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The differential expression of mRNAs and long noncoding RNAs between ectopic and eutopic endometrium provides new insights into adenomyosis

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The differential expression of mRNAs and long noncoding RNAs between ectopic and eutopic endometrium provides new insights into adenomyosis

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Abstract

Background: Adenomyosis, defined as ectopic endometrial tissue within the myometrium, can often be misdiagnosed as multiple uterine leiomyomata or endometrial thickening. We therefore performed a combined mRNA and long noncoding (lnc)RNA microarray and bioinformatic analysis of eutopic and ectopic endometrium in women with adenomyosis to better understand its pathogenesis and help in the development of a semi-invasive diagnostic test.

Results: A total of 586 mRNAs were increased and 305 mRNAs decreased in ectopic endometrium of adenomyosis compared with eutopic endometrium, while 388 lncRNA transcripts were up-regulated and 188 down-regulated in ectopic compared with paired eutopic endometrial tissue.

Conclusion: mRNA and lncRNA expression was comparable in eutopic and ectopic endometria. Bioinformatic analysis suggested a series of metabolic and molecular abnormalities in adenomyosis, which have many similarities with endometriosis. Furthermore, our study constitutes the first known report of lncRNA expression patterns in human adenomyosis ectopic and eutopic endometrial tissue.

Keywords:

Adenomyosis,microarray, long noncoding RNA, lymphangiogenesis

Background

Adenomyosis' characteristics is the presence of ectopic endometrial mucosa

within the myometrium and the invagination of endometrium at a depth of at least 2.5 mm below the basal layer of the endometrium. It is a common benign pathology that is asymptomatic in one third of cases. Clinical signs, consisting mainly of menometrorrhagia, dysmenorrhoea, and pelvic pain, are non-specific, often leading to the misdiagnosis of multiple uterine leiomyomata or endometrial thickening, both of which have different prognoses and treatments[1].

The pathogenesis of adenomyosis is poorly defined. Its diagnosis is confirmed by hypertrophy of smooth muscle caused by the invagination of endometrium [2], and it is often associated with hormone-dependent pelvic lesions such as myoma, deep pelvic endometriosis, polyps, or endometrial hyperplasia[3, 4].

Endometriosis is defined as the presence of endometrial glands and stroma in ectopic locations, mainly the pelvic peritoneum, ovaries, and rectovaginal septum. The clinical signs of endometriosis include dysmenorrhea, dyspareunia, chronic pelvic pain, irregular uterine bleeding, and/or infertility[5]. A number of similarities exist between adenomyosis and endometriosis, both of which were originally described as adenomyoma. The two conditions have been recognized as distinct entities since the retrograde menstruation theory became widely accepted to explain the pathogenesis of endometriosis, because it does not explain adenomyosis[6]. However, magnetic resonance

imaging and laparoscopy showed that the two conditions often co-exist, at least in some subgroups[7].One study reported that 27% of women with endometriosis had concomitant adenomyosis[8]. Moreover, adenomyosis is thought to be a significant factor of infertility in endometriosis, probably because of changes in the uterine transport of spermatozoa[9].

Investigations into the pathophysiology of endometriosis have revealed several well-sustained molecular hallmarks of this disease, such as genetic predisposition, estrogen dependence, progesterone resistance, and inflammation[10]. At the same time, eutopic and ectopic endometria of adenomyosis were reported to show a series of metabolic and molecular abnormalities which include increase angiogenesis and proliferation, decrease apoptosis, allow local production of estrogens, create progesterone resistance, and impair cytokine expression[11]. However, the mechanism responsible for the survival and subsequent implantation of the translocated endometrium remains unknown. Moreover, the origin of adenomyosis and endometriosis is still controversial, which not only influencing our understanding of uterine function and pathophysiology, but also of clinical management and treatment. Long noncoding RNAs (lncRNAs) are a class of ncRNAs up to 200 nucleotides in length. Through observations of the transcriptional activation ripple effect, they were long disregarded as transcriptional noise because of their low expression levels[12]. However, recent accumulating evidence suggests that

lncRNAs function in biological processes through diverse mechanisms[13], and many studies have reported their roles in genetic imprinting, immune responses, cell differentiation, human diseases, and other biological processes[14-16]. It has also been suggested that their expression can be cell specific and regulated by common transcription factors[17, 18].

