Differential Expression Pattern of \textit{linc-ROR} Spliced Variants in Pluripotent and Non-Pluripotent Cell Lines

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Abstract

Objective: The human large intergenic non-coding RNA-regulator of reprogramming program (\textit{linc-ROR}) is known as a stem cell specific linc-RNA. \textit{linc-ROR} counteracts differentiation via sequestering microRNA-145 (miR-145) that targets OCT4 transcript. Despite the research on the expression and function, the exact structure of \textit{Linc-ROR} transcripts is not clear. Considering the contribution of alternative splicing in transcripts structures and function, identifying different spliced variants of \textit{linc-ROR} is necessary for further functional analyses. We aimed to find the alternatively spliced transcripts of \textit{linc-ROR} and investigate their expression pattern in stem and cancer cell lines and during neural differentiation of NT2 cells as a model for understanding \textit{linc-ROR} role in stem cell and differentiation.

Materials and Methods: In this experimental study, \textit{linc-ROR} locus was scanned for identifying novel exons. Different primer sets were used to detect new spliced variants by reverse transcription polymerase chain reaction (RT-PCR) and direct sequencing. Quantitative PCR (qPCR) and RT-PCR were employed to profile expression of \textit{linc-ROR} transcripts in different cell lines and during neural differentiation of stem cells.

Results: We could discover 13 novel spliced variants of \textit{linc-ROR} harboring unique array of exons. Our work uncovered six novel exons, some of which were the product of exonized transposable elements. Monitoring expression profile of the \textit{linc-ROR} spliced variants in a panel of pluripotent and non-pluripotent cells exhibited that all transcripts were primarily expressed in pluripotent cells. Moreover, the examined \textit{linc-ROR} spliced variants showed a similar down-regulation during neural differentiation of NT2 cells.

Conclusion: Altogether, our data showed despite the difference in the structure and composition of exons, various spliced variants of \textit{linc-ROR} showed similar expression pattern in stem cells and through differentiation.

Keywords: \textit{linc-ROR}, Pluripotency, Spliced Variants, Stem Cell

Introduction

One of the main achievements of genomic era was the discovery of myriads of long non coding RNA transcripts (lncRNAs) that show state specific expression in different samples and biological processes. This finding suggested lncRNAs as new regulators of diseases and biological events (1, 2). lncRNAs could be detected in the nucleus and cytoplasm and they could exert their regulatory functions through a broad range of mechanisms entailing hybridization to RNA (3) or DNA sequences (4), interaction with transcription factors (5), epigenetic regulators (6). One functional manifestation that showcased the dependency of lncRNAs on interactions with different molecules, was discovery of lncRNAs that operate as competitive endogenous RNA (ceRNA) to compete with mRNAs for binding to regulatory miRNAs (7). However, there are still incomplete experimental evidences to validate the exact mechanisms of lncRNA-disease associations (8). Previous studies demonstrated that some long noncoding RNAs such as metastasis associated lung adenocarcinoma transcript 1 (\textit{MALAT1}) and psoriasis susceptibility 1 candidate 3 (\textit{PSORS1C3}) were able to exert their various regulatory roles at transcriptional and post-transcriptional levels through production of different transcript spliced variants (9-11).

Among lncRNAs, long intergenic non coding RNAs (linc-RNAs) are located between two protein coding genes and usually display high expression levels. lincRNAs exhibit specific expression patterns in different cell types and tissues. They are involved in cellular processes like stemness maintenance, cell cycle regulation and differentiation, however, their exact mechanism of function is still unresolved (12, 13).

The large intergenic non-coding RNA-regulator of reprogramming (\textit{linc-ROR}, \textit{lincRNA-ROR}), was firstly
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introduced in 2010 by Loewer et al. (14) as a 2.6 kb long transcript. This linc-RNA modulates reprogramming of the human induced pluripotent stem cells by sequestering miR-145 (15). Deregulation of linc-ROR expression is associated with tumorigenesis in various malignancies such as esophageal (16), pancreatic (17), gastric (18), colon (19), ovarian (20) and breast cancers (21). However, complete transcript repertoire of this IncRNA has not been clarified yet.

