Integrated analysis of cancer stem cells-associated lncRNA-miRNA-mRNA network for ovarian cancer via microarray and Gene Expression Omnibus database

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Research

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Abstract

Background: Cancer stem cells (CSCs) are associated with the recurrence, metastasis and chemoresistance of epithelial ovarian cancer. Competing endogenous RNAs (CeRNAs) play an important role in maintenance of ovarian cancer stem cell-like cells (OCSCs) characteristics. To construct a ceRNA regulatory network for OCSCs, microarray technology and Gene Expression Omnibus (GEO) database had been used. Human serous epithelial ovarian carcinoma cell line COC1 cells were treated with cisplatin and paclitaxel then maintained in stem cell conditions for 6 days to obtain *CD117+/CD133+* cells (OCSCs). We identified the differentially expressed miRNAs (DEMs), lncRNA (DELs) and mRNA (DEGs) between OCSCs and COC1 by microarray and combined them with representative microarray profiles in GEO Database.

Results: According to the combination, 28 DEMs were identified at first, and 452 DEGs were obtained combining with the predicted targets of these miRNAs and our mRNA microarray results. Up-regulated DEGs of them were significantly enriched in 'p53 signaling pathway', 'FoxO signaling pathway' and 'MicroRNAs in cancer', whereas down-regulated DEGs were significantly enriched in 'Adherens junction' and 'Hepatitis C' pathway. 29 transcripts of 17 lncRNAs should be the ceRNAs of 10 of these miRNAs according to bioinformatics predicted results and lncRNA microarray. Finally, we obtained ceRNA network with 10 DEMs, 21 DEGs, and 25 transcripts of 13 DELs which should play an important role in maintenance of OCSCs characteristics. *LINC00665-miR-146a-5p-NRP2* should be one of ceRNA pathways of the network. The qPCR results indicated that the expression of *miR-146a-5p* in OCSCs was lower than that in COC1, and *LINC00665* shows the opposite trend. These results were consistent with the results of microarray partially. When *LINC00665* expression was up-regulated in COC1, the cell proliferation ability enhanced, apoptosis rate reduced, and the percentage of G2/M phase cells increased.

Conclusions: The ceRNA network we constructed may be involved in the stem cell characteristics maintenance of OCSCs and provide directions for further OCSCs research in the future, so as to assist the development and treatment of ovarian cancer.

Background

Epithelial ovarian cancer is the most lethal gynecologic malignancy GD Aletti, MM Gallenberg, WA Cliby, A Jatoi and LC Hartmann [2], and it's the fourth most common cause of death from cancer in 40–59 years old women [59]. According to statistics, it's predicted that the increase of incidence should be 22530 in the US in 2019, which accounts for 2.5% of all estimated new tumor cases in women, and 13980 of deaths should occur, accounting for almost 5% of the total of cancer deaths in women [60]. The most common therapy is the optimal cytoreductive surgery combined with 6–8 cycles of platinum-based chemotherapy. However, after optimal cytoreductive surgery performance, many patients emerged secondary chemoresistance within one year with chemotherapy, resulting in a recurrence rate of serous ovarian cancer of up to 80% [30].

It is reported that many mechanisms play important role in regulation of chemoresistance in ovarian cancer chemotherapy, such as enhancement of repair of DNA damage, reduction of drug accumulation in cells, loss of drug function and inhibition of apoptosis. While cancer stem cells (CSCs) theory gives a new understanding in the development of drug-resistant tumors [12]. CSCs are one unique subpopulation of cells with stem cell proprieties in tumors. This kind of cells remain in a dormant and quiescent state, therefore, exposure to a drug that targets divising cells at DNA replication and mitosis could not reduce the number of CSCs efficiently [42, 64]. On the contrary, chemotherapy drugs may increase the ratio of CSCs in tumor tissues because of its inhibitory effect on differentiated tumor cells [30]. And the expression of drug membrane transporters such as ABCG2 make it difficult for chemotherapy drugs to accumulate in CSCs [40]. CSCs also can expand the pool of stem cells through symmetric division, and differentiate cancer cell causing tumor recurrence after treatment through asymmetric division, during which stem cells give rise to daughter cells of different fates, proliferative potential, size, or other characteristics [43]. Sometimes these two kinds of division can be transformed into each other. Some studies have suggested that asymmetric division was associated with CSC in early and highly differentiated cancers, while inhibition of it was associated with advanced and highly proliferative cancers[4]. In summary, CSCs are a kind of ‘self-renewing cells within a tumor that can cause the heterogeneous lineages of cancer cells that comprise the tumor’ [8]. The existence of CSCs means that there is a small population of cells with stem cell properties proliferating slowly and carrying primary resistance to chemotherapy in tumor tissues and these cells can be selected during the period of chemotherapy and pass the drug resistant property to their daughter cells [53]. It is also one of the important reasons for ovarian cancer recurrence after chemotherapy. Therefore, CSCs theory, as a way to result in CSCs-dependent cancer recurrence during the treatment of ovarian cancer, is worthy of further study.

