Temporal expression profiles of lncRNA and mRNA in human embryonic stem cell-derived motor neurons during differentiation

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Abstract

Background: Human embryonic stem cells (hESC) have are an invaluable research tool to study motor neuron development and disorders. However, transcriptional regulation of multiple temporal stages from ESCs to spinal motor neurons (MN) has not yet been fully elucidated. Thus, the goals of this study were to profile the time-course expression patterns of lncRNAs during MN differentiation of ESCs and to clarify the potential mechanisms of the lncRNAs that are related to MN differentiation.

Methods: We utilized our previous protocol which can harvest motor neuron in more than 90% purity from hESCs. Then, differentially expressed lncRNAs (DElncRNAs) and mRNAs (DEmRNAs) during MN differentiation were identified through RNA sequencing. Bioinformatic analyses were performed to assess potential biological functions of genes. We also performed qRT-PCR to validate the DElncRNAs and DEmRNAs.

Results: Total 441 lncRNAs and 1068 mRNAs at day 6, 443 and 1175 at day 12, and 338 lncRNAs and 68 mRNAs at day 18 were differentially expressed compared with day 0. Bioinformatic analyses identified that several key regulatory genes including POU5F1, TDGF1, SOX17, LEFTY2 and ZSCAN10, which involved in the regulation of embryonic development. We also predict 283 target genes of DElncRNAs, in which 6 mRNAs were differentially expressed. Significant fold changes in lncRNAs (NCAM1-AS) and mRNAs (HOXA3) were confirmed by qRT-PCR. Then, through predicted overlapped miRNA verification, we construct lncRNA NCAM1-AS-miRNA-HOXA3 network.

39   **Conclusion:** This study comprehensively identified lncRNAs and mRNAs during MN
40   differentiation of hESCs, providing the theoretical basis for further study of the regulatory role of
41   lncRNAs in MN differentiation.
42
43   **Keyword**s: Embryonic stem cells; Long non-coding RNA; Motor neuron; Differentiation; RNA
44   sequencing
45

46   **Introduction**
47   Generation of specific cell types from human embryonic stem cells (hESCs) in vitro have
48   provided powerful platforms to study human disease and to understand fundamental biological
49   processes. Highly efficient directed differentiation of hESCs into spinal motor neurons have been
50   used to explore not only MN development but also MN disorders mechanism [1, 2]. Motor
51   neurons are responsible for innervating skeletal muscles in the periphery and controlling
52   movement. Transcription factors (TFs) regulate precise temporal and spatial gene expression in
53   motor neuron specification and differentiation [3, 4]. The TFs Olig2 and Ngn2 function in
54   opposition to regulate gene expression in MN progenitors in the pMN domain and the TFs Isl1
55   and Lhx3 are crucial for specifying MN identity [5-7].
56   LncRNAs, ranging in length from 200 nt to 100 kb, are highly expressed in the central nervous
57   system. Accumulating evidence suggested that lncRNA played crucial roles in numerous
58   biological and pathological processes at the chromatin remodeling level, transcriptional level and
59   post-transcriptional level [8, 9]. Notably, lncRNAs function as key regulators of cell
60   differentiation and development, especially in neurogenesis. Particularly, lncRNAs can regulate
61   ESC pluripotency and control multiple lineage differentiation by association with miRNAs,
62   RNA-binding proteins, and epigenetic modifiers [10]. LncRNA-1604 functioned as competing
63   endogenous RNAs (ceRNAs) of miR-200c and indirectly regulated the core TFs ZEB1 and
64   ZEB2 during neural differentiation from mouse ESCs [11]. LncRNA Haunt functions as a
65   genetic enhancer and an epigenetic repressor of HOXA gene activation during ESC
66   differentiation [12]. *Dlk1-Dio3* locus-derived lncRNAs play a critical role in maintaining
67   postmitotic MN cell fate by repressing progenitor genes and they shape MN subtype identity by
68   regulating Hox genes [13]. Nevertheless, at present very little functional characterization of
69   lncRNAs in human motor neuron differentiation has been elucidated.
70   We used spinal motor neuron differentiation to profile the temporal changes without further
71   purification steps. We combined highly efficient MN differentiation of hESCs in vitro with
72   RNA-seq analysis to reveal the expression profiles of lncRNAs and mRANs. Our findings may
73   provide a new theoretical basis for further studies on lncRNAs modulation of motor neuron
74   differentiation.

Materials and Methods

Cell cultures form embryonic stems cells to spinal motor neurons

H9 (WA09, NIH registry 0046) hESC lines were obtained from WiCell Research Institute (Madison, WI). Human ESCs were maintained on irradiated mouse embryonic fibroblasts and differentiated as described before [2]. Briefly, ESCs were cultured using MN differentiation medium containing DMEM/F12, Neurobasal medium, N2, B27, ascorbic acid, Glutamax and penicillin/streptomycin (All from Gibco). The medium was additionally supplemented with chemical compounds: 3μM CHIR99021 (Torcris), 2μM DMH-1 (Torcris) and 2μM SB431542 (Stemgent) for 6 days differentiated into neuroepithelial progenitors; 1μM CHIR99021, 2μM DMH-1, and 2μM SB431542, 0.1μM RA (Sigma) and 0.5μM Purmorphamine (Pur, Sigma) for 6 days differentiated into MN progenitors; 0.5μM RA and 0.1μM Purmorphamine for 6 days differentiated into MNs.