In this study, we experimentally validated novel transcript variants for linc-ROR (Fig.1A, B). We also monitored expression pattern of some of these novel spliced variants in different cells and during the neural differentiation of Ntera-2 cells to see if they behaved differently.

Materials and Methods

The study was approved by Research Ethics Committee of Rajaie Cardiovascular Medical and Research Center (IR.RHC.REC.1397.016).

Bioinformatic analysis

Using the UCSC genome browser, linc-ROR genomic locus was scanned for the conserved regions and existence of TE elements specifically long interspersed nuclear elements (LINE) and short interspersed nuclear elements (SINE) repeats.

Fig.1: Genomic region of linc-ROR and its spliced transcript variants. A. The genomic location of linc-ROR in Chr.18:q21.31. The conserved regions in linc-ROR sequence along with UNE, SINE, ERV1, conventional and new exon sequences are represented here. Orientations of the primers were depicted in linc-ROR sequence. B. linc-ROR spliced transcript variants. Some isoforms contain new exons (gray) besides the conventional ones (black).
Cell culture

Dr. Peter Andrews, University of Sheffield was generously provided the human embryonic stem-like cell line NTERA2cl.D1 (NT2). The human embryonic stem cell lines, including hESC-RH5, hESC-RH6 (22), human induced pluripotent stem cell line 1 and human induced pluripotent stem cell lines 4 (hiPSC1 and hiPSC4 respectively) were obtained from Royan institute (Tehran, Iran) and cultured as described previously (23).

Human cell lines emanated from bladder carcinoma (5637), breast adenocarcinoma (MCF-7), hepatocellular carcinoma (HepG2), prostate cancer (PC3), prostatic adenocarcinoma (LNCAP), colorectal adenocarcinoma (HT-29), malignant glioma cell lines (U-87MG, A172), brain astrocytoma (1321N1), medulloblastoma (DAOY), cervix adenocarcinoma (Hela), hepatoblastoma (Huh-7), colon adenocarcinoma (SW480), esophageal squamous cell carcinoma (KYSE-30) and gastric carcinoma (AGS), were obtained from Pasture Institute of Iran (Tehran, Iran).

The human embryonic kidney 293 (HEK293T), human lung adenocarcinoma (A549), human USSC (unrestricted somatic stem cells) and fibroblast cells were purchased from the Stem Cell Technology Company (Tehran, Iran).

The cells were cultivated to reach 70% confluency before collection at 37°C with 5% humidified CO₂ in RPMI 1640 (for U-87MG, A172, 1321N1, DAOY) or high glucose Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, USA) supplemented with 10% FBS (Invitrogen), 100 U/MI penicillin, 100 mg/ml streptomycin and 25 ng/ml amphotericin B.

RNA isolation and cDNA synthesis

Total RNA was extracted from cell lines using TRIzol reagent (Invitrogen, USA), according to the manufacturer’s instructions. RNase free DNaseI (TaKaRa, Japan) treatment was applied to remove any possible traces of DNA contamination. Reverse transcription of RNA was primed using an oligo (dT) primer and random hexamer by applying the PrimeScript TM Reagent kit (TaKaRa, Japan). Each sample had a no-reverse transcription (No-RT) control in parallel with the DNase-treated RNA to detect any potential non-specific amplification of genomic DNA.

Quantitative and qualitative reverse-transcriptional polymerase chain reaction

The GeneRunner (version 3.02, Hastings Software Inc., USA), PerlPrimer v1.1.16 and Oligo v 6.54 softwares were utilized to design the specific amplifying primers for qualitative and quantitative reverse-transcription PCR (RT-PCR) of both lin-ROR (GenBank accession numbers NR_048536 (HQ315778), AB844430, AB844431, AB844432, AB844433, AB908956 and AB932951) and β2 microglobulin (β2M, as an internal control; GenBank accession number: NM_000246). RT-PCR reactions were carried out using 2 µl of the synthesized cDNA, 0.5 mM of each primer and 10 µl of Taq DNA polymerase master mix RED (Ampliqon, Denmark).