This study was performed to establish the differential mRNA and lncRNA expression in the eutopic and ectopic endometria of adenomyosis, with the aim of furthering our understanding of the pathogenesis of adenomyosis and developing a semi-invasive diagnostic test. We collected eutopic and ectopic endometria from women with adenomyosis and conducted microarray and bioinformatic analyses.

Results

HE staining of surgical specimens

Normal endometria contained a few uterine glands, numerous matrix cells, reticular fibers, and blood vessels. Adenomyosis was diagnosed when ectopic endometrial mucosa were observed within the myometrium. See Figure 1.

Differentially expressed mRNAs and lncRNAs

A total of 891 mRNAs (see Additional file 1:Table S1) and 576 lncRNAs (see Additional file 2:Table S2) were identified by microarray analysis after quantile normalization and data filtering steps. Of the mRNAs, 586 were up-regulated and 305 were down-regulated (fold-change >2, $P < 0.05$) in ectopic compared

with eutopic endometria, respectively. Of the lncRNAs, 388 were up-regulated, and 188 were down-regulated (fold-change >2, $P < 0.05$), respectively.

Quantitative real-time PCR validation

The expression levels of three randomly chosen differentially expressed mRNAs (*NRP2*, *TGFBR1*, and *ITGB3*) and three lncRNAs (uc004dwe.2, ENST00000454594, and NR_003521) were verified by quantitative (q)RT-PCR. The relative fold-changes in expression, as detected by qPCR, were consistent with the microarray data. See Figure 2.

Gene Ontology (GO) and pathway analysis

Genes showing greater than a 2-fold change in expression level were analyzed by GO. Those genes up-regulated in ectopic compared with eutopic endometrial samples were found to be involved in a variety of pathways including vasculogenesis, cell–matrix adhesion, cell differentiation, and the regulation of cell proliferation, apoptosis, and muscle contraction. By contrast, those genes down-regulated in ectopic compared with eutopic endometrial samples were involved in immune response pathways, and the positive regulation of the I-kappaB kinase/NF-kappaB cascade. See Figure 3a,b.

The major pathways thought to involve mRNAs up-regulated in ectopic versus eutopic endometria include the adhesion junction, MAPK signaling, and cytokine–cytokine receptor interaction, while mRNAs down-regulated in ectopic versus eutopic endometria are thought to be involved in pathways of

androgen and estrogen metabolism, among others. See Figure3c,d.

Signal network analysis

Ingenuity pathway analysis was used to identify pathways of significantly differentially expressed genes. A network of genes was built according to the relationships among the genes, proteins, and compounds of the KEGG database[19, 20]. This identified many candidate genes and their interactions that may play an important role in the pathogenesis and development of adenomyosis. See Figure 3e.

lncRNA and mRNA coexpression profiles and lncRNA function prediction

To associate lncRNAs with the regulated expression of target mRNAs, we superimposed lncRNA target predictions onto the lncRNA–mRNA correlation network. The resulting network was defined as an lncRNA–mRNA regulatory network, with direct connections between an lncRNA and an mRNA represented by solid lines. A total of 24 lncRNAs were co-expressed with many different mRNAs in the co-expression profiles. See Figure 4. This enabled us to predict the functions of lncRNAs through the GO and KEGG pathway annotations of their co-expressed mRNAs.

Discussion

The establishment of adenomyosis requires several key steps, including epithelial–mesenchymal transition (EMT), survival and growth of ectopic tissue

in the myometrium, myometrial hypertrophy and hyperplasia, and induction of an immunosuppressive microenvironment[21]. We used microarray analysis to show that 891 mRNA and 576 lncRNA transcripts are dysregulated in the ectopic endometrium of adenomyosis compared with paired eutopic endometrium. Our GO analysis data show that the different genes between eutopic and ectopic endometrium of adenomyosis are involved in pathways of cell–matrix adhesion, cell differentiation, vasculogenesis, the positive regulation of cell proliferation and the I-kappaB kinase/NF- kappaB cascade, and the regulation of apoptosis, immune responses, EMT, and muscle contraction, it's worth noting that,all of above have previously been reported to participate in the pathogenesis and pathophysiology of endometriosis[22-24]. It has been hypothesized that adenomyosis and endometriosis share a common pathogenesis[25-27], which is supported by our data, although more research is needed to confirm this hypothesis.

