ESP-induced M2 macrophages and polarized M1 macrophages. Red plots represent differentially expressed lncRNAs and mRNAs.

Fig. 2. Statistics of lncRNAs, mRNAs and UCRs obtained during the differentiation from *F. gigantica* ESP-induced M2 to polarized M1 phenotype macrophages. (A) and (B) Total numbers of lncRNAs, mRNAs and UCRs, and differentially expressed lncRNAs, mRNAs and UCRs were presented, respectively. (C, D, E, F) Six types of lncRNAs and UCRs were found in total and differentially expressed lncRNAs (C and D) and UCRs (E and F), respectively. Only those fold changes > 2.0 and $p < 0.05$ were counted as differentially expressed genes. (G) Heatmap of UCRs obtained during macrophage differentiation.

lncRNAs and 22,201 mRNAs were obtained (Figs. 2 A, B), and among these, 2,844 lncRNAs and 1,782 mRNAs were differentially expressed (Figs. 2 A, B). In the differentially expressed lncRNAs, 1,579 transcripts were upregulated and 1,265 transcripts were downregulated. Furthermore, 789 and 993 mRNAs were up- and downregulated, respectively (Figs. 2 A, B). In particular, one type of lncRNAs, known as ultra-conserved RNAs (UCRs), was observed during macrophage polarization. Figures 2 A, B, and G show that a total of 243 UCRs were differentially expressed, of which 52 were upregulated and 191 were downregulated. In total, six types of lncRNAs and UCRs were identified during polarization (Figs. 2 C-F). The up- and downregulated lncRNAs (absolute fold change ≥ 2) identified in the microarray analyses are presented in Table 1 and 2.

Validation of the microarray data by qPCR

qPCR analysis was performed to verify the reliability of the microarray results, and the expression levels of randomly selected 34 lncRNAs and mRNAs in ESP-induced M2 and polarized M1 macrophages were detected. All primers are presented in Table 3. The qPCR results indicated that 30 transcripts out of 34 selected genes were positively amplified, of which 20 were used in generating the Pearson correlation coefficient (PCC) in M1 and M2 macrophages. Fig. 3 A shows the 12 transcripts (Ch25h, Ube4b, Rragd, Scd1, Gpr55, Btk, ENSMST00000154000, ENSMST00000117590, ENSMST00000168470, ak043175.1, uc0008hps.1, and ENSMST00000118407) that were verified by the qPCR assay and coincided with the PCC results ($r = 0.726$ in M1 and $r = 0.684$ in M2) between the qPCR value and the microarray results in both M1 and M2 cells (Fig. 3 B).

Fig. 3. QPCR validation of candidate lncRNAs and mRNAs among *F. gigantica* ESP-induced M2 macrophages and the polarized M1 macrophages. The relative expression levels of 34 candidate lncRNAs and mRNAs were detected by qPCR in ESP-induced M2 macrophages and the polarized M1 macrophages. The ΔC_t values of the lncRNAs and mRNAs were determined by subtracting the β -actin ΔC_t value. A smaller ΔC_t value is, the higher expression level indicates. (A) Twelve lncRNAs and mRNAs were presented to represent the results of qPCR validation. * indicates $p < 0.05$. Three duplicated wells of each phenotype of macrophage were collected for qPCR assay ($n = 3$). (B) Ten lncRNAs and mRNAs in M1 and M2 macrophages, respectively were selected randomly to evaluate the relationship of microarray results and qPCR value which was analyzed by Spearman's coefficients. (C) Heatmaps of nine picked lncRNAs and their neighboring mRNAs, and (D) presented their relationships analyzed by Spearman's coefficients.

The correlation of lncRNAs and associated coding genes

Previous studies have indicated that lncRNAs can affect the expression of neighboring protein-coding genes. Based on the UCSC database (<http://genome.ucsc.edu/>), our microarray provided data on nearby protein-coding genes (Tables 1 and 2). To investigate the association between lncRNA expression and associated protein-coding genes, we randomly selected 10 paired lncRNAs and mRNAs for qPCR and PCC analyses. Figures 3 C and D show that the expression profiles of the 10 paired lncRNAs and mRNAs were positively correlated ($p < 0.05$), and the correlation index was 0.758 in M1 and 0.831 in M2, respectively, thereby indicating good consistency between qPCR values and the microarray results in both the M1 and M2 cells.