There are many methods for CSCs isolation, such as identifying SP cells from ovarian cancer cells by efflux of Hoechst 33342 dye which could be blocked by exposure to verapamil [62], or isolating CSCs by detecting the transmembrane pluripotent marker protein such as *CD44+*, *CD133*+ [3, 31] and *CD117*+ [71] at the use of flow cytometry. *CD133* has been used as a means of detecting CSCs in many malignancies, including epithelial ovarian cancer (EOC)[54]. In ovarian cancer, *CD133* + cancer cells have stronger tumorigenicity than *CD133*- cancer cells and are more likely to develop chemoresistance [29]. While *CD117*, which is also known as c-Kit, is commonly used to identify CSCs among EOC cells[70]. *CD117* is tyrosine kinase type receptor, which is involved in cell differentiation, proliferation, apoptosis, migration and other biological processes. It has been suggested that human ovarian cancer cells with CD 117 + phenotype had properties similar to those of CSCs, such as self-renewing, differentiation, high tumorigenicity and chemoresistance [39]. In some studies, CSCs were obtained by recognizing *CD117+/CD133 +* cells. The *CD117+/CD133 +* CSCs subpopulation of HO8910, which was the human EOC cell line, were identified to be more likely to develop resistance to conventional chemotherapy agents including cisplatin, doxorubicin, and mitoxantrone[68]. Li Ma, et al had obtained *CD117+/CD133 +* cells identified stem cell properties with cisplatin and paclitaxel selection and under the stem cell culture

condition from serous ovarian cancer cell line SKOV3 [40]. According to their protocol, we selected *CD117+/CD133 +* cells from serous ovarian cancer cell line COC1 for our further study.

Competitive endogenous RNA (ceRNA) theory indicates that specific RNAs can isolate miRNAs to bind to target genes, impair miRNAs activity, and thus up-regulate miRNA target genes expression. In addition to being involved in the occurrence and development of tumors, this regulation mechanism plays an important role in the stem characteristics maintenance of CSCs. For example, *miR-193a* targeted *E2F6* and *c-Kit*, and there was an estrogen-mediated *E2F6* ceRNA network which epigenetically and competitively inhibited *miR-193a* activity, up-regulated OCSCs marker *c-Kit* expression, and promoted ovarian cancer stemness and tumorgenesis[6]. The latest study also found that as an oncogenic lncRNA in cervical cancer, *LINC00337* affected the expression of *KLF5* and maintained the CSC-like properties by down-regulating *miR-145*[23]. At present, there are many studies on the involvement of lncRNAs as ceRNAs in the occurrence and development of ovarian cancer, but fewer in the cancer stem cell characteristics regulation of ovarian cancer. There even has been no report on the constructed lncRNA-miRNA-mRNA interaction network that were associated with stemness and progression of ovarian cancer. Herein, we analyzed the differentially expressed miRNAs, lncRNAs and mRNAs by microarray, and obtained the key differentially expressed miRNAs (DEMs) by combining with representative microarray profiles in GEO Database. The predicted target genes of these DEMs (identified by PicTar [5], Targetscan [1], DIANA [55] and starbase [32]) and the differentially expressed mRNAs (DEGs) of our microarray and GEO database were combined to identify key DEGs that were differentially expressed and had the same trend in all these datasets. Protein-protein interactions (PPI) of these DEGs were constructed by STRING[61] and Cytoscape[58] to find hub genes in OCSCs. Differentially expressed lncRNAs (DELs) that may interact with these DEMs were obtained by combining the predicted results of lncBase [47] and starbase with our lncRNA microarray results. Finally, we obtained the ceRNAs network constructed by miRNA, lncRNA and mRNA in OCSCs. *MiR-146a-5p* and *LINC00665* of this network were selected for partial validation. Through bioinformatics methods, we constructed ceRNAs network that might be involved in the stem characteristics maintenance of OCSCs. The study of OCSCs can help to reveal the CSCs related way in which ovarian cancer develops chemoresistance and provide a theoretical basis for the research on reducing or reversing chemoresistance during the treatment of ovarian cancer.

Results

1. Preparation of *CD117+/CD133 +* ovarian cancer cells and detection of stem cell characteristics

Cells treated with cisplatin (40 mol/L) and paclitaxel (10 mol/L) were cultured under stem cell conditions for 6 days to form cell spheres. Western blot analysis of sphere cells showed that the expression of *SOX2, OCT4* and *NANOG* were higher than those of COC1, and the expression of *ALDH1* and *LGR5*, which were considered to be OCSCs-specific stem cell markers, were also increased. Flow cytometry detection showed that the percentage of *CD117+/CD133 +* cells in sphere was significantly higher (78.49%) than that in COC1 (27.78%). Cell cycle detection showed that the percentage of M stage of cells was

significantly higher (84.9–95.6%) than that in COC1 (3.2%). Therefore, *CD117+/CD133 +* cells were considered to be ovarian cancer stem cell-like cells (OCSCs). (Fig. 1)

2. MiRNA-target regulatory network was established by combining miRNA microarray and GEO datasets results

The results of miRNA microarray were combined with GSE107155-SKOV3 and GSE107155-Kuramochi to find common DEMs. 28 DEMs were identified. In our miRNA microarray results, 15 of them up-regulated and 13 down-regulated in OCSCs. In GSE107155-SKOV3 dataset, 24 of them showed increased expression, while only 4 DEMs showed decreased expression. While the results of GSE107155-Kuramochi dataset suggested that all of these 28 DEMs were up-regulated. According to our miRNA microarray results, *miR-1268a* was the most significantly up-regulated miRNA and *miR-324-5p* was the most significantly down-regulated one. *MiR-630* was the most significantly upregulated miRNA in GSE107155-SKOV3 and -Kuramochi datasets. *MiR-221-3p* was the most significantly downregulated one in GSE107155 SKOV3 dataset. (Additional file 1)

After analysis by PicTar, Targetscan, DIANA and Starbase, potential target genes of these DEMs were combined with the mRNA microarray results to find common DEGs. Finally, 285 up-regulated and 167 down-regulated DEGs were identified. (Table 2) These DEGs were the predicted target genes for 24 of the 28 DEMs.

Table 2

Obtained potential target DEGs by combining mRNA microarray results with PicTar, Targetscan, DIANA and Starbase predicted results of targets of the 28 DEMs (Only the top 25 DEGs with the greatest expression difference were listed in each group. All the DEGs listed in the table showed significant differences, $P < 0.05$. And the fold changes of each gene was more than 2.)