Our findings support those of a number of other studies about endometriosis and adenomyosis. For example, Mechsner reported that epithelial and stromal cells in endometriosis develop from persistent coelomic epithelial cells by metaplasia [28], which is in line with our GO analysis including of cell differentiation and the positive regulation of cell proliferation . Similarly, our results support the theory that persistent estrogenic stimulation leads to EMT[29], myometrial smooth muscle cell hypertrophy, and hyperplasia of

adenomyosis[30]. A previous study suggested that uterine myometrial growth can be modulated by prolonged mechanical stretch and contraction increasing the intrauterine pressure and leading to smooth muscle cell hypertrophy and hyperplasia of adenomyosis [31]. And, the most widely accepted hypothesis is that adenomyosis is caused by defects of the inner myometrial layer of the uterus[32], and that invagination of the stroma together with microenvironmental factors stimulate smooth muscle cell growth[21].

Indeed, many of the genes shown to be differentially expressed by our microarray data have been reported to be associated with the development of endometriosis. These include *CDH1*, *SGPP2*, *MET*, and *SLC2A1*, which demonstrate 2.5- to 4.7-fold reductions in the ectopic versus eutopic endometria of women with adenomyosis in our study, with the observed reduction in *CDH1* and *SGPP2* expression being consistent with the literature[33, 34]. Moreover, the expression of *VEGFA*, *PDGFA*, *KDR*, *ACTN4*, *CD44*, and *JAM2* was increased 2.3- to 3.2-fold in ectopic versus eutopic endometria of women with adenomyosis, with the up-regulation of *VEGFA*, *PDGFA*, *KDR*, and *ACTN4* being in line with earlier reports[35-38].

To the best of our knowledge, this study presents the first mRNA and lncRNA microarray analysis of eutopic and ectopic endometria in women with adenomyosis. Our findings show that mRNA expression differs between the

two endometrial types, with ectopic endometria more closely associated with pathways of focal adhesion, tight junctions, regulation of the actin cytoskeleton, the calcium signaling pathway, the transforming growth factor-beta signaling pathway, androgen and estrogen metabolism, the cell cycle, EMT, positive regulation of mesenchymal cell proliferation, and regulation of muscle contraction, all of which are related to the development of adenomyosis[21].

Although our GO and pathway analysis reveal many similarities between the development of adenomyosis and endometriosis, adenomyosis may also have some unique molecular mechanisms, and some differentially expressed lncRNAs may play a role in its pathogenesis and development by affecting their coexpressed mRNAs.For example,the most widely accepted theory of retrograde menstruation of endometriosis isn't suitable to explain adenomyosis. The detection of endometrial stromal cells within lymph nodes in a baboon model of induced endometriosis [39] and in 6–7% of women at lymphadenectomy [40] supports the possibility of the lymphatic dissemination of endometrial cells.

It's a great encouraging detection of the high over-expression of the neuropilin 2 gene (*NRP2*) in ectopic versus eutopic endometria which was verified by our qRT-PCR. This gene demonstrates tissue-specific distribution in glandular and surface epithelia, venous and lymphatic endothelia, and a leukocyte

subpopulation, with low levels reported in endometrial stromal cells[41].

NRP receptors and tyrosine kinase receptors such as vascular endothelial cell growth factor (VEGFR) are cell surface receptor families of the VEGF family that are important mediators of angiogenesis and lymphangiogenesis[42]. Our GO analysis also confirmed that the differentially expressed genes are more related to many pathways including vasculogenesis. NRP2 is important in modulating developmental lymphangiogenesis and mediating VEGF-C-induced lymphatic sprouting[43]. The previous observation of endometrial tissue in intramyometrial lymphatics [44] together with our microarray findings of NRP2' high over-expression in ectopic endometrium indicate that lymphatic vessels are a possible route for the invagination or dissemination of the basal endometrium in adenomyosis.