Total RNA isolation and RNA sequencing

Total RNA from samples was extracted using the Trizol reagent (Invitrogen). RNA quantity and purity of total were confirmed using NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was assessed by Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Ribosomal RNA was removed. Sequencing libraries were constructed. The dTTP were replaced by dUTP in the reaction buffer during second strand cDNA synthesis. Products were purified and library quality was assessed on the Agilent Bioanalyzer 2100 system. RNA Sequencing was performed on the Illumina HiSeq ((Illumina, USA) by KangChen Biotechnology Corporation (Shanghai, China) using next-generation sequencing analysis.

Immunocytochemistry

The primary antibodies information was used: SOX1 (gIgG, 1:1000, R&D), HOXA3 (mIgG, 1:1000, R&D), OLIG2 (rIgG, 1:1000, Millipore), HB9 (rIgG, 1:1000, Millipore), NF-200 (mIgG, 1:400, CST). Image J was used to perform cell counting (NIH, USA).

Quantitative real-time PCR

Total RNA was extracted using the Trizol reagent (Invitrogen). qRT-PCR was then performed and the $2^{-\Delta\Delta Ct}$ method was calculated for quantification. The GAPDH was used as an internal control. The primer sequences used are listed in Table 1.

Bioinformatic analysis

Gene Ontology (GO) analysis was used to investigate differentially expressed mRNAs with GO categories. The predicted target genes above were conducted using the DAVID database (http://david.abcc.ncifcrf.gov/). GO terms with a P value < 0.05 were considered as significantly enriched. PPI networks was used STRING database (https://string-db.org/) and Cytoscape. The networks were visualized in CytoHubb plug-in of Cytoscape. LncRNAs and mRNAs possessing

114   microRNA recognition elements (MREs) for the targeted miRNAs were predicted using the
115   miRanda and TargetScan.
116
117   **Statistical analysis**
118   All qRT-PCR results are expressed as the means ± SEM of at least three independent
119   experiments. Statistical analyses were performed with SPSS statistics software version 22.0. P
120   values < 0.05 was considered statistically significant. All graphs were made with GraphPad
121   Prism 8.
122

Results

Differentiation of high purity motor neuron from human embryonic stem cells

In this study, we used hESCs to differentiate into spinal cord MNs in vitro. The MNs were generated using chemical protocol described as method part [2] (Figure 1A). The ESC can differentiate into SOX1 neuroepithelial progenitors at day 6, OLIG2 motor neuron progenitors at day 12, and HB9 motor neuron at day 18 high efficiently by using a combination of small molecules (Figure 2).

Patterns of gene expression changes from hESCs to motor neuron

High throughput sequencing is an efficient approach for investigating the biological function of RNAs. All the differently expressed DElncRNAs and DElncRNAs were statistically significant ($P < 0.05$) with fold change (FC) > 2.

   A total of 441 DElncRNAs (192 up-regulated and 249 down-regulated), 443 DElncRNAs (198 up-regulated and 245 down-regulated) and 338 DElncRNAs (164 up-regulated and 174 down-regulated) were identified in D6 *vs* D0 (neuroepithelial progenitors, NEP), D12 *vs* D0 (motor neuron progenitors, MNP), and D18 *vs* D0 (motor neuron, MN) respectively (Additional file 1). The volcano plots of expression profile for all the detected transcripts showed the relationship between the fold change and the significance (Figure 2A). To determine the key RNAs in human MN differentiation, we analyzed DElncRNAs in Venn diagram form. Veen analysis revealed that 33 lncRNAs were simultaneously up regulated and 78 were down regulated on D6, D12 and D18 (Figure 2B). Meanwhile, a total of 1068 DEmRNAs (360 up-regulated and 708 down-regulated), 1175 mRNAs (444 up-regulated and 731 down-regulated) and 68 DEmRNAs (34 up-regulated and 34 down-regulated) were identified in D6 *vs* D0, D12 *vs* D0 and D18 *vs* D0 respectively (Additional file 2). The volcano plots was shown in Figure 3A. Veen analysis revealed that 8 mRNAs were simultaneously up-regulated and 34 down-regulated (Figure 3B).

Validation of lncRNAs and mRNAs expression

To verify the results of the RNA sequencing, five strongly expressed DElncRNAs and DEmRNAs during MN differentiation with FC＞40 were selected for qRT-PCR validation. As shown in Figure 4A, the expression of ZSCAN10, OCT4 and VRTN were down regulated, while HOXA3 and SP9 were up regulated. Moreover, the expression of ENST00000454596 (lncRNA rp11.001), ENST00000419695 (lncRNA rp11.003) and ENST00000583521 (lncRNA rp11-c9-001) were down regulated, while NCAM1-AS were up regulated (Figure 4B). However, lncRNA H9 increased significantly at D12, but decreased at D18. Our results agreed with the data of RNA-sequencing generally.