Up-regulated DEGs		**Down-regulated DEGs**	
Gene name	**Fold change**	**Gene name**	**Fold change**
CHRD	24.64	*LONRF1*	-3443.92
BHLHE41	11.39	*TLE4*	-373.14
PRDM1	10.33	*PRR9*	-9.36
YPEL5	10.05	*SLC2A14*	-9.32
TSC22D3	8.86	*PRKCDBP*	-8.67
NLGN2	7.32	*NAV3*	-8.01
NR1D1	7.27	*MAT2A*	-7.93
CAPS	6.91	*TET2*	-7.45
RFFL	6.75	*SYNCRIP*	-6.44
RASA4	6.50	*PSEN2*	-6.39
PCDH18	6.38	*MAT2A*	-6.27
NLN	6.35	*PCDHGC4*	-6.17
DIO2	6.30	*PPP2R2C*	-6.15
SMIM14	6.07	*HOMEZ*	-6.00
AK7	6.02	*MRPS25*	-5.68
PLD6	5.87	*USP6NL*	-5.60
CLMN	5.80	*EGFR*	-5.58
ADAM10	5.76	*ZSWIM5*	-5.57
NF1	5.54	*CLDN2*	-5.51
BBX	5.54	*KLHL6*	-5.21
EIF4A2	5.48	*UBA5*	-4.89
PIGV	5.44	*AP1S3*	-4.82
NRP2	5.36	*UBTF*	-4.64

Up-regulated DEGs		Down-regulated DEGs	
TMEM154	5.29	*NLGN1*	-4.46
KIAA0430	5.26	*EPHB3*	-4.36

GO functional enrichment analysis was performed on these 452 DEGs. It was indicated that these genes were significantly enriched in 'regulation of GTPase activity' (BP, GO: 0043087), 'cytosol' (CC, GO: 0005829) and 'protein binding' (MF, GO: 0005515). 285 up-regulated genes were significantly enriched in 'cell migration' (BP, GO: 0016477), 'nucleus' (CC, GO: 0005634) and 'ATP binding' (MF, GO: 0005524), et al. 167 down-regulated genes were significantly enriched in 'positive regulation of transcription from RNA polymerase II promoter' (BP, GO: 0045944), 'nucleoplasm' (CC, GO: 0005654) and 'protein binding' (MF, GO: 0005515), et al. (Fig. 2)

KEGG pathway analysis found that these genes were significantly enriched in 'Salivary secretion', 'Ras signaling pathway', 'p53 signaling pathway' and 'AMPK signaling pathway', et al. The tumor-related pathways in which 285 up-regulated genes were significantly enriched included 'p53 signaling pathway', 'FoxO signaling pathway' and 'MicroRNAs in cancer', et al. There were 2 KEGG pathways which the 176 down-expression genes significantly riched in, such as 'Adherens junction' and 'Hepatitis C', but for the other pathways, 'Notch signaling pathway' and 'Endometrial cancer', the P values were > 0.05. (Fig. 2)

Using STRING and cytoHubba, we had obtained the top 10 key genes of these DEGs, ranked by Maximal Clique Centrality (MCC) degree. (Table 3) According to MCODE analysis, there were 16 clusters, among which the most significant cluster included 11 nodes and 55 edges. The top 10 key genes were all included in this cluster. *UNKL, GNAQ, NMD3, CPD, YPEL5, GAPVD1, VIM, EIF4EBP2, RND3, CALB2, STX16, NHLRC3, TNFRSF21* and *OAS2* were seed genes of these clusters. (Fig. 3)

Table 3
The top 10 key genes of PPI network of the target DEGs, ranked by Maximal Clique Centrality (MCC) degree

Rank	Name	Fold change	Score
1	*FBXO32*	2.44	3628810
2	*UBE2L6*	2.03	3628806
3	*RNF14*	2.17	3628802
3	*UBE2H*	3.39	3628802
3	*UBA5*	-4.89	3628802
3	*FBXL16*	2.52	3628802
3	*LONRF1*	-3443.92	3628802
8	*RNF115*	-2.24	3628801
8	*HECTD1*	2.07	3628801
8	*TRIM4*	2.63	3628801

3. MiRNA-lncRNA regulatory network was established by combining miRNA, lncRNA microarray, lncBase and starbase results

LncBase and Starbase were applied to predict the lncRNA transcripts that could bind to the 24 DEMs mentioned above. Combined with our lncRNA Microarray result, we found that 29 transcripts of 17 lncRNA could bind to 14 of the 24 DEMs. (Additional file 2) 22 transcripts were up-regulated and 7 transcripts were down-regulated in OCSCs compared with common ovarian cancer cells according to our lncRNA microarray analysis.

4. Functional enrichment analysis, establishment of protein-protein interactions (PPI) network and and identification of hub genes in OCSCs

Microarray analysis of DEGs was combined with GSE80373 and GSE28799 datasets. As a result, 105 differentially expressed genes were identified in all the three datasets, including 72 up-regulated genes and 33 down-regulated genes in OCSCs compared with ovarian cancer cells. (Fig. 4)

GO functional enrichment analysis on these genes indicated that these DEGs were significantly enriched in ‘negative regulation of cell proliferation’ (BP, GO:0008285), ‘cytosol’ (CC, GO:0005829) and ‘ferroxidase activity’ (MF, GO:0004322). 72 up-regulated genes were significantly enriched in ‘doxorubicin metabolic process’ (BP, GO:0044597), ‘extracellular exosome’ (CC, GO:0070062) and ‘geranylgeranyl reductase activity’ (MF, GO:0045550). 33 down-regulated genes were significantly enriched in ‘intracellular

ribonucleoprotein complex' (CC, GO:0030529). Although they were enriched in 'positive regulation of cell growth' (BP, GO:0030307) and 'poly(A) RNA binding' (MF, GO:0044822), but the enrichments were not significant (P > 0.05). (Fig. 4)