Our present study findings suggest that 24 lncRNAs are involved in the establishment of adenomyosis. Although the functions of many lncRNAs are poorly understood, it is of interest that our lncRNA microarray analysis showed that lncRNA uc004dwe.2 is over-expressed in ectopic compared with eutopic endometria. This is in line with our real-time PCR result and is highly correlated with the expression of NRP2, suggesting that lncRNA uc004dwe.2 may play a role in the pathophysiology of adenomyosis through modulating the function of NRP2.

Conclusions

In conclusion, our study is the first to investigate genome-wide lncRNA expression patterns in human adenomyosis tissue, and identified several mRNAs and lncRNAs differentially expressed between paired, autologous, ectopic and eutopic endometria in women with adenomyosis. Several metabolic and molecular abnormalities detected in this study have previously been reported to participate in biological pathways related to endometriosis. The lncRNAs identified may participate in the pathophysiology of adenomyosis through diverse mechanisms. Further research is required in this area because of the limited current understanding of lncRNA functions in adenomyosis.

Methods

Patients and samples

Endometrial samples were collected from women of childbearing years undergoing hysterectomies for adenomyosis. Patients using the oral contraceptive pill (combined or progesterone only), those on chronic medication, or those having undergone surgery in the 6 months prior to sample collection were excluded. Specimens of ectopic endometria were isolated away from areas of obvious uterine adenomyoma. Eutopic endometria were full-thickness, 1-cm biopsies of the uterine wall. Harvested samples were divided into two parts: one half was snap-frozen in liquid nitrogen and prepared

for RNA extraction. The other half was fixed and used for morphological examination. Sectioning and hematoxylin and eosin staining of surgical specimens were carried out by the pathological department of the Wuxi Maternity and Child Health Hospital (Jiangsu Province, China).

All patients had signed a written informed consent prior to recruitment and the study protocol was approved by the Medical Ethics Committee of Wuxi Maternity and Child Health Hospital.

Microarray analysis

mRNA and lncRNA microarray analysis was undertaken in three paired samples of uterine adenomyoma versus matched eutopic endometrial tissues using the Agilent（California, USA） microarray platform. Sample preparation and microarray hybridization were performed according to the manufacturer's standard protocols with minor modifications. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and purified to remove rRNA using the NucleoSpin® RNA Clean-up kit (Macherey-Nagel, Cat # 740.948.250). Then, each sample was amplified and transcribed into fluorescent (Cy5 and Cy3-dCTP) labeled cRNA along the entire length of the transcripts without 3′ bias using a reaction with random primers. Labeled cRNAs were hybridized onto the Agilent Human LncRNA+mRNA Array V2.0 (4×180K format), which contains probes interrogating about 39,000 human lncRNAs and about 32,000

human mRNAs assembled from databases such as RefSeq, Ensembl, the Chen Runsheng Laboratory (Institute of Biophysics, Chinese Academy of Science), and other related literature. The arrays were scanned on the Agilent Scanner G2565CA after washing the slides.

Agilent Feature Extraction software version 10.7 (Arraystar, USA) was used to analyze the acquired array images. Quantile normalization and subsequent data processing were performed using the GeneSpring GX v11.5.1 software package (Arraystar). Microarray data were selected using threshold values of >2 and <−2 fold-change under false discovery rate (FDR) protection (*P* < .05). Hierarchical clustering was performed to show distinguishable expression patterns of lncRNAs and mRNAs.

RNA labeling, and microarray hybridization and analysis were performed by CapitalBio (Beijing, China).

Isolation of total RNA and Quantitative Real-time PCR

Total RNA was isolated from ectopic and eutopic endometria tissues as described above. The RNA yield was determined using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Rockford, IL, USA), and its integrity was evaluated using agarose gel electrophoresis stained with ethidium bromide. Reverse transcription was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) for 15 min at 37°C, followed by heat inactivation for

5 s at 85°C.