Bioinformatics analysis during MN differentiation

To identify the key factors that regulated MN differentiation, GO analysis was performed on DEmRNAs. The top 15 GO terms related to biological processes are shown in Figure 5A**.** These genes were enriched in cell migration involved in gastrulation, somatic stem cell population

163   maintenance, positive regulation of transcription from RNA polymerase II promoter, positive
164   regulation of cell proliferation, cardiac cell fate determination and anterior/posterior pattern
165   specification. Genes associated with cell migration involved in gastrulation were SOX17,
166   MIXL1 and CER1. Genes associated with positive regulation of cell proliferation were EPHA1,
167   ETS1, HOXA3, POU3F3, FLT1, and TDGF1.
168   Furthermore, the Protein-Protein Interaction (PPI) network of DEmRNAs contained 28 nodes
169   and 39 edges (Figure 5B). The topological analysis of the network was carried out by Network
170   Analyzer in Cytoscape. PPI network was imported into Cytohubba to determine the hub
171   transcription factors with high degree of connectivity between the nodes. The top ten hub genes
172   were POU5F1, TDGF1, SOX17, LEFTY2, ZSCAN10, CER1, ZFP42, MIXL1, L1TD1 and
173   ESRP1 shown in Figure 5B.
174

175   **Analysis of lncRNAs target mRNAs**
176   LncRNA may regulate nearby protein-coding genes by cis-regulatory effects, and we searched
177   the mRNAs transcribed within 100-kb window upstream or downstream of DElncRNAs. Next,
178   we analyzed potential function of the target genes of DElncRNAs at D21 *vs* D0. Total 338
179   DElncRNAs had 289 mRNAs target genes (Additional file 3). Then we performed GO analysis
180   on DElncRNAs target genes to explore their potential biological functions. The top 15 GO terms
181   related to biological process (BP) were shown in Figure 6A. Veen diagram analysis indicated
182   that 6 mRNAs targeted by DElncRNAs were found at the interaction of DEmRNA at D18 *vs* D0
183   (Figure 6B). Moreover, lncRNAs and their *cis* target DEmRNAs were up-regulated at D21,
184   including two well-known TFs HOXA6 and HOXC9. We also performed the topological
185   analysis of PPI network on these target genes (Figure 6C).
186

187   **Construction of lncRNA -miRNA-mRNA interaction network**
188   LncRNAs can regulate gene expression by acting as ceRNAs to sponge miRNAs [14].
189   Thererfore, we constructed a ceRNA interaction network from verified DE mRNAs and DE
190   lncRNAs based on previous qRT-PCR data. As the RNA sequencing and PCR data shown, the
191   transcription factors HOXA3 and SP9, exhibited continuous up-regulation in the transition from
192   ESCs to MN stages, and especially showed a sharp up-regulation coincident with MNs
193   specification. Interestingly, the expression of LncRNA NCAM1-AS showed same trend as
194   HOXA3 and SP9. The NCAM1-AS is antisense lncRNA with two exons, produced from gene
195   NCAM1, which is involved in cell adhesion, axonal outgrowth, synapse formation during
196   development and differentiation, and highly expressed in the developing central and peripheral
197   nervous systems [15]. Bioinformatics analysis of same putative target miRNAs of HOXA3 and
198   lncRNA NCAM1-AS identified a direct binding site of has-miR-338-3p. Thus, lncRNA-miRNA-
199   mRNA pathway was constructed including: lncRNA NCAM1-AS-miR-338-3p-HOXA3.
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Discussion

Motor neuron differentiation is precisely regulated and orchestrated by combinatorial expression of TFs during embryogenesis [4]. Accumulating evidence suggested that lncRNAs could interact with transcription factors to regulate cell differentiation [16-18]. In this study, we profiled mRNAs and lncRNAs expression from our highly efficient ESC-derived MN differentiation protocol to study the development of MNs. Our analysis focused on the identification of transcription factors and lncRNAs that are strongly involved in the temporal development of MNs.

ESCs, derived from the inner cell mass of blastocyst stage embryos, can both self-renew and differentiate into other cell types [19]. The balance between self-renewal and differentiation is regulated by a complex interaction network of translation factors. Pluripotent genes such as Oct4, Nanog, Sox2, Klf4, and Myc [20], activated in ESCs, were inhibited during cell differentiation, whereas expression of differentiation marker genes increases gradually.

Notably, we found the well-known pluripotency-associated transcription factors POU5F1 (also known as OCT4) and TDGF1 which were hub downstream-regulated genes upon MN differentiation in our study. It has been reported that lincRNA linc-RoR, may function as a key ceRNA to link the network of miRNAs and core TFs OCT4, SPX2, and NANOG, thus regulating ESC maintenance and differentiation [21]. As a previous study [22], hub transcription factor ZSCAN10 ,verified by qRT-PCR, also down-regulated in our study and could regulate ESCs gene expression and differentiation. OCT4 can directly regulate expression of ZSCAN10 and TDGF1 [23, 24].

Here, we also identified *cis* regulatory target genes HOXA6 and HOXC9 of lncRNA HOXA-AS3 and HOXC-AS2 at MN stage, respectively. Spinal MNs acquire specialized pool identities that guide their axons to target muscles in the limb, and the specificity of these precise connections[25]. MNs could express many HOX genes specifying MN pool identity and connectivity [25, 26]. HOX6 paralog group genes (HOXA6, HOXC6, and HOXB6) contributed to diverse aspects of motor neuron subtype differentiation, and determined lateral motor column (LMC) fate at forelimb levels of the spinal cord [26]. In addition, HOXC9 determined thoracic level MN population fates, including preganglionic column (PGC) and hypaxial motor column (HMC) neurons [27]. The LncRNA HOXA-AS3 was found to inhibit osteogenic differentiation and promote adipogenic differentiation [28]. The up-regulated lncRNAs HOXA-AS3 and HOXC-AS2 at human MN derived from ESCs was first identified in our study. The two lncRNAs might be involved in MN differentiation by *cis*-regulating HOXA6 and HOXC9, which needs further study.