KEGG pathway analysis indicated that these genes were significantly enriched in 'FoxO signaling pathway'. 72 up-regulated genes were significantly enriched in 'FoxO signaling pathway', 'Pentose and glucuronate interconversions', 'Mineral absorption' and 'Glutathione metabolism'. There were 2 KEGG pathways which the 33 down-expression genes were enriched in, such as 'RNA transport' and 'Ras signaling pathway', while the P value of the latter one is > 0.05. This might be due to the relatively small number of down-regulated genes. (Fig. 4)

Using STRING and cytoHubba, we had obtained the top 10 key genes of these DEGs, ranked by MCC degree. They were *CAT, EGFR, GSR, EEF1A1, IDH1, CLU, TGFB1, AKR1B1, MT1X, MT1E*. (Table 4, Fig. 4) According to MCODE analysis, there were 3 clusters,including 3 nodes and 3 edges, 10 nodes and 13 edges and 9 nodes and 11edges respectively. *MT1E* and *FBXO32* were seed genes of these clusters. The *GSR, EEF1A1, IDH1, CLU, TGFB1, AKR1B1, MT1X* and *MT1E* of top 10 key genes were included in these clusters. (Fig. 4)

Table 4
The top 10 key genes of the PPI network of the 105 DEGs, ranked by Maximal Clique Centrality (MCC) degree

Rank	Name	Fold change	Score
1	*CAT*	2.15	32
2	*EGFR*	-5.58	30
3	*GSR*	2.40	13
4	*EEF1A1*	2.13	8
4	*IDH1*	2.10	8
4	*CLU*	2.66	8
4	*TGFB1*	6.10	8
4	*AKR1B1*	26.08	8
9	*MT1X*	2.98	6
9	*MT1E*	3.44	6

5. Construction of ceRNA regulatory network of OCSCs

21 DEGs were identified after combination of the 452 DEMs target genes and the 105 DEGs, including 11 up-regulated and 10 down-regulated DEGs. Finally, these DEGs were predicted as target genes for 10 of the 24 DEMs. Among them, the up-regulated miRNA were *miR-1287-5p, miR-193b-3p, miR-423-5p* and

miR-374b-5p, and the down-regulated miRNA were *miR-425-5p, miR-96-5p, miR-26a-5p, miR-30e-5p, miR-183-5p* and *miR-146a-5p*. A total of 25 transcripts of 13 lncRNA were predicted as the ceRNA of these miRNAs, including 21 up-regulated transcripts and 4 down-regulated ones. (Table 5, Additional file 3) Fig. 5 showed the obtained ceRNA regulatory network.

Table 5
MiRNAs, mRNAs and lncRNAs of the ceRNA network of OCSCs

miRNA	Predicted targets		lncRNA	
	up	down	up	down
miR-1287-5p up	*NRP2*	*GFRA1, EGFR*	*NEAT1-202*	*AC040162.3-201*
miR-193b-3p up	*TLE4, BCL6*	*CAMTA1, SLC7A5, LONRF1*	*NEAT1-202*	*LINC01184-208*
miR-423-5p up		*RASAL2*	*PVT1-212*	*SNHG20-203*
miR-374b-5p up	*AFF1*, *PAPD4*		*NEAT1-202*	
miR-425-5p down		*HNRNPD*	*MALAT1-201* *NEAT1-202*	
miR-96-5p down	*BRWD1, GNE*, *FBXO32*	*TNS3*, *GFRA1*	*MAPKAPK5-AS1-205* *MAPKAPK5-AS1-207* *MAPKAPK5-AS1-206* *MALAT1-201* *MAPKAPK5-AS1-204* *MAPKAPK5-AS1-201* *MALAT1-202*	

miRNA	Predicted targets		lncRNA	
	up	down	up	down
miR-26a-5p down	*ADM, PAPD4*	*ATP11C*	*LINC00665-207* *GAS5-212* *GAS5-206* *LINC00665-202* *MALAT1-202* *LINC00665-204* *NEAT1-202* *SNHG5-210* *SNHG5-206* *SNHG5-202*	*LINC00665-205*
miR-30e-5p down	*PRDM1, PAPD4*	*RASAL2, HMGB3*	*LINC01089-210* *LINC01089-211* *SNHG16-208* *NEAT1-202* *AC008124.1-201*	
miR-183-5p down	*NRP2*		*NEAT1-202*	
miR-146a-5p down	*CASK, NRP2*	*HNRNPD*	*LINC00665-207* *NEAT1-202*	

6. Preliminary validation of this bioinformatics research results

Through Kaplan-Meier (Km) curve analysis, there were 5 miRNAs, 12 mRNAs and 5 lncRNAs of which the differential expressions were significantly related to survival probability in ovarian cancer. Among these genes, the most significantly related lncRNA was *LINC00665*, of which the expression was up-regulated in OCSCs. According to the ceRNA network, one of *LINC00665* transcripts was considered to be the ceRNA of *miR-146a-5p*. In this study, its expression showed a downward trend in OCSCs. *NRP2* was one of its predicted target genes, which was the most significantly up-regulated mRNA of in the OCSCs ceRNA network. Km curve analysis showed that ovarian cancer patients with reduced *mir-146a-5p* expression had shorter survival time, while patients with reduced *LINC00665* and *NRP2* expression tended to have longer survival time. QPCR confirmed the microarray results that *miR-146a-5p* expression in OCSCs was

lower than that in COC1, while *LINC00665* expression was higher than that in COC1. ($P < 0.05$) *LINC00665* was further overexpressed in COC1, and it was found that the proliferation ability of *LINC00665*+ COC1 was increased, apoptosis was decreased, and percentage of G2/M cells was increased. (Fig. 6, $P < 0.05$) In future studies, we need to further clarify the roles of *LINC00665*, *miR-146a-5p* and *NRP2* in OCSCs and the regulatory relationships among them. (Fig. 6 and Additional file 4)

The procedure of our study was shown in Fig. 7.