Real-time PCR was performed using a StepOnePlus Real-Time PCR Instrument (Applied Biosystems) in a 20-μL reaction including 2-μL of template DNA (100 ng/μL), 10 μL 2×SYBR Green Master Mix (Vazyme), 0.8 μL forward primer (5 μM), 0.8 μL reverse primer (5 μM), 0.4 μL 50×ROX Reference Dye 1, and 6 μL nuclease-free water. Reactions were incubated in a 96-well optical plate (Applied Biosystems) at 95°C for 6 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Each sample was run in triplicate. Melting curve analysis was performed to validate the specific generation of the expected PCR product. Primer sequences were synthesized by Generay Biotech (Shanghai, China) on the basis of the noncoding RNA sequences obtained from the National Center for Biotechnology Information (NCBI) database. Primer sequences are listed in Table Ⅰ. lncRNA and mRNA expression levels were normalized to those of glyceraldehyde 3-phosphate dehydrogenase and calculated using the $2^{-\Delta\Delta Ct}$ method[45].

Bioinformatics analysis

All mRNA and lncRNAs microarray data were given by Capital Bio Corp. (Beijing, China). Raw microarray signals underwent $\log_2$ transformation after being normalized using Agilent GeneSpring software. Differential expression of mRNA or lncRNA was defined by an absolute value of fold-change (FC) >2 (eutopic endometrium=1) and $P < 0.05$ (unpaired *t*-test).

Gene ontology (GO) analysis of the main function of the differentially expressed genes was conducted according to the GO database, which provides the key functional classifications for the NCBI [46] . Fisher's exact test and the χ^2 test were used to classify the GO category, and the FDR[47] was calculated to correct the *P*-value: the smaller the FDR, the smaller the error in judging the *P*-value. *P*-values were computed for the GOs of all differential genes. Enrichment provided a measure of the significance of the function: as the enrichment increased, the corresponding function became more specific, which helped identify those GOs with a more concrete function description in adenomyosis. Within the significant category, the enrichment Re was given by:

$$\mathrm{Re} = (n_f / n) / (N_f / N),$$

where n_f is the number of differential genes within the particular category, n is the total number of genes within the same category, N_f is the number of differential genes in the entire microarray, and N is the total number of genes in the microarray [48].

Similarly, ingenuity pathway analysis was used to identify pathways involving significantly differentially expressed genes according to the Kyoto Encyclopedia of Genes and Genomes (KEGG). Fisher's exact test and the χ^2 test were used as statistical tests, and the threshold of significance was defined by the *P*-value and FDR. The enrichment Re was calculated using the equation mentioned in a previous study [49].

Co-expression network and functional prediction of lncRNAs

Because most lncRNAs in current databases have not yet been functionally annotated, the prediction of their functions relies on the functional annotations of their co-expressed mRNAs, i.e., neighboring protein-coding genes, which may influence each other during biogenesis [50]. We constructed co-expression networks of genes with specific expression levels according to their normalized signal intensities. The Pearson's correlation coefficient was calculated for each pair of genes, and significantly correlated pairs were chosen to construct the network.

Availability of supporting data

The microarray data sets supporting the results of this article are included within the article and its additional files.

Additional files

Additional file 1:Table S1. Fold-changes of differentially expressed mRNAs in ectopic endometria compared with eutopic endometria.

Additional file 2 :Table S2. Fold-changes of differentially expressed lncRNAs in ectopic endometria compared with eutopic endometria.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CZ and RH carried out the mRNA and lncRNA expression analyses. ZS performed the statistical analysis of microarray data.CZ and JZ performed the statistics and drafted the manuscript. XN and TZ participated in the study design and helped to draft

the manuscript. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1 Hematoxylin and eosin staining of eutopic and ectopic endometria in adenomyosis. (a) Normal uterine endometrium. (b) Ectopic endometrium within the myometrium. G, gland.

Figure 2 Relative fold-changes in expression between ectopic and eutopic endometria, as detected by quantitative PCR (light gray bars), were in agreement with microarray data (dark gray bars).

Figure 3 Bioinformatic analysis for differentially expressed mRNAs and lncRNAs. (a) GO analysis of mRNAs up-regulated in ectopic endometria. (b) GO analysis of mRNAs down-regulated in ectopic endometria. (c) Top pathway annotations of up-regulated mRNAs may involved in. (d) Top pathway annotations of down-regulated mRNAs may involved in. (e) The interaction network of differentially expressed genes (Signal-net). Circles represent important functional genes in adenomyosis (red: up-regulated genes; blue: down-regulated genes). Solid arrow represents activation; dotted arrow represents indirect effect; solid line represents compound or binding/association.