Bioinformatic analysis of differentially expressed lncRNAs

To explore the potential functions and mechanism of the lncRNAs during polarization from M2 to M1 macrophages, we conducted GO and pathway analyses for the target genes of the differentially expressed lncRNAs. The biological process (Figs. 4 A, B), molecular function

Fig. 4. GO analysis and pathway analysis of the differentially expressed lncRNAs. Top ten GO terms of biological processes (A and B) and molecular function (C and D) for lncRNAs upregulated and downregulated during the differentiation from *F. gigantica* ESP-induced M2 to polarized M1 phenotype macrophages. (E and F) Top ten pathways corresponding to the lncRNAs upregulated and downregulated during the differentiation from *F. gigantica* ESP-induced M2 to polarized M1 phenotype macrophages.

(Figs. 4 C, D), and cellular component (unpublished) were predicted by the GO Project. Biological processes revealed that the associated protein-coding gene functions of upregulated lncRNAs (M1 vs. M2) were primarily involved in positive regulation of endopeptidase and peptidase activity, cell fate determination, and establishment of planar polarity (Fig. 4 A). In addition, the associated protein-coding gene function of the downregulated lncRNAs primarily included post-translational protein modification, response to organophosphorus, adult behavior, and response to purine-containing compounds (Fig. 4 B). In terms of molecular function, the genes of upregulated lncRNAs primarily included peptidase activator activity, enzyme regular activity, cysteine-type endopeptidase regulator activity involved in apoptotic process, and glucose binding. The genes of downregulated lncRNAs were primarily involved in transition metal ion binding, cation binding, C-C chemokine receptor activity, and metal ion binding. Pathway analysis showed that these gene products were involved in several signaling pathways. Regarding the upregulated transcripts (M1 vs. M2), the major pathways included butyrosin and neomycin biosynthesis, galactose metabolism, fructose and mannose metabolism, and carbohydrate digestion and the absorption signaling pathway (Fig. 4 E). The downregulated transcripts were mainly involved in RNA degradation, glycosphingolipid biosynthesis (ganglio series), fatty acid elongation, and the maturity onset diabetes of the young signaling pathway (Fig. 4 F).

Fig. 5. Coding and non-coding gene (CNC) network example of the lncRNA and mRNA. Totally eight significantly expressed lncRNAs and mRNAs were conducted to build the CNC network. (A) represented the CNC network of two mRNAs and (B) represented the CNC network of lncRNAs. In each network, dozens of mRNAs or lncRNAs were connected. The value of interaction represents the likelihood that the two genes are co-expressed. The bigger of the valve, the stronger of the interaction. The degree value represents the number of genes that can interact with. The higher the degree, the more centrally the mRNA or lncRNA locates in the network.

Coding and non-coding gene (CNC) analysis

To explore the relationship of coding mRNA and its non-coding genes, we performed the CNC analysis by calculating the PCC between the expression levels of coding and non-coding genes. In total, eight differentially expressed mRNAs and lncRNAs were selected, and the co-expression network was built and illustrated using Cytoscape (v3.4.0). As shown in Fig. 5 A, two mRNA and two lncRNAs were used to illustrate CNC relationship. Rragd had 24 while Gimap1 had 28 differentially expressed lncRNAs, as indicated by statistically significant *p* values. Among these, 16 and 13 genes were downregulated, and 8 and 15 were upregulated, corresponding to Rragd and Gimap1, respectively. The other two selected mRNAs, Btk and Scd1, had 55 and 130 differentially expressed lncRNAs, respectively (unpublished). For lncRNAs, ENSMUST00000119016 and ENSMUST00000121286 were selected to show their relationships with corresponding mRNAs (Fig. 5 B), and had 24 and 28 differentially expressed mRNAs based on the *p* value, respectively. The other two selected lncRNAs, ENSMUST00000143435 and human lincRNA1907, had 55 and 130 differentially expressed mRNAs, respectively (unpublished).