Discussion

Failure of ovarian cancer treatment was often associated with the development of chemoresistance, including refractoriness (inherent resistance) and resistance (recurrence occurred within 6 months after response to complete treatment) [44], which was more common in ovarian cancer chemotherapy. It is of great significance to study OCSCs to clarify the formation and reversal of chemoresistance in ovarian cancer. Therefore, the study on the stem characteristic maintenance of OCSCs is expected to partially reveal the causes of ovarian cancer drug resistance and help find a way to inhibit or reverse the chemoresistance of ovarian cancer through the OCSCs pathway. The application of microarray technology and GEO database helps us to screen differential genes in OCSCs and to further find important genes related to maintain stem characteristics, so as to identify important regulatory genes that may influence drug resistance in ovarian cancer.

In order to construct important ceRNA regulatory networks, *CD117+/CD133 +* cells were first screened in the sphere from ovarian cancer cells COC1. The elevation of stem cell markers revealed that this group of cells had maintained stem cell conditions. The DEMs, DELs and DEGs between *CD117+/CD133 +* cells (OCSCs) and COC1 were further identified by microarray technology. Combining the microarray results with GEO datasets, we found that the target DEGs of DEMs of OCSCs were enriched in cancer related pathway, such as ‘Ras signaling pathway’, ‘p53 signaling pathway’, ‘AMPK signaling pathway’ and ‘FoxO signaling pathway’. It was suggested that these DEMs were involved in the regulation of the occurrence and development of OCSCs. While the DEGs of OCSCs were enriched in cancer related pathway, such as ‘FoxO signaling pathway’, ‘RNA transport’, ‘Pentose and glucuronate interconversions’, ‘Mineral absorption’ and ‘Glutathione metabolism’. It was also suggested that these DEGs were involved in the regulation of the occurrence and development of OCSCs by metabolic pathways. Combining with these two groups of DEGs had contributed to narrow the range of important mRNAs that regulate the characteristics of OCSCs. Meanwhile, lncRNAs that might be ceRNAs of these DEMs were found by bioinformatics analysis combined with lncRNA microarray result. Finally, 10 key (4 up-regulated and 6 down-regulated miRNAs) miRNAs and 21 predicted target mRNAs (11 up-regulated and 10 down-regulated mRNAs) were identified. 25 transcripts of 13 lncRNAs (21 up-regulated and 4 down-regulated transcripts) should be ceRNAs of these miRNAs. According to Km curve analysis, we found that the 5 DEMs, 12 DEGs and 5 DELs were significantly related to survival probability in ovarian cancer.

We selected the lncRNA with the highest correlation with survival probability, *LINC00665*, and its related miRNA,*miR-146a-5p* for preliminary study. QPCR confirmed microarray results that the expression of *LINC00665* was increased and *miR-146a-5p* was decreased in OCSCs compared with COC1. Further regulation of the expression of *LINC00665* indicated that *LINC00665* was associated with cell proliferation and apoptosis in ovarian cancer cells. The target genes of *MiR-146a-5p* in our ceRNA network included *NRP2*, *CASK* and *HNRNPD*, and the differential expression of these target genes was statistically significant with survival probability. However, the expression trend of *HNRNPD* was the same as that of *miR-146a-5p*, while among the three genes, the expression difference of *NRP2* mRNA was the largest in our microarray result. Therefore, we focused on analyzing the possible roles of *miR-146a-5p*, *LINC00665* and *NRP2* in the regulation of OCSCs stem cell characteristics in the following sections.

MiR-146a-5p is one of the anti-inflammatory miRNAs, which is considered to be a therapeutic target for inflammation-related diseases such as perinatal cardiomyopathy [22] and obesity [51]. It acts as a single miRNA regulatory factor inhibiting the function of regulatory T (Treg) cells, maintaining immune homeostasis of Treg cells, and its absence leads to *IFNγ*-dependent immune-mediated fatal lesions or tumors in a variety of organs [38, 41]. Its rs2910164 SNP genotype may also influence the age of onset of cancer and is associated with early onset of cancer [48]. The abnormal expression of *miRNA-146a* in CSCs has been found in many studies. And its effects in different tumors might be completely opposite [56]. As an exosomal miRNA in colorectal cancer, *miR-146a-5p* could target *NUMB* to activate wnt signaling pathway to generate and maintain cancer stem cells characteristics for promotion of tumor formation [7, 26]. While in cervical cancer and breast cancer CSCs, *miR-146a-5p* played a completely opposite role [14, 34]. It has been indicated that the expression of *miR-146a-5p* in tumors was also related to chemoresistance and metastasis of tumor cells [56]. *MiR-146a-5p* could enhance the induction effect of platinum on apoptosis so as to prohibit cell proliferation in EOC cells [11, 33]. Patients with lower expression of *miR-146a-5p* had reduced survival probability, shortened progression-free survival (PFS), and were prone to platinum chemoresistance [66]. At present, there is no study on *miR-146a-5p* in OCSCs, but the *miR-146a-5p* derived from mesenchymal stem cells exosome has been reported to be able to enhance the sensitivity of ovarian cancer cells to docetaxel and taxane [50]. According to our study, *miR-146a-5p* might play an inhibitory role in OCSCs.