Figure 4 lncRNA–mRNA core network consisting of 24 lncRNAs and their

mRNAs with the most credibility.

Table 1 Primers used in real-time PCR.

ID	Forward	Reverse
GAPDH	CAGCCGCATCTTCTTTTGCG	AGTTAAAAGCAGCCCTGGTGA
UC004dwe.2	GATCCCAGCCAGCCTAGAAG	AAATGCTGGAAAGGCCCTCAT
ENST00000454594	CGTTCGTTGTACAGAGGAGGA	CTGCAAGTGGGAGCGGAAA
NR_003521	GCTGCGTCAGGTAAGCGAG	CCGGCTCCTCCCCATCAG
NRP2	CAAGACAGGCTCTGAAGATTGC	GGGTTTGGCCAGGATGGTAA
TGFBR1	TTCCGTGAGGCAGAGATT	GCAATACAGCAAGTTCCATT
ITGB3	AAGAGCCAGAGTGTCCCAAG	GTGCCCCGGTACGTGATATT

Figure
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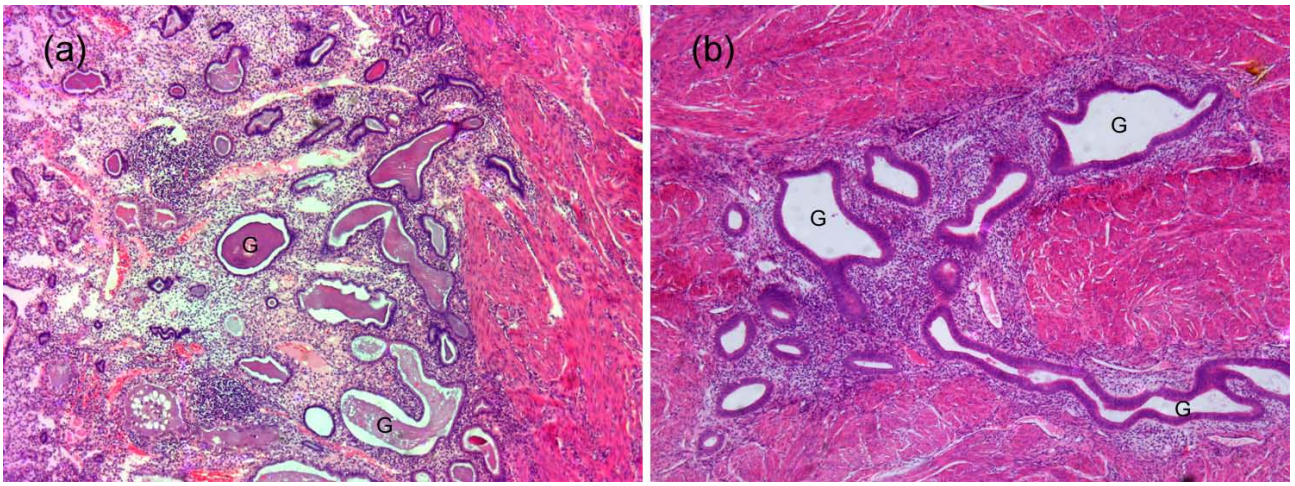
Figure 1