Construction of competing endogenous RNAs (ceRNA) network

Because mRNAs and lncRNAs can interact with miRNAs through their MREs within a ceRNA network [17], we initially selected four mRNAs based on qPCR validation results and searched for putative MREs of Gimap1, Rragd, Scd1, and Btk by RNA22 and PITA analyses. As shown in Fig. 6 A, a total of 38 lncRNAs, 8 mRNAs, and 41 miRNAs were predicted to be involved in the Gimap1 ceRNA networks. CK375702, AK135613, mouselincRNA1395+, and AK133468 were the top four lncRNAs with the highest number of MREs shared with ceRNA and Gimap1. Moreover, 41 miRNA binding sites were shared with ceRNA and Gimap1. For each miRNA, 1 to 6 MREs were found to bind to Gimap1, respectively. Two examples of the MREs binding site are shown in Figs. 6 A, B, in which there were eight MREs in seed sequences. The ceRNA networks of Rragd included 7 lncRNAs and 180 miRNAs (Fig. 6 B). In total, 180 MREs were shared with ceRNA and Rragd. HumanlincRNA0957-, humanlincRNA0490-, humanlincRNA2090+, and humanlincRNA1159- were the top four lncRNAs with the highest number of MREs that were shared between ceRNA and Rragd. For each miRNA, 1 to 4 MREs were found to bind to Rragd, respectively. We further conducted ceRNA network for Scd1 and Btk and found that 34 lncRNAs and 192 miRNAs were involved in the Scd1 ceRNA network, while 241 lncRNA, 65 mRNAs, and 47 miRNAs were involved in the Btk ceRNA network (unpublished).

Discussion

Fasciola ESP-induced M2 cells exhibit common immunobiological features [5, 6]. Previous studies have examined *Fasciola* ESP-induced M2 macrophage polarization in mice and human [18, 19]. In the current study, our objective was to identify potential lncRNAs and mRNAs that might be novel targets for preventive, diagnostic or therapeutic strategies against fasciola infections. We hypothesized that the identification of molecular targets that can regulate the M1 and M2 switching, may be important in discovering ways in which *F.gigantica* infections may be controlled by genetic engineering. LncRNAs have been shown to play important roles in macrophage polarization and activation [14-16]; therefore, we explored whether lncRNAs extended this function to *F. gigantica* ESP-induced macrophage polarization. In our study, immunological reactions in which mice responded to ESP stimulation *in vivo* were simulated, and we detected the differentially expressed lncRNAs and mRNAs in *F. gigantica* ESP-induced M2 cells and compared these with IFN- γ + LPS-induced M1 cells. Our data showed that some lncRNAs and their target genes significantly increased or decreased during the phenotype switching, suggesting our strategy was meaningful. To the best of our knowledge, this is the first report on lncRNA expression profiling in *F. gigantica* ESP-induced M2 polarization into M1 cells, providing clues in the elucidation of the mechanism of helminth-induced M2 regulation in helminth-related microenvironment.

Compared to protein-coding genes, lncRNAs exhibit spatio-temporal specific expression profiling, which makes lncRNAs a promising molecular tool for decoding biological process-specific mechanisms. For example, lncRNAs were found to be highly dynamic and showed cell-specific expression patterns during T cell polarization. Specifically, lncR-Ccr2-5'AS, together with GATA-3, is essential in the regulatory circuit in gene expression specific to the migration of Th2 cells [20]. Such roles of lncRNAs were also found in CD8⁺ T cell [21] and CD4⁺ T cell polarization [22, 23]. More importantly, lncRNAs also play essential roles on macrophage polarization. For instance, knocking down TCONS_00019715 following the activation of THP-1 cells using IFN- γ and LPS diminished the expression of M1 markers and elevated the expression of M2 markers [16]. Another lncRNA, lnc-MC, acted as a ceRNA to miR-199a-5p and alleviated repression of activin A receptor type 1B (ACVR1B), a regulator of monocyte/macrophage polarization [14]. In our study, 1, 579 and 1, 265 lncRNAs and 789 and 993 mRNAs were upregulated and downregulated, respectively, in *Fasciola* ESP-induced M2 cells differentiated into IFN- γ + LPS-induced M1 cells. Among the lncRNAs, 52 upregulated and 191 downregulated UCRs were differentially expressed. We compared these UCRs with human T-UCRs, and found that five UCRs (uc.172, uc.352, uc.101, uc.131, and uc.306) were expressed in both human T-UCRs and our database, suggesting these UCRs are conserved and may be important for macrophage polarization. This indicated that lncRNAs indeed play essential roles on the biological process of *Fasciola* ESP-induced M2 cells polarization into M1 cells.