In our study, it had been found that a transcript of *LINC00665* could be a ceRNA to participate in the regulation of *miR-146a-5p* on target genes. Studies on *LINC00665* have been increasing in recent years. It has been considered to play an important regulatory role in various tumors. In 2018, Dong-Yue Wen et al. found an abnormally elevated *LINC00665* expressed in hepatocellular carcinoma (HCC) data in the Cancer Genome Atlas (TCGA) database, which could interact with the cell cycle regulatory proteins to promote the development and progression of HCC[65]. Following studies suggested that *LINC00665*, as the ceRNA of multiple miRNAs, could play an important role in the regulation of cell biological behaviors and chemoresistance in various malignant tumors [10, 37, 49, 57, 69]. In high-grade serous ovarian cancer, a ceRNA regulatory network correlated with lymphocyte infiltration had been established by using TCGA, GTEx and GEO datasets, and it was suggested that *LINC00665* was positively correlated with lymphocyte infiltration [67]. *LINC00665* also plays a role in tumor regulation by maintaining protein

stability. For example, by activating and maintaining *PKR* stability, *LINC00665* could promote hepatocellular carcinoma progression [13]. It also could promote lung adenocarcinoma angiogenesis by stabilizing YB-1 protein [9]. Encoding small peptides is another way in which *LINC00665* participates in the regulation of tumor progression [21]. *LINC00665* could also participate in the regulation of malignant biological behaviors of tumor cells by *STAU1*-mediated mRNA Degradation (SMD), which was an important post-transcriptional regulation of mRNA stability [52]. In conclusion, *LINC00665* has a clear regulatory role in many tumors, but there has been no study on its influence on the stem cell characteristics of CSCs, including OCSCs. In addition, we found that not all transcripts of *LINC00665* showed an upward trend in the constructed ceRNA regulatory network. Therefore, the function of *LINC00665* is worthy of further study.

NRP2 protein, a member of the NRPs family, is related to the development of the nervous system. It has the function of affecting signal receptors as a co-receptor. It is a transmembrane glycoprotein, which contains four extracellular domains capable of binding to ligands and one short cytoplasmic domain. Many microarray analyses revealed that *NRP2* might be a hub gene for the development of a variety of tumors [36, 72, 73]. Its function was either dependent or independent of the vascular endothelial growth factor receptor (VEGFR). Vascular endothelial growth factors (VEGFs) have the function of receptor tyrosine kinases activity to promote angiogenesis and increase vascular permeability by binding with VEGFR, and are involved in maintaining the stem cell characteristics of CSCs in many tumors [28, 35]. While the co-receptor *NRP2* can form a complex with VEGFR to enhance its affinity with VEGF and enhance tumor-promoting effect of VEGF [46]. In addition, *NRP2* can bind to integrins to regulate CSCs generation and stem characteristics maintenance through VEGF/*NRP2* signaling [17, 18, 20]. At present, most of anti-VEGF drugs (such as bevacizumab), which only block the binding of VEGF and VEGFR but not *NRP2*, cannot inhibit CSCs effectively, but promote the enrichment [16]. Therefore, in addition to anti-VEGF therapy, combining anti-VEGF/*NRP2* therapy may enhance the benefits of anti-VEGF therapy alone [19]. *NRP2* can also act without VEGF signaling in CSCs. It has been indicated that *NRP2* could bind to PDZ protein through PDZ domain in its cytoplasmic part, participating in receptor transport and signal transduction in tumor development regulation M Katoh [27]. Other studies have shown that *NRP2*b, an alternative splicing isomer of *NRP2*, could bind to *PTEN* to increase the resistance of non-small-cell lung cancer (NSCLC) cells to EGFR inhibitor gefitinib [15]. Although *NRP2* wasn't the hub genes of PPI networks according to our analysis, but the up-regulated trend was the most obvious among mRNA in the ceRNA network in OCSCs. Km curve suggested that the survival probability of patients with high *NRP2* expression decreased faster than that of patients with low expression. All the above suggested the importance of *NRP2* in tumor development. According to our study, *NRP2* might play an important role in OCSCs. Further study of the ceRNA mechanism regulating *NRP2* expression in OCSCs can better reveal the mechanism of stem cell characteristics maintenance in OCSCs.

CeRNA is an important post-transcriptional regulation mode of genes. At present, studies on ceRNA regulation in various CSCs have been carried out extensively. However, as one of cancer stem cell-related tumors, the establishment of ceRNA regulatory network in ovarian cancer hasn't been reported yet. According to our study, a ceRNA regulatory network of OCSCs has been initially established by microarray

analysis and bioinformatics research, but further clinical and molecular biology studies are required. In the ceRNA network constructed, it was indicated that the protein encoding genes had less direct interaction with each other according to PPI constructed by STRING and Cytoscape. However, through the construction of ceRNA network, these protein encoding genes with no direct interactions were connected indirectly by lncRNA-miRNA-mRNA network. Our further studies are expected to find out the important sites and regulatory ways to maintain the characteristics of stem cells in OCSCs, providing new theoretical basis and experimental guarantee for ovarian cancer research.

Conclusion

The ceRNA network we constructed may be involved in the stem cell characteristics maintenance of OCSCs and provide directions for further OCSCs research in the future, so as to assist the development and treatment of ovarian cancer.

Methods

1. Cell culture and drug screening

Human serous epithelial ovarian carcinoma cell line COC1 was bought from China Center for Type Culture Collection (CCTCC). The cells were seeded in 6-well plate and cultured in the 37 ℃, 5%CO2 incubator, feeding with RPMI-1640 medium (Gibco, 31800-014, US) supplement with 10% fetal bovine serum (Hyclone, SH30084.03, US). Centrifuged and then changed the medium after 24 h culture. The cells were feeded with the same medium above until they grew to 80% confluence. After treated with 40 μmol/L cisplatin and 10 μmol/L paclitaxel, the cells were cultured for another 5 days [63]. Then passed the cells after centrifuging and maintained them under stem cell conditions, which was composed of RPMI-1640 medium (Gibco, 31800-014, US), 5 μg/ml recombinant human insulin (Solarbio, 11061-68-0, China), 10 ng/ml recombinant human epithelial growth factor (*EGF*, Sino Biological Inc, 10605-HNAE, China), 10 ng/ml basic fibroblast growth factor (*bFGF*, Sino Biological Inc, 10014-HNAE, China) and 12 ng/ml leukemia inhibitory factor (*LIF*, Cloud-Clone Corp, RPA085Hu01, US) [40]. Changed the medium every other day by centrifuging at 1000 rpm for 5 min, and then collected the cells after 6 days for further gene expression and protein detection.