Our sequencing results suggested a series of mRNAs and lncRNAs significantly changed during the transition from ESC to motor neurons. HOXA3, SP9 and lncRNA NCAM1-AS verified by PCR were observed dramatically up-regulated, especially at period of motor neuron. HOXA3 and HOXB3 are necessary for the specification of Pax6-and Olig2-dependent somatic MN progenitors [29]. In addition, HOX1 was reported to be involved in mediating both the role of RA-signaling in specification of hindbrain MNs [30]. In our sequencing data, HOXB3 was up-

243   regulated at NEP and MNP stages but not altered at MN stage. The neuronal differentiation
244   marker NCAM was involved in motor neurons functionally expanding synaptic territory [31].
245   Additionally, the potential function of lncRNA NCAM1-AS has been originally identified in
246   human MN differentiation from ESC. The lncRNAs could function as miRNA sponges and
247   might compete against other endogenous RNAs to regulate mRNA expression levels and
248   maintain normal biological function. Bioinformatics analysis indicated lncRNA-miRNA-mRNA
249   pathway: lncRNA NCAM1-AS-miR-338-3p-HOXA3. A recent study found that miR-338-3p
250   targeted and inhibited HOXA3 in breast cancer [32]. Thus, the upregulated lncRNA NCAM1-AS
251   might inhibit the expression of miRNA by acting as a miRNA sponge, and in turn, increasing the
252   expressions of MN differentiation-associated mRNAs HOXA3. The dysregulated lncRNAs may
253   regulate gene expression through many ways and play a critical role in the processes of neuronal
254   differentiation.
255

Conclusions

257   In conclusion, we utilized our highly efficient ESC-derived MNs differentiation protocol and
258   next-generation sequencing to provide new insights into understanding the molecular
259   mechanisms underlying MN differentiation and modulating lineage commitment of ESCs. The
260   understanding of MN differentiation could ultimately offer the early diagnosis and novel
261   therapeutic tools of MN-related diseases. More importantly, additional experiments will be
262   needed to further validate those lncRNAs functions.
263

264

Declarations

Acknowledgements
We acknowledge Professor Su-Chun Zhang of University of Wisconsin–Madison for his
assistance during the development of this work.

Competing interests
The authors declare that they have no competing interests.

Funding
This study was supported in part by the National Key Research and Development Project of
China (2016YFA0102500), the National Natural Science Foundation of China (81801066),
the China Postdoctoral Science Foundation (2018M640706).

Author contributions
Xue-Jiao Sun was responsible for assembly of data, data analysis and interpretation, and
manuscript writing. Ming-Xing Li and Chen-Zi Gong were responsible for the preliminary
data search, selection and manuscript revision; Jing Chen and Ya-Jie Li, collection of data
and data analysis. Mohammad Nasb, Sayed Zulfiqar Ali Shah and Rehan Muhammad were
responsible for manuscript revision. Hong Chen were responsible for conception, experiment
design and final approval of manuscript. All authors read and approval the final manuscript.

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384

Primer sequences of five lncRNAs and mRNAs.

Table 1 primer sequences of five lncRNAs and mRNAs

Gene	Forward	Reverse
GAPDH	GGAAGCTTGTCATCAATGGAAATC	TGATGACCCTTTTGGCTCCC
ENST00000454596	CAGCCCAAGGAACATCTCACC	TCTTGCCAACTTGAGTGTCCAT
ENST00000419695	ATCGGACTGTTCAACTCACCTG	TCAGCCGCTAAGCCAAGAAG
NCAM1-AS	TGAGATGCGAGACCTCCAGAC	CTCCAACTGCCTCATTATCCG
ENST00000583521	GGGGGCTGGAAACCAACTTAT	CATCCCAAGTCCAGCGTGAA
H19	CGGCCTTCCTGAACACCTTA	GTGTCTTTGATGTTGGGCTGATG
ZSCAN10	GCCACCGTTTCCGCAATA	GCAGGTGTCGCAGCAGATT
CST1	CCCCAAGGAGGAGGATAGGAT	AGTTGGGCTGGGACTTGGTA
VRTN	TCCCGCTCAACCTACTATGCC	CGTTTGAAGCAGCGATAGGG
OCT4	TCTATTTGGGAAGGTATTCAGCC	CCTCTCACTCGGTTCTCGATACT
HOXA3	CTCAGAATGCCAGCAACAACC	G
SP9	CCAAGCAGTTTTTCCGAGCAG	ACAGGTAGCGGTTGAAGTGGA
		GGCTCGTGTTGCCGATCTT

Figure 1

Differentiation of hESCs into MNs.