Figure
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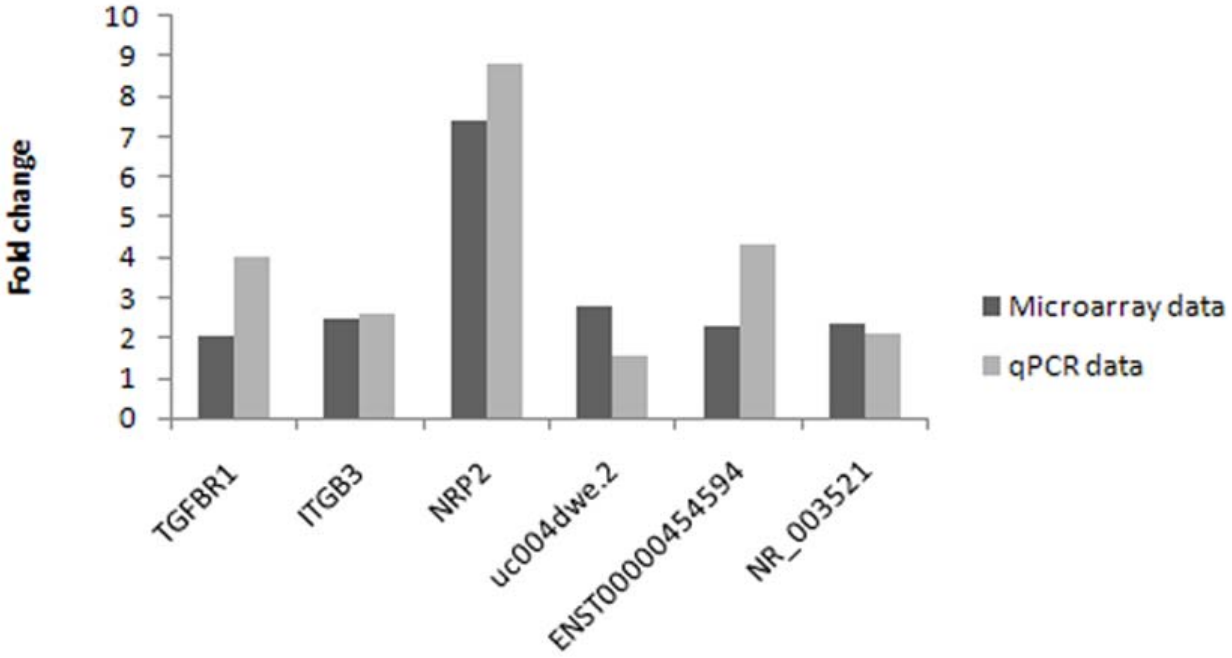
Figure 2



Figure
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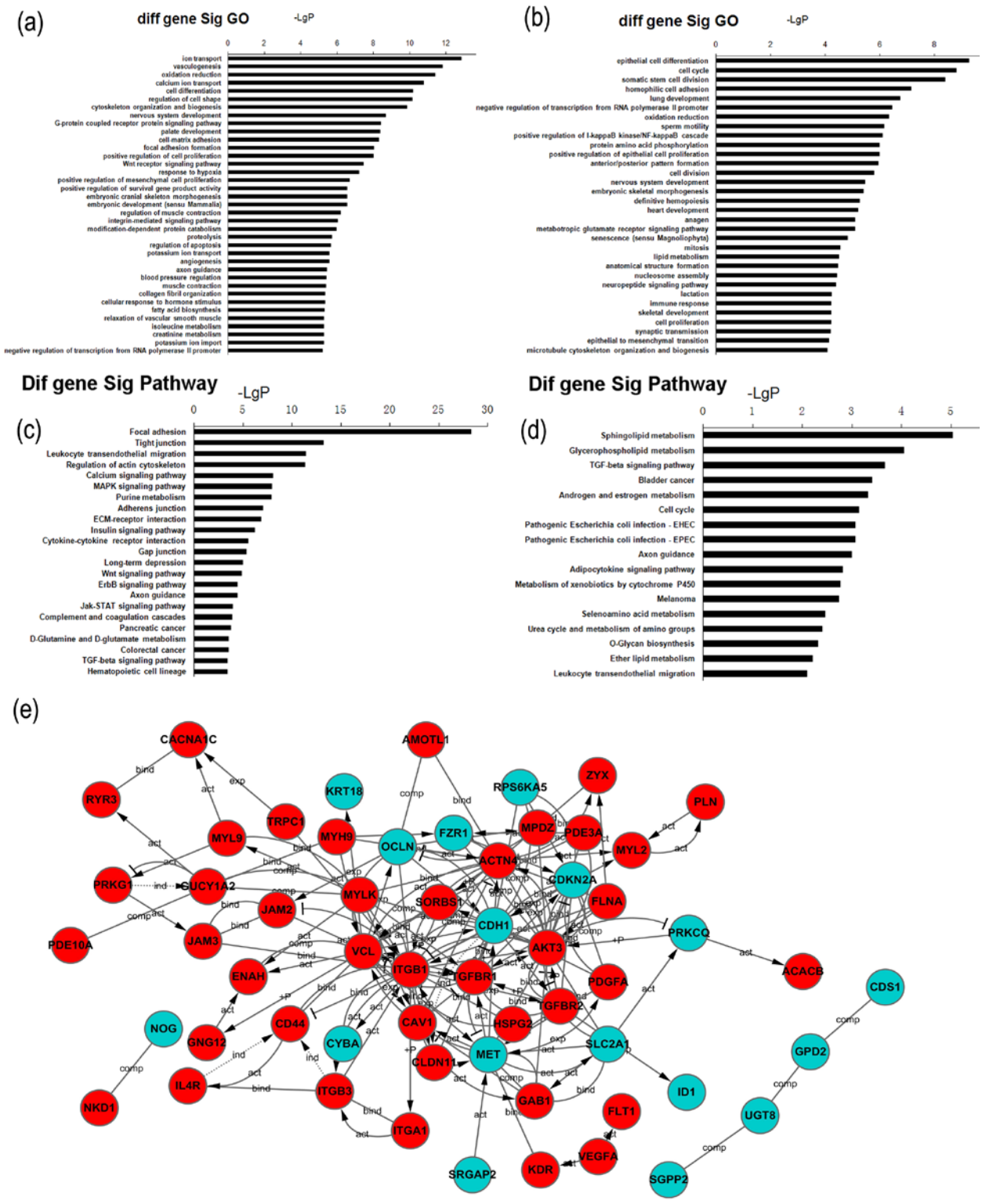
Figure 3