2. Sphere formation assay

The floating sphere cells were dissociated by incubation with 0.02% trypsin–EDTA for 1–2 min at 378C and 100 cells per well were plated in 96-well culture dishes in 200 ml of growth medium; 25 ml of medium per well was added every 2 days. The number of dissociated spherical cells for each well was evaluated after 7-day culture.

3. Flow cytometric analysis

The treated cells were centrifuged at 1000 rpm for 5 min and then yielded. Wash the cells two times by phosphate buffered solution (PBS, Doublehelix, P10033, China) and collected the cells 1000rmp at 1000 rpm for 5 min. After centrifugation, the supernatant was discarded. The grouped 1×10^6 cells were resuspended with 100 μl PBS supplement with anti-*CD133* (eBioscience, 12-1339-41, US), anti-*CD117* (eBioscience, 11-1178-41, US) and control antibody, respectively. The percentage of *CD133+/CD117 +* cells was performed by flow cytometric analysis after incubated in dark.

4. RNA extraction and real-time quantitative PCR (qPCR)

Total RNA was extracted according to the protocol of total RNA kit (BioTeke, RP1201, China). The concentration of the RNA extracted was calculated, then 1 μg of total RNA was added into 19 μl reverse transcription mixture for reverse transcription polymerase chain reaction (RT-PCR) according to the protocol of PrimeScript™ RT reagent Kit (Perfect Real Time) (Takara, RR037Q, China). The gene expression was detected according to the protocol of SYBR Green (Solarbio, SY1020, China) method by using the ExicyclerTM 96 fluorescent quantitative machine (BIONEER, Korea). The primers sequences are showed in Table 1. The reaction cycle is: 94 ℃, 10 min, 94 ℃, 10 s, 60 ℃, 20 s, 72 ℃, 30 s, repeated 40 cycles, 72 ℃, 2 min 30 s, 40 ℃, 5 min 30 s, 60 ℃ to 94 ℃, 1.0 ℃ per 1 s, 25 ℃, 1 min. The data were analyzed by $2^{-\Delta\Delta CT}$ method and the CT (threshold cycle) value means the number of cycles after which a signal of each sample could be detected. And β-actin was used as internal reference control for normalization.

Table 1
Primes of qPCR

Name	F	R
miR-146a-5p	TGAGAACTGAATTCCATGGGTT	GTGCAGGGTCCGAGGTATTC
LINC00665	GGTGCAAAGTGGGAAGTGTG	AGTCCGGTGGACGGATGAGAA
miR-183-5p	GCGGCTATGGCACTGGTAGAA	GTGCAGGGTCCGAGGTATTC
miR-96-5p	TTTGGCACTAGCACATTTTTGCT	GTGCAGGGTCCGAGGTATTC
β-actin	GGCACCCAGCACAATGAA	CGGACTCGTCATACTCCTGCT
U6	GCTTCGGCAGCACATATACT	GTGCAGGGTCCGAGGTATTC

5. Western blot

Cell lysis buffer (Beyotime, P0013, China) was used to lyse COC1 and spheroid cells at 4 ℃ for 5 min. The debris was removed by centrifugation at 12000 rpm, 4 ℃ for 10 min and the supernatant protein extract was analyzed for western blot. The quantitative determination of protein was made according to the manufacturer's instructions (BIOTEK, ELX-800, US). The protein sample diluted by 5 × Loading Buffer and PBS was boiled in boiling water for 5 min. The volume of each sample was 20 μl containing 40 μg

protein. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed after protein loaded at 80 V for 2.5 h. Then the protein was transferred to a polyvinylidene fluoride (PVDF, Millipore, IPVH00010, US) membrane by electroblotting. The transferred PVDF was put into the 5% (w/v) milk diluted by TBST buffer supplemented with 0.005% Tween 20 for 1 h to block nonspecific reaction. The membrane was incubated with rabbit anti-human *OCT4* antibody (1:500, Abcam, ab18976, UK), rabbit anti-human *SOX2* antibody (1:500, Abcam, ab97959, UK), rabbit anti-human *NANOG* antibody (1:500, Abcam, ab80892, UK), goat anti-human *ALDH1* antibody (1:500, Abclonal, A0157, China) and goat anti-human *LGR5* antibody (1:500, Abclonal, A12327, China) overnight at 4 ℃, followed by incubated with goat anti-rabbit secondary antibody (1:5000, Abcam, ab7090, UK). ECL western blot detection reagents (Pierce) was used for protein imaging according to the manufacturer's instructions by Gel-Pro-Analyzer (Beijing Liuyi, WD-9413B, China). β-actin (wanleibio, WL01845, China) was used as an internal reference.

6. Bioinformatics research methods

Agilent Human miRNA, Release 21.0 (8*60K, Design ID:070156) was used in this study for analysis of DEMs. Agilent Human lncRNA V5 (4*180K, Design ID:076500) was used in this study for analysis of DGLs and DEGs. Experiments were performed according to the instruction of the manufacturer. The criteria for differentially expressed miRNA is |Score (d)| ≥2. We idenfied the DEMs by combining with representative microarray profiles in GEO Database (GSE107155-SKOV3 and -Kuramochi). PicTar, Targetscan, DIANA and Starbase were used to predict the target genes of these DEMs. The results of each group were analyzed to find the common genes as the DEMs prediction target genes dataset in OCSCs. The obtained target genes were further combined with our microarray results to identify a set of miRNA target DEGs. At the same time, we identifed the common DEGs by combining our microarray profiles with GEO Database (GSE80373 and GSE28799). The obtained DEGs were further combined with the miRNA target DEGs to identify common DEGs in both sets. Differentially expressed lncRNAs (DELs) that may interact with DEMs were obtained by lncBase [47] and starbase, and combined with our lncRNA microarray results.