To select lncRNAs and mRNAs that play important roles in *Fasciola* ESP-induced M2 cells polarization into M1 cells, differentially expressed lncRNAs and mRNAs between *Fasciola* ESP-induced M2 cells and IFN- γ + LPS-induced M1 cells were selected and used in validating the microarray results with qPCR analysis. Our qPCR results indicated that most of the lncRNA and mRNA expression profiles coincided with the microarray results, and were further supported by the CNC profiling results. The results of the CNC analysis and qPCR assays consistently revealed that lncRNAs play roles during M2 cell polarization into M1 cells. Our results, to some extent, are also consistent with those of Huang et al [16]. However, because no lncRNAs patterns during helminths ESP-induced macrophages polarization have been reported to date, we cannot directly compare our data to those of Huang et al., and thus we are unable to elucidate common underlying mechanisms. In addition, the objective of our study differed from theirs. Our aim was to investigate lncRNA profiles during macrophage polarization from ESP-induced M2 to M1 (M1 vs. M2), whereas the data from Huang et al. examined lncRNA expression during macrophage polarization from M0 to M1 and M2 (M1 vs. M0, M2 vs. M0, M2 vs. M1). Moreover, the major difference in both studies are the stimulators. In the present study, the ESP was used to induce the M2 phenotype, whereas the IL4 was used by Huang et al. Furthermore, we obtained M2 macrophages *in vivo*, that is because only the real physiological and immunological conditions can represent the real immunological reactions in which mice respond to ESP stimulation. Therefore, the lncRNAs produced in both studies during the biological process were different. Because macrophages are quite plastic cells, it is quite possible that different lncRNAs are produced in different immunological conditions.

Our qPCR results showed that some mRNAs such as Rragd, Scd1, Gimap1, and Btk show good PCC relationship with their corresponding lncRNAs, including ENSMUST00000119473, uc008hps.1, ENSMUST00000155906, and ENSMUST00000117590. This findings suggests that differentially expressed mRNAs and lncRNAs may be co-regulated by certain networks. We therefore investigated the functions of Rragd, Scd1, Gimap1, and Btk. Rragd (Ras-related GTP-binding D) that might be involved in transmitting signals within macrophages. Ras-regulated signalling pathways control processes such as cell proliferation, polarization, adhesion, and migration. Scd1 (stearoyl-coenzyme A desaturase 1) is the rate-limiting enzyme in the synthesis of monounsaturated fatty acids. Nakaya et al. reported that macrophage-specific SCD1 overexpression promoted overall reverse cholesterol transport through increased cholesterol efflux of high-density lipoproteins [24]. Gimap1 (GTPase, IMAP family member 1) has been suggested as essential for the development of mature B and T lymphocytes [25]

and its expression in human T cell may vary under Th1 or Th2 polarization conditions *in vitro* [26]. Btk (bruton agammaglobulinemia tyrosine kinase) has been reported to regulate macrophage polarization in response to LPS [27]. Furthermore, Feng et al. demonstrated that macrophages express calreticulin and that TLR signaling through Btk results in its trafficking to the cell surface, where it induces macrophage-mediated programmed removal of tumor cells [28]. Given these genes functionally act in macrophages and their corresponding lncRNAs co-regulate with them in some networks, these lncRNAs may logically play crucial roles in macrophage polarization, and thus contribute to helminth-induced immunological progresses in which macrophages play essential roles. Although gain-of-function and loss-of-function experiments on these lncRNAs and mRNAs were not been performed in this study, their expression patterns in M1 and M2 cells provide clues that they may be crucial in the regulation of M2 or M1 polarization.