PCR cycling parameters were comprised of initial cDNA denaturation of 5 minutes at 94°C, followed by 45 seconds at 94°C, 45 seconds at annealing temperature 60°C and DNA extension for 1 minute at 72°C for 35 and 26 cycles (for lin-ROR and β2M amplification, respectively). A final extension step was performed at 72°C for 10 minutes. All PCR reactions incorporated no template controls or no RT reaction. Sequences of the designed oligos were listed in Table 1. PCR products were electrophoresed on 1% or 1.5% agarose gel electrophoresis, followed by staining with ethidium bromide and visualization through UV light exposure.

Quantitative RT-PCR (qRT-PCR) was carried out applying 2 µl of the synthesized cDNA, 10 µl of SYBR-Green ready mix (TaKaRa, Japan), 0.1 µl of Rox and 0.5 µM of each specific primer. β2M gene was utilized as an internal control, and expression of the other genes was normalized to its expression level using the 2-∆Δct method.

ABI 7500 real-time PCR system (Applied Biosystems, USA) was employed to execute the PCR reactions using the following cycling conditions: initiation at 95°C for 15 minutes, amplification for 40 cycles with denaturation at 95°C for 15 seconds, annealing at 62.5°C for 30 seconds and extension at 72°C for 30 seconds. Melt curves were analyzed to validate the PCR products and amplified products were sequenced.

Cloning constructs and sanger sequencing

All lin-ROR PCR products were purified from agarose gel with ExpiTM combo kit (GeneAll, South Korea). Then, PTG19-T vector (Vivantis, Malaysia) was applied to clone the purified products using T4 DNA ligase (Fermentas, USA) and transformed to DH5α competent cells (TaKaRa, Japan).

Recombinant colonies with resistance to ampicillin (Sigma-Aldrich, USA) were selected as positive ones. Universal M13 primers were employed to select different variants of the lin-ROR gene via colony check PCR. Sanger sequencing (Microgen, South Korea) was utilized to validate identity and validity of the PCR products.

Induction of neural differentiation of NT2 cells

Peter Andrews protocol, which was described before (24), was applied to induced neural differentiation of NT2 cells. Concisely, NT2 cells were treated with 10-5 M all-trans-retinoic acid (RA, Sigma-Aldrich, USA) for up to 21 days. Then, the cells were passaged and re-cultured without retinoic acid for additional two weeks. NT2 cells were also treated with 1% DMSO (RA solvent) as a control group. The cells were then collected at different time points (3rd, 7th, 14th, 21st, 25th and 32nd days) for further analyses.
## Table 1: Sequence of the utilized primers in the study