Figure 1

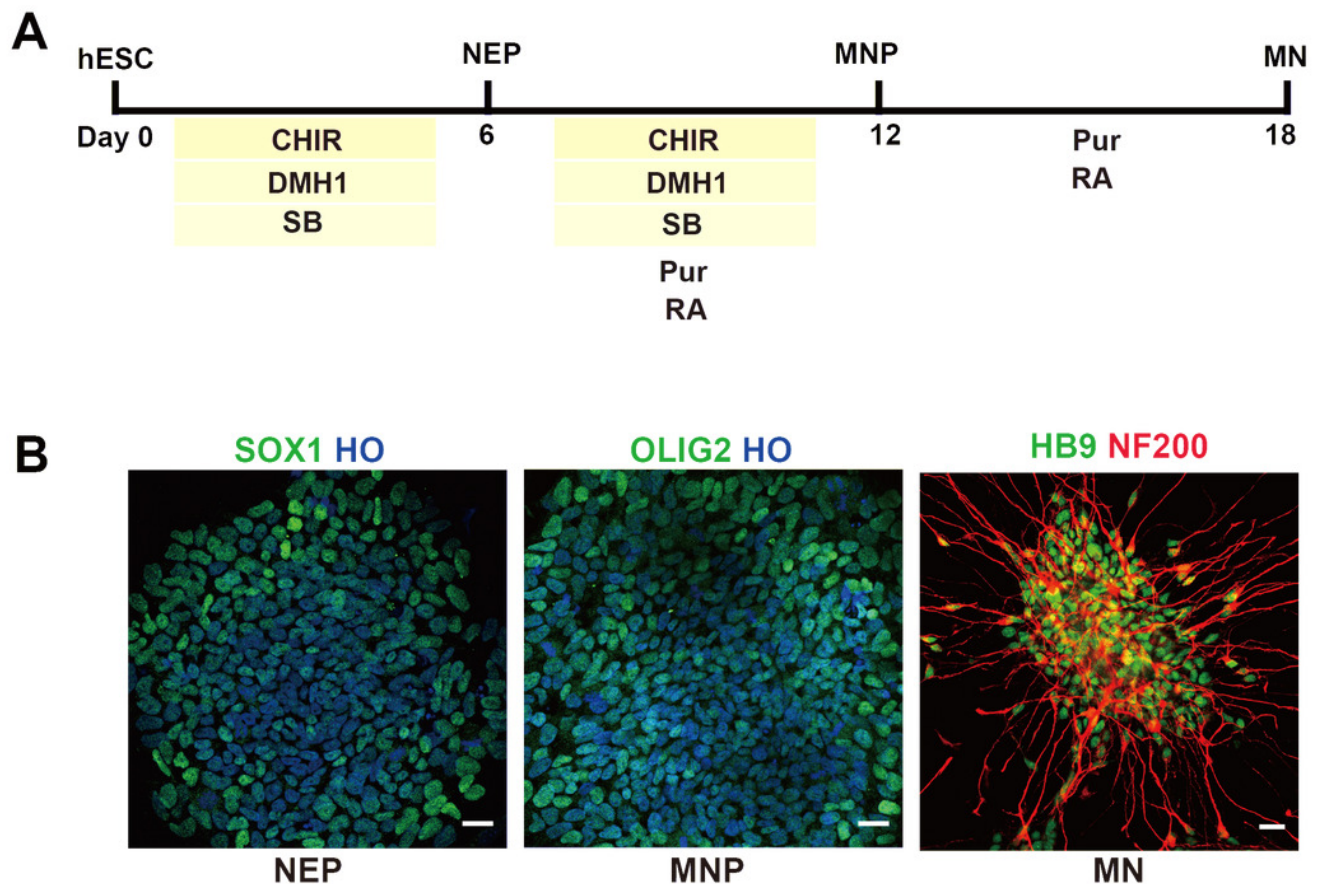



Figure 2

Expression Profiles of lncRNAs.