Figure
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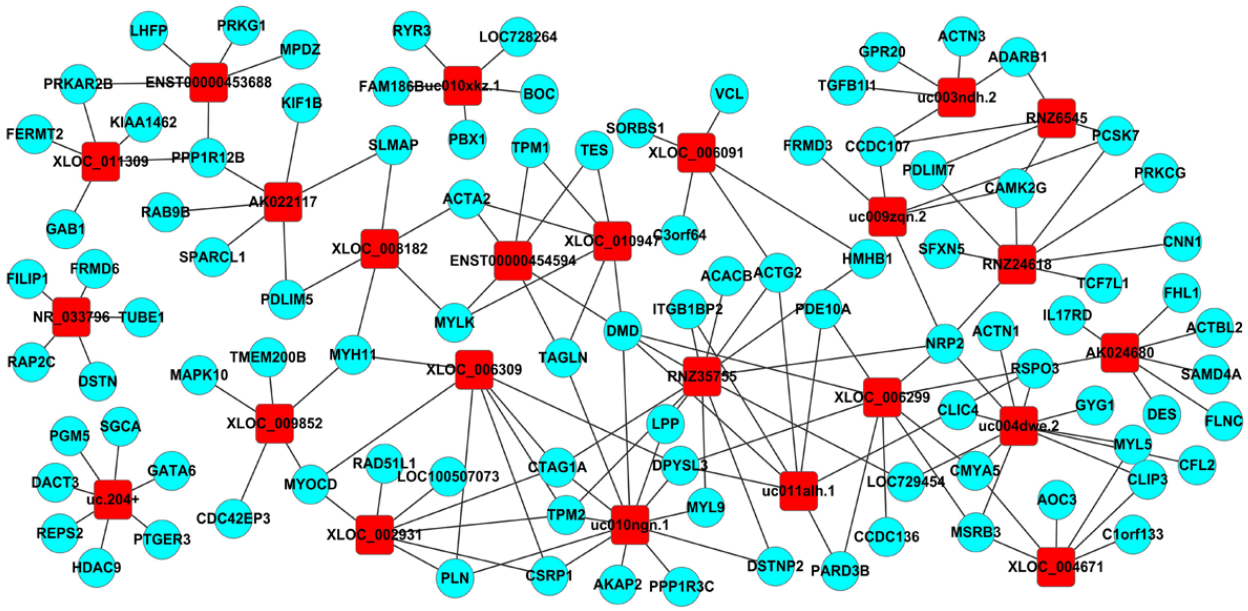
Figure 4



Supplementary Material

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Supplementary Material

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