| Gene                  | Name 1   | Sequences (5’-3’)                       | Products (bp) |
|-----------------------|----------|------------------------------------------|---------------|
| linc-ROR variant 6    | F1       | GCCATGTT CTCACACAAAG                    | 1149          |
|                       | R3       | CTCATGGCTAATTGCACTTG                   |               |
| linc-ROR              | F3       | ACCAATTTCACATTCAGACC                   | 320           |
|                       | R1       | TCTTACTTAGCAATGCGGCACTC                |               |
| linc-ROR              | F3       | ACCAATTTCAATTCAGACCG                   | 787           |
|                       | R2       | TTTGAGTTGCTGCTGTAGAG                   |               |
| linc-ROR              | F4       | ACAAGGAGGAAAGGGCGTGAC                  | 124           |
|                       | R4       | TTCTGGAAGCTAAGTGCACTTC                |               |
| OCT4A                 | F        | CTTCTGCACCCCTCCTACCTG                  | 496           |
|                       | R        | AAATAGAACCCACGGGTTGAGC                 |               |
| OCT4B1                | F        | AGACTATTCTCTGGGCGCCACCC               | 272           |
|                       | R        | CTTAGAGGGAGATGCCTGCA                   |               |
| OCT4B                 | F        | AGACTATTCTCTGGGCGCCACCC               | 244           |
|                       | R        | GGCTGAAATACCTTCCAAAATGAG               |               |
| Nanog                 | F        | AGAGGGCGAGGAAGAGTACTAC                 | 253           |
|                       | R        | ACTCTGCTTTTACCAAATG                   |               |
| SOX2                  | F        | CATGCTCTGCTGCTGCTG                    | 160           |
|                       | R        | GCTGGAATTTTCTGCTG                     |               |
| GAPDH                 | F        | GTGGAACCATGAAAGATGACAACC              | 123           |
|                       | R        | CATGAGTCCTCTCCACAGATACC               |               |
| B2M                   | F        | GGGTTTCATCCATCCAGCAGTTG               | 167           |
|                       | R        | TGGTCAACAGGCGACAGCAGAATAC             |               |
| miR-145               | F        | TAIAGCTAAGCTCGGCGGCGATG                | 202           |
|                       | R        | GCTCATTGTGAAGGTGTGTTG                 |               |
| OCT4A                 | F        | TGCCCATCCAGTCAAATCTCA                 | 444           |
|                       | R        | TCCAGAGACGGCAAGCAAG                   |               |
| linc-ROR              | F        | CGTAGGGAAGGGAGGAAAC                   | 124           |
|                       | R        | GTACACGTGAAATGCGAGGTTT                |               |
| linc-ROR variant 4    | FB22     | TATCGTCAGAGTGTAGGCAGGT                | 251           |
|                       | R2NEW    | TACTTACCGCAATGCCCAC                    |               |
| linc-ROR variant 3    | FC3      | AGATCACACACCTGCACCTC                  | 145           |
|                       | RC3      | AAGGGATGAGTTGATGCTT                   |               |
| linc-ROR              | F11      | TGGTGAAGTGATCAGTGGATAGG               | 448           |
|                       | R22      | TCTTACTTAGCAGAATGCCCATC               |               |
| linc-ROR              | FEA1     | AAGCCAGCTGCTGACCTTGG                  | 288           |
|                       | R2       | TCTTACTTAGCAGAATGCCCATC               |               |
| linc-ROR (whole transcript) | Rex   | GGTGAATAAACACACCGCATGTT            | 2580          |
|                       | Rex      | GAGGAACGTGCATACCAGT                   |               |

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Statistical analysis

All experiments were replicated at least three times. GraphPadPrism 8 software (GraphPad Software, USA) was employed to perform Student T test. All values were presented as means ± standard error of mean (means ± SEM) and P<0.05 were considered statistically significant.

Results

*linc-ROR* spliced variants has different patterns of expression in various cell lines

To profile expression of *linc-ROR* in different stem and cancer cell lines, we firstly designed a primer set on the IncRNA terminal exon (F4R4 primers, Fig.1A) with the idea to detect all possible spliced variants (Fig.1B). Our initial investigation on the expression of *linc-ROR* transcripts by qRT-PCR revealed that NT2 cells had the highest expression levels for *linc-ROR* (Fig.2A). Among the cancer cells, Huh-7, HEK293T and DAOY showed a relatively higher expression level for *linc-ROR*. Expression of *linc-ROR* was undetectable in fibroblast cells representing somatic normal cells.

Our bioinformatics analysis on the *linc-ROR* locus revealed that this lncRNA was overlapped with different transposable elements (TEs, Fig.1A). Based on the previous researches, TEs could contribute to create new alternatively spliced variants since they provide splice site acceptors and donors and they can be exonized into the transcript (25). To find out whether *linc-ROR* transcripts contained any TE driven exons, we specifically designed different sets of oligos mapping on different TEs to capture those potential transposon containing exons (Fig.1A, B).

Primers, designed on the junction of *linc-ROR* first and second exons (F3) and reverse primers on exon 3 (R1), were used to capture transcripts with possible TEs as exon. These primers were used in RT-PCR on NT2 cells since they showed the highest expression level of *linc-ROR*. In this experiment, we could detect several amplicons with different sizes that differ from what we expected to see from the RefSeq sequence (320 bp, Fig.2B).

To uncover the identity and structure of these bands, they were isolated from the gel, cloned, sequenced and aligned against the human genome (hg19) and transcript sequences. The results demonstrated existence of five novel *linc-ROR* spliced variants retaining different parts of the intron 2 sequence, as novel exons, some overlapped with TE elements. To examine whether we could also detect transcripts with extra exons in different cancer cells, we carried out RT-PCR analysis with the same set of primers in a panel of different cells. The result revealed a diverse pattern of expression for different variants in those cell lines (Fig.2C). The HT-29 and 5637 cells displayed almost the same expression pattern as NT2 cells.