Figure 2

A

lncRNAs D6 vs D0   lncRNAs D12 vs D0   lncRNAs D18 vs D0

B

up-regulated lncRNAs

- D6 vs D0 — 97
- 35
- 27
- 33
- D12 vs D0 — 106
- 24
- D18 vs D0 — 80

down-regulated lncRNAs

- D6 vs D0 — 87
- 69
- 15
- 78
- D12 vs D0 — 75
- 23
- D18 vs D0 — 58

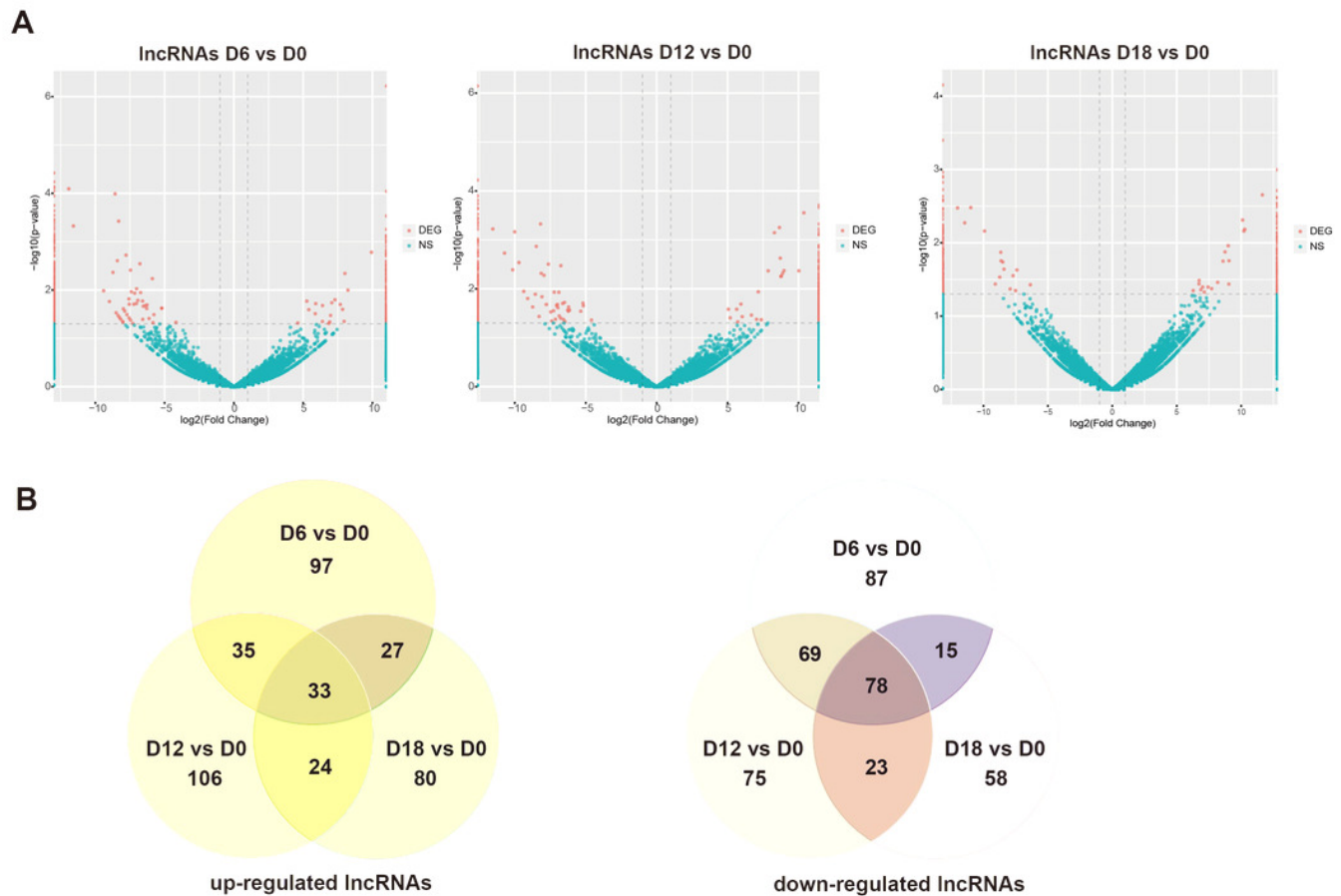



Figure 3

Expression Profiles of mRNAs.

(A) Plot indicated up-regulated and down-regulated mRNAs at different stages points of MN differentiation from ESCs. (B) Venn diagram showed the number of overlap mRNAs during the different stages of MN differentiation from ESCs.

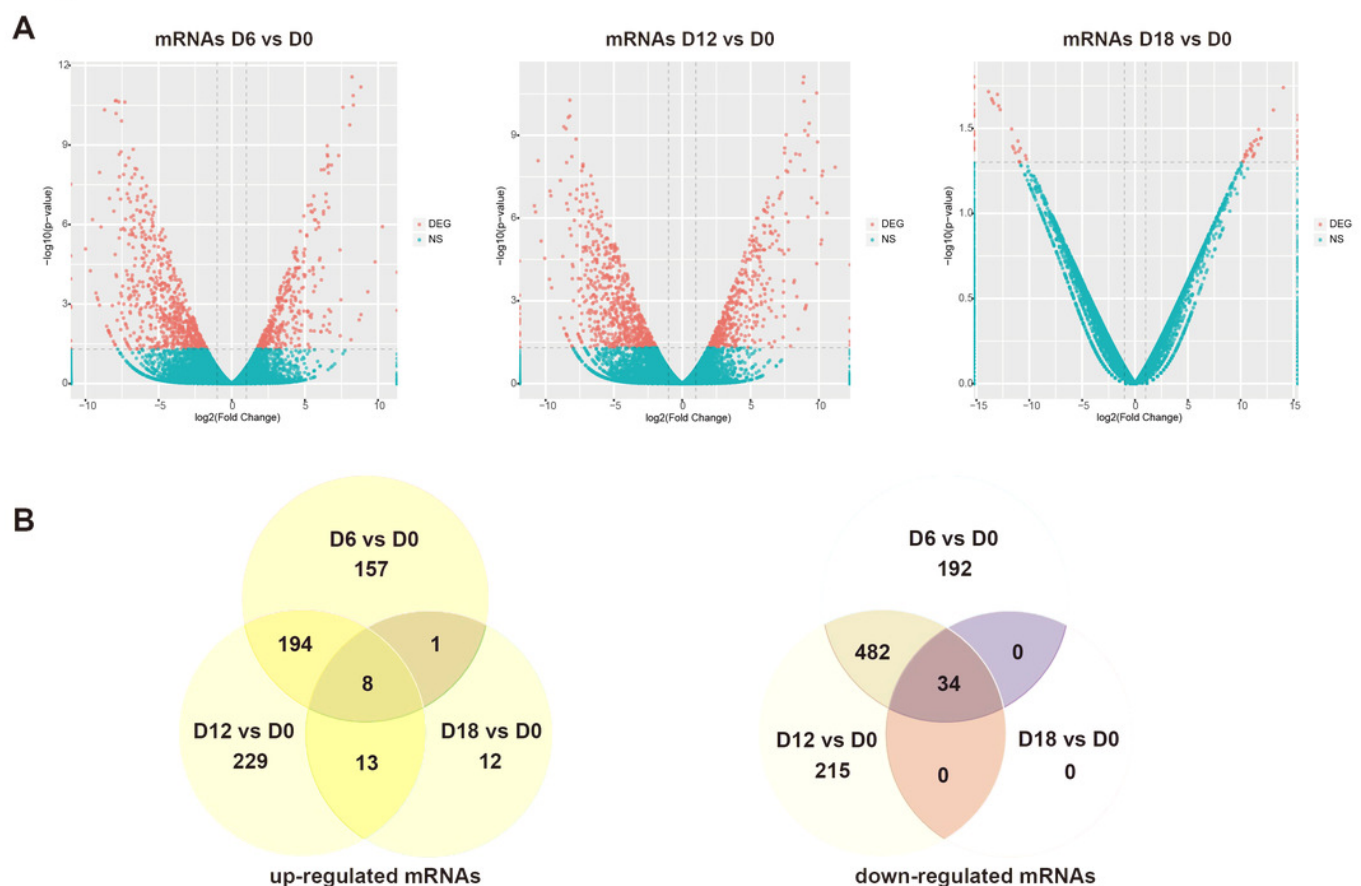



Figure 4

Validation of DElncRNAs and mRNAs in MN differentiation.