GO and KEGG analyses were performed for each group of DEGs [24, 25]. GO includes three parts: Biological Process (BP), Cellular Component (CC) and Molecular Function (MF). The number of DEGs included in each GO entry was counted, and the significance of enrichment of DEGs in each GO entry was calculated by statistical test. $P < 0.05$ indicated that the DEGs were enriched in this GO entry. Pathway analysis of DEGs was carried out using KEGG database and statistical test was used to calculate the significance of enrichment of DEGs in each pathway entry. $P < 0.05$ indicated that the DEGs were enriched in this pathway. The relationship between the expression and survival probability of key genes was analyzed by Km Curve [45]. Finally, we analysed the ceRNA network constructed by miRNA, lncRNA and mRNA in OCSCs by STRING and Cytoscape.

7. Statistical analysis

Data analysis was performed using SPSS 16.0 (IBM) statistical software. Numerical data were presented as mean ± standard deviation (SD), and categorical data were presented as percentage. To compare the

mean values of two related groups, homogeneity of variance test and unpaired samples t-test were performed. The Bonferroni test was used for pairwise comparisons of three data sets. $P < 0.05$ was considered statistically significant.

List Of Abbreviations

DEM: differentially expressed miRNAs; DELs: differentially expressed lncRNA; DEGs: differentially expressed mRNA.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Y Z designed the overall experimental scheme, performed bioinformatics analysis and manuscript modification, and acquired fund support. Z L: was a major contributor in writing the manuscript. Z L and Z W performed the molecular biology experiments. All authors read and approved the final manuscript.

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Figures

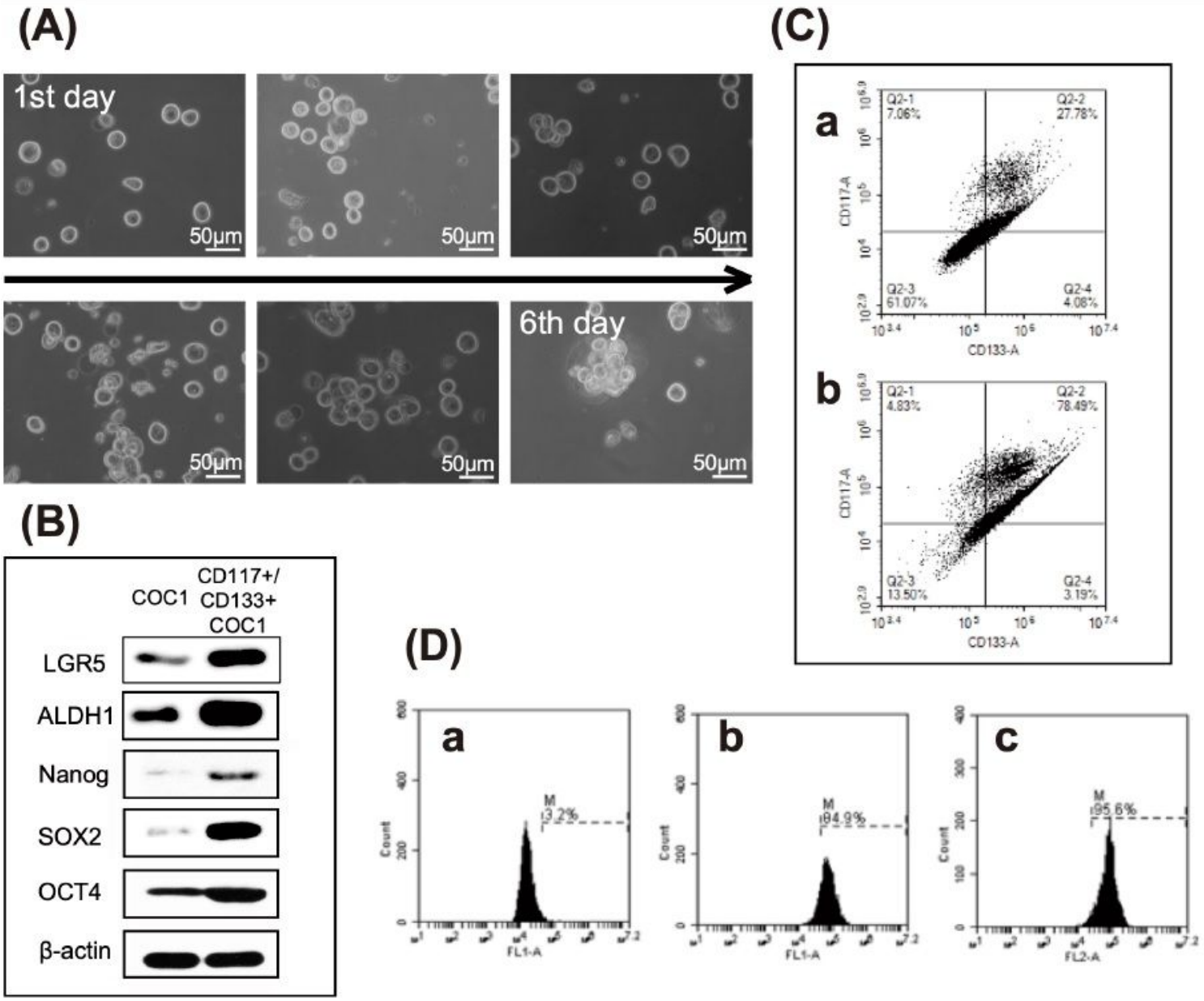