A large number of lncRNAs were identified in our study. However, the functions of these lncRNAs remain unclear. To infer the possible functional roles of the lncRNAs between *F. gigantica* ESP-induced M2 cells and IFN- γ + LPS-induced M1 cells, GO and pathway analyses were initially used for the functional analysis of the lncRNA target gene pool. GO analysis revealed that differentially expressed lncRNAs were most highly enriched in "regulation of peptidase activity", "metabolism", and "post-translational protein modification". Moreover, pathway analysis indicated that "galactose metabolism", "fructose and mannose metabolism", "carbohydrate digestion and absorption signaling pathway", "glycosphingolipid biosynthesis", and "fatty acid elongation" were involved in the processes of *F. gigantica* ESP-induced M2 polarization into M1 cells. Therefore, it is reasonable to infer that these differentially expressed lncRNAs act mainly by regulating the peptidase activity and carbohydrate metabolism, which in turn affects the regulation of peptidase activity and metabolism of macrophages. Moreover, lncRNAs have been well known to regulate cell cycle progression, imprinting, cell polarization, and development in response to cellular and/or developmental signals [29]. However, whether lncRNAs are involved in peptidase activity and carbohydrate metabolism remains unclear. Nonetheless, some studies have reported that lncRNAs can regulate carbohydrate metabolism progression in cancers. For instance, lncRNA GCASPC regulate glycolysis in cancer cells by directly binding with key glycolytic enzymes [30] and lncRNA NRCP is an intermediate binding partner between STAT1 and RNA polymerase II, leading to increased expression of glucose-6-phosphate isomerase [31]. Zhao et al. reported that LINC00092 interacts with 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 2 (PFKFB2) and is involved in increased glycolysis levels in ovarian cancer metastasis [32]. Moreover, GAL lncRNAs (galactose-related gene in budding yeast *Saccharomyces cerevisiae*) promote induction from a repressed state that could have biological relevance to galactose metabolic adaptation [33]. Although no lncRNA studies have been reported on the regulation of peptidase activity and carbohydrate metabolism in macrophages or even parasite ESP-induced macrophage polarization, our current study provides evidence that ESP-induced lncRNAs could regulate macrophage polarization by modulating peptidase activity and/or carbohydrate metabolism, which may be meaningful in the elucidation of a new mechanism underlying parasite-induced macrophage phenotype differentiation.

Increasing literatures have confirmed that ceRNAs play key roles in many biological processes and immune responses [34, 35], and miRNAs have been documented to be involved in the regulation of macrophage polarization [36, 37], similar to lncRNAs [14-16]. We were interested in identifying whether an mRNA-related ceRNA network existed during *F. gigantica* ESP-induced M2 cell polarization into M1 cells. We selected four differentially expressed mRNAs (Gimap1, Rragd, Scd1, and Btk) and built respective ceRNA networks since these mRNAs were identified and confirmed by qPCR and CNC analyses. We found that almost three types of RNAs were included in each ceRNA network, implying a complicated regulation network modulate each mRNA. Given that these mRNAs play essential roles on ESP-induced macrophage polarization, lncRNAs and miRNAs might also play regulatory roles during this differentiation process. Although we did not confirm the functions of the lncRNAs and miRNAs in our study, we found that some miRNAs in the ceRNA network were possibly involved in

macrophage function. For example, miR-6955-5p, miR-6953-5p, and miR-1894-5p were the top three miRNAs of Gimap1 and miR-3076-5p, miR-346-5p, and miR-6381 were the top three miRNAs of Rragd, all of which have more than 4 binding sites on their corresponding mRNAs, thus may likely function as “molecular sponges” that compete with corresponding lncRNAs. CeRNAs have been widely implicated in various biological processes, and studies have confirmed that disorders involving the ceRNA network can induce tumorigenesis [38]. Although ceRNA has not been reported in parasite ESP-induced macrophage polarization, ceRNA may be involved in M2 macrophage differentiation. Nevertheless, the ceRNA network presented in this study highlights an unknown ceRNA regulatory network in ESP-induced macrophage polarization.

Although our work is the first report on lncRNA profiling in *F. gigantica* ESP-induced M2 polarization into M1 cells, we realize that some limitations should be addressed. One such shortcoming might be the lack of large number of lncRNA candidates that have been identified as therapeutic targets, thus comparisons are incomplete. Another weakness might be the non-deep verification of the ceRNAs regulatory networks due to lack of specific cell lines and animal models. Nonetheless, we believe that these limitations will be addressed in our future studies focusing on the functions and therapeutic potentials of the discovered lncRNAs and mRNAs in the present study.

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Honglin Luo designed and coordinated the study, analyzed the data, and wrote the manuscript. Yaoyao Zhang performed cell culture and qPCR identification. Zhaoan Sheng participated in qPCR identification and data preparation. Tao Luo, Jie Chen and Junjie Liu developed the methodologies for cell culture, bioinformatics analysis, and mouse keeping. Huifeng Wang, Miao Chen and Yunliang Shi collected experimental materials and analyzed the data. Lequn Li conceived the study, drafted the manuscript, and provided crucial advice on data analysis. All authors read and approved the manuscript.

Disclosure Statement

The authors declare to have no conflict of interests.

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