Furthermore, our bioinformatic analysis displayed that there are three highly conserved regions approximately in the middle of the second intron of *linc-ROR* and we hypothesized that maybe these parts also could take part in creating new variants as well. Our RT-PCR outcomes using F1R3 primers in NT2 cells exhibited a novel spliced variant of *linc-ROR* (Fig.2D) with the AB908956 accession number (variant 6) that contained exon 1, 2 and a part of the conserved sequence in the second intron (Fig.1A, B).

To find whether we could detect the transcripts containing novel exons in full length, we designed two sets of primers, located on the beginning (Fex) of exon 1 and the end of exon 4 (Rex), followed by performing RT-PCR on human pluripotent cells (hiPSC1, hiPSC4, hESC-RH5 and hESC-RH6). The expected band for the transcript containing all exons was about 2.6 kb. Our results exhibited that there were additional bands with alternative sizes (Fig.2E). To confirm identity of the bands and presence of novel exons, all bands were extracted from the gel, cloned, sequenced and aligned to the human genome and transcript sequences (UCSC genome browser, hg19). Aside from validating the spliced variants that we first detected in NT2 cells, we identified eight novel variants for *linc-ROR*. Blat analysis exhibited two of the newly identified variants, retained some parts of LINE and SINE sequences which are located in intron 2 of the *linc-ROR* gene (Fig.1A, B).

*linc-ROR* variants are downregulated following differentiation

To scrutinize the correlation between the expression of *Linc-ROR* spliced variants and the undifferentiated state of human embryonic stem and embryonic carcinoma cells, NT2 human cells were treated with all-trans-retinoic acid (24). qRT-PCR results (using primers which could detect all transcripts) demonstrated that expression of *linc-ROR* was downregulated in the course of neural differentiation. Surprisingly, decline in *linc-ROR* expression was detected before downregulation of master regulators of pluripotency *OCT4A, SOX2, NANOG* and *miR-302b*. Adversely, expression of *miR-145* was upregulated gradually during differentiation (Fig.3A).

To see if the observed downregulation for *linc-ROR* was a trend affecting different variants, we used semi-qRT-PCR. Our results revealed that all detected *linc-ROR* spliced variants were downregulated the same way in the course of neural differentiation (Fig.3B, C), a pattern that was not seen in tumor samples, where some isoforms showed upregulation and some exhibited downregulation [unpublished data, (16)].
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**Fig. 2:** linc-ROR expression pattern in different cell lines. **A.** Expression profile of linc-ROR in a variety of pluripotent and non-pluripotent cell lines (F4R4; normalized to B2M). Expression of linc-ROR reaches the highest level in NT2 cell line. **B.** Reverse transcription polymerase chain reaction (RT-PCR) detection of linc-ROR isoforms in NT2 cell line (F3R1). **C.** Semi-quantitative RT-PCR results of linc-ROR detection in different pluripotent and non-pluripotent cell lines (F3R1). **D.** RT-PCR products in NT2 cell line using primers for variant 6 (F1R3), and **E.** RT-PCR products of linc-ROR spliced variants in human pluripotent cells using Fex and Rex oligos.

**Fig. 3:** Expression of linc-ROR and its spliced variants during NT2 cells differentiation. **A.** Expression levels of linc-ROR (normalized to B2M), miR-145, miR-302b, SOX2, NANOG and OCT4A during differentiation. **B.** Expression pattern of linc-ROR spliced variants and linc-ROR variant 6 during differentiation of NT2 by means of semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). DMSO; Dimethyl sulfoxide.
Discussion