(A) qRT-PCR analysis of the 5 DElncRNAs during MN differentiation. (B) qRT-PCR analysis of the 5 DEmRNAs up-regulated during MN differentiation.

Figure 4

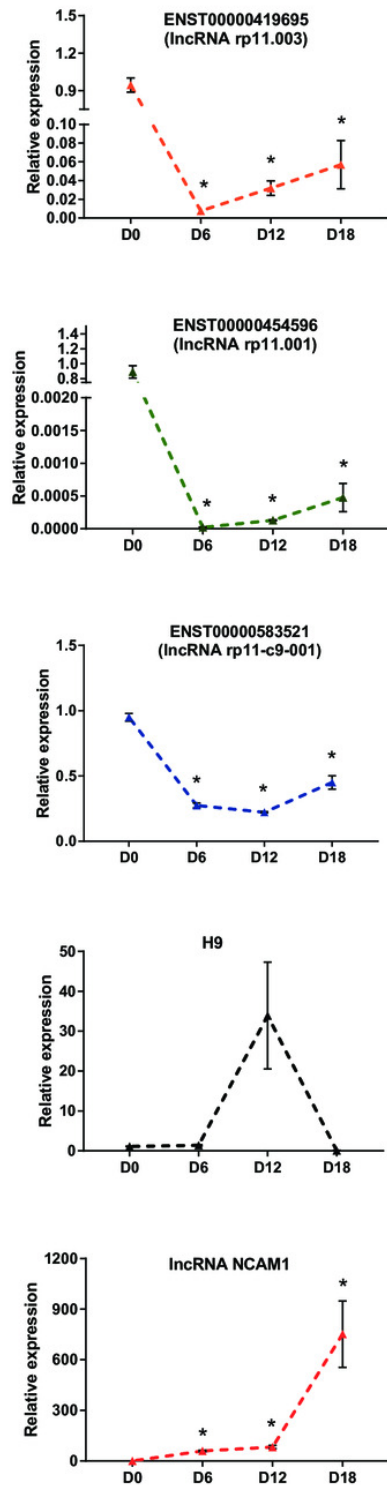
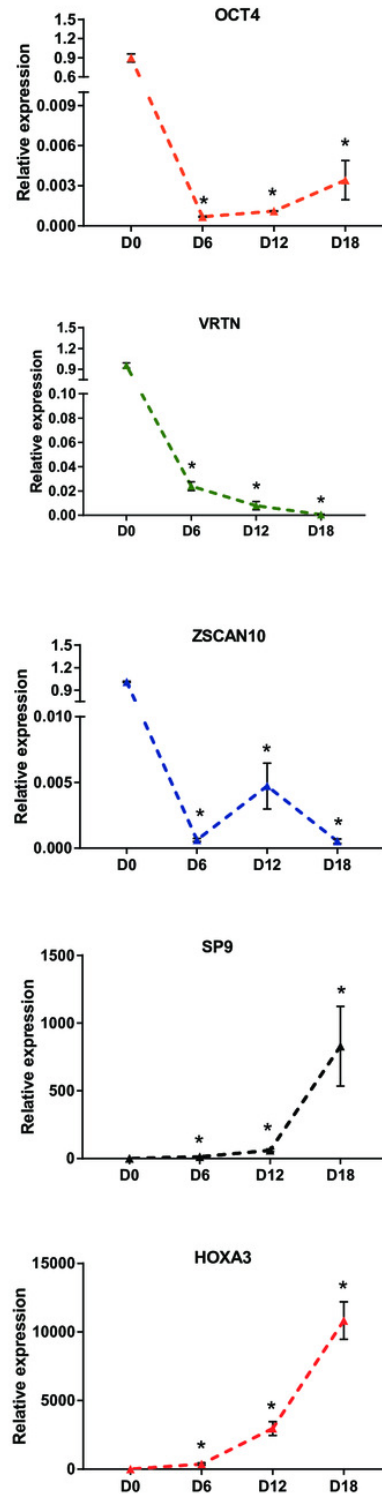
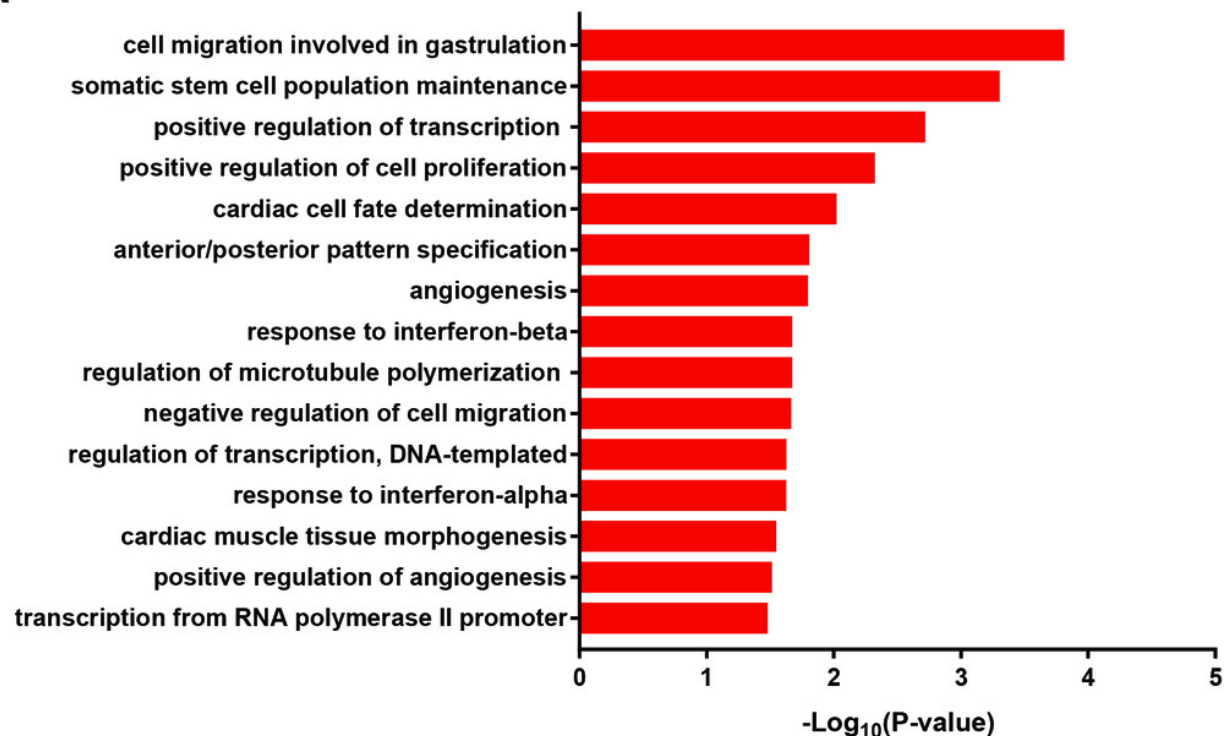



Figure 5

Bioinformatic analysis of DEmRNAs the biological function.

(A) Go analysis of shared DEmRNAs at D6, D12 and D18 compared with D0. (B) Protein interaction network analysis of shared DE mRNAs at D6, D12 and D18 compared with D0.

Figure 5

A

B

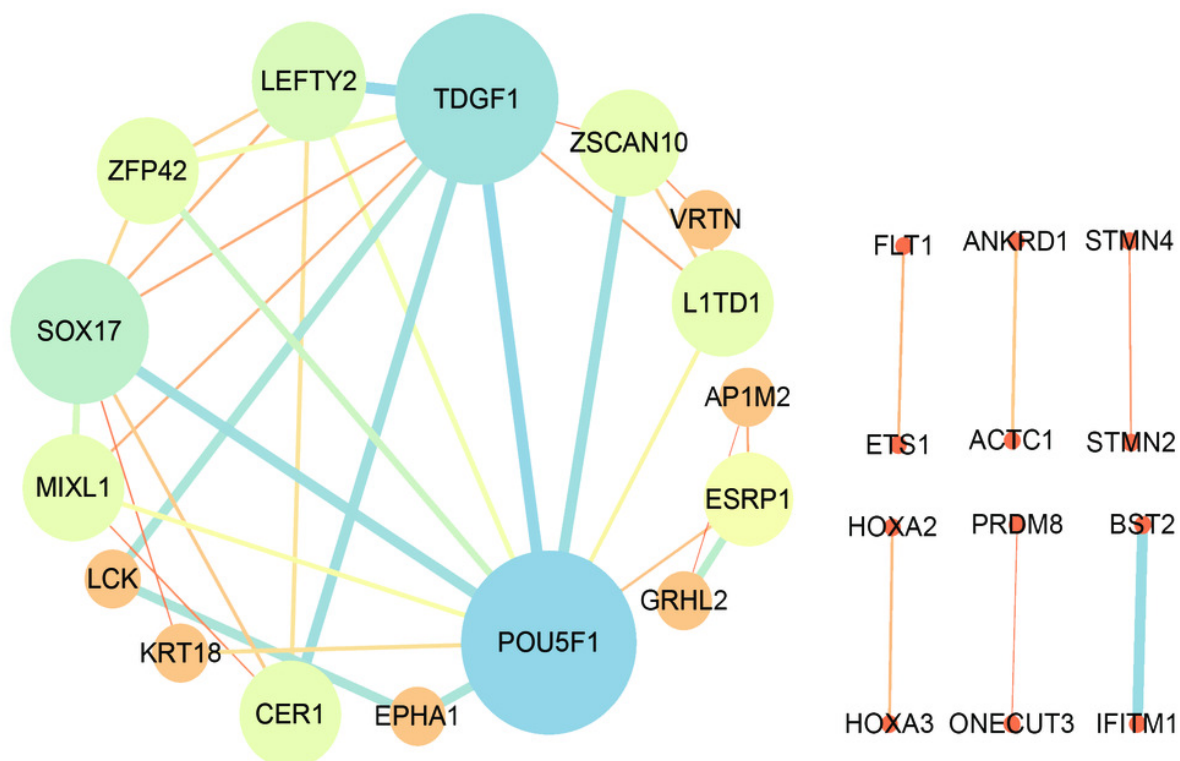



Figure 6

Bioinformatic analysis of DElncRNAs the biological function.

Go analysis of DEmRNAs at MN stage. Venn diagram showed mRNAs tagerted by DElncRNA and DEmRNAs at MN stage. Protein interaction network analysis of DE mRNAs at MN stage.

Figure 6