*linc-ROR* plays a pivotal role in regulating self-renewal and reprogramming of pluripotent stem cells (12, 15). *linc-ROR*, some of the major regulators of pluripotency and self-renewal such as OCT-4 share common expression signatures in some tumors and cancer cell lines (26) which advocate the hypotheses of the involvement of cancer stem cells and potential association of these factors in tumorigenesis. By scanning the genomic location of *Linc-ROR* which harbored transposon elements, we predicted there must be some transcript spliced variants of *Linc-ROR*. Our study has introduced and confirmed existence of the 14 different spliced variants of *lincROR* expressing in different stem and non-stem cells and in the course of differentiation. Our group previously reported differential expression of the three spliced variants of *linc-ROR* in human esophageal squamous cell carcinoma (16). In this study, we introduced 13 novel spliced variants for *linc-ROR*. Our investigations showed these variants were highly expressed in stem-like cells and differentially detectable in different cancer cell lines. For instance, *linc-ROR* expression level was not similar in different glioblastoma cell lines that we examined in this research. This could be due to differences in cancer stem cell pool, represented grade and stage and intra population heterogeneity of these cell lines.

The evolutionary role of alternative splicing in fine-tuning gene function and transcriptome dynamics in eukaryotes has been established previously (27) and it is known for many protein coding (28) and noncoding genes (10) with different transcript spliced variants. For instance, *OCT4*, a master regulator of pluripotency, has various isoforms with different expression and distinct functional roles (10, 26, 29). Our team also previously reported this phenomenon for long noncoding RNA *PSORS1C3*, which has 24 spliced variants with different expression pattern and function in pluripotent and non-pluripotent cells (10, 11, 30). Another example is *MALAT1*, one of the well-studied IncRNAs with various spliced variants that differ greatly in their expression patterns and functions in different cells and tissues (9, 31). There is an increasing number of publications on the function of *linc-ROR* and its expression. However, differential expression of its spliced variants in different cell types and tissues needs to be elucidated.

Here we detected new variants of *linc-ROR* transcripts in pluripotent stem cells and cancer cells, showing their expression alteration during neural differentiation. Our data indicated that many of *linc-ROR* spliced variants behaved similarly and their abrupt downregulation upon differentiation fit with their suggested role as guardians of stemness circuits.

Furthermore, the genomic location of *linc-ROR* contained transposon elements (TE), which are considered as selfish genomic parasites (32). Several studies argued that TE were involved in different transcriptional regulatory networks (32-34), as well as harboring splicing signals, leading to being spliced as new exons (25, 35). Our result exhibited that these elements could contribute to *linc-ROR* transcripts as new exons. TE derived sequences are considered as functional domains of IncRNAs which enable them to interact with and regulate RNA species and proteins (36). It is possible that *linc-ROR* exonized TEs are also involved in different functional abilities of their harboring transcripts. To validate this, further investigations are required.

Here, we showed that *linc-ROR* had higher expression level in NT2 cells, as a pluripotent human embryonal carcinoma cell line. This data is in line with the published association of *linc-ROR* with stemness state (14). Our data also pinpointed the significant decrease in *linc-ROR* and its transcript spliced variants expression throughout neural differentiation in both quantitative and semi-quantitative approaches which is in line with previous findings on the contribution of *linc-ROR* in self renewal and pluripotency through regulating core stemness factors (15, 37-39).

Mis-regulation of mRNA splicing can affect signaling pathways and contribute to diseases like cancer (40). The alternative splicing of IncRNAs might also impact various cellular processes, however, understanding the exact molecular effect of alternatively spliced exons needs to be further investigated.

Conclusion

The detailed picture of *linc-ROR* regulatory network is still missing. We identified 13 novel splice variants for *linc-ROR* that were expressed in pluripotent and some cancer cells. Our results, together with those from previous studies, depicted new insights into investigations of the molecular repertoire of IncRNAs, suggested a new angle for the scrutinizing IncRNA genes and showed the missing pieces that are needed for IncRNAs expression and function investigations.

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Authors’ Contributions

M.M., S.J.M; Contributed to experimental design and supervision, finalized the draft. F.M.A., E.T.B., P.N., A.R., M.S.A., M.B.M., A.Gh.; Participated in experimental...
work and statistical analysis. M.M., F.M.A., E.T.B., P.N., A.R., M.S.A., M.B.M., A.Gh.; Contributed to writing and submitting the manuscript. All authors participated in the finalization of the manuscript and approved the final draft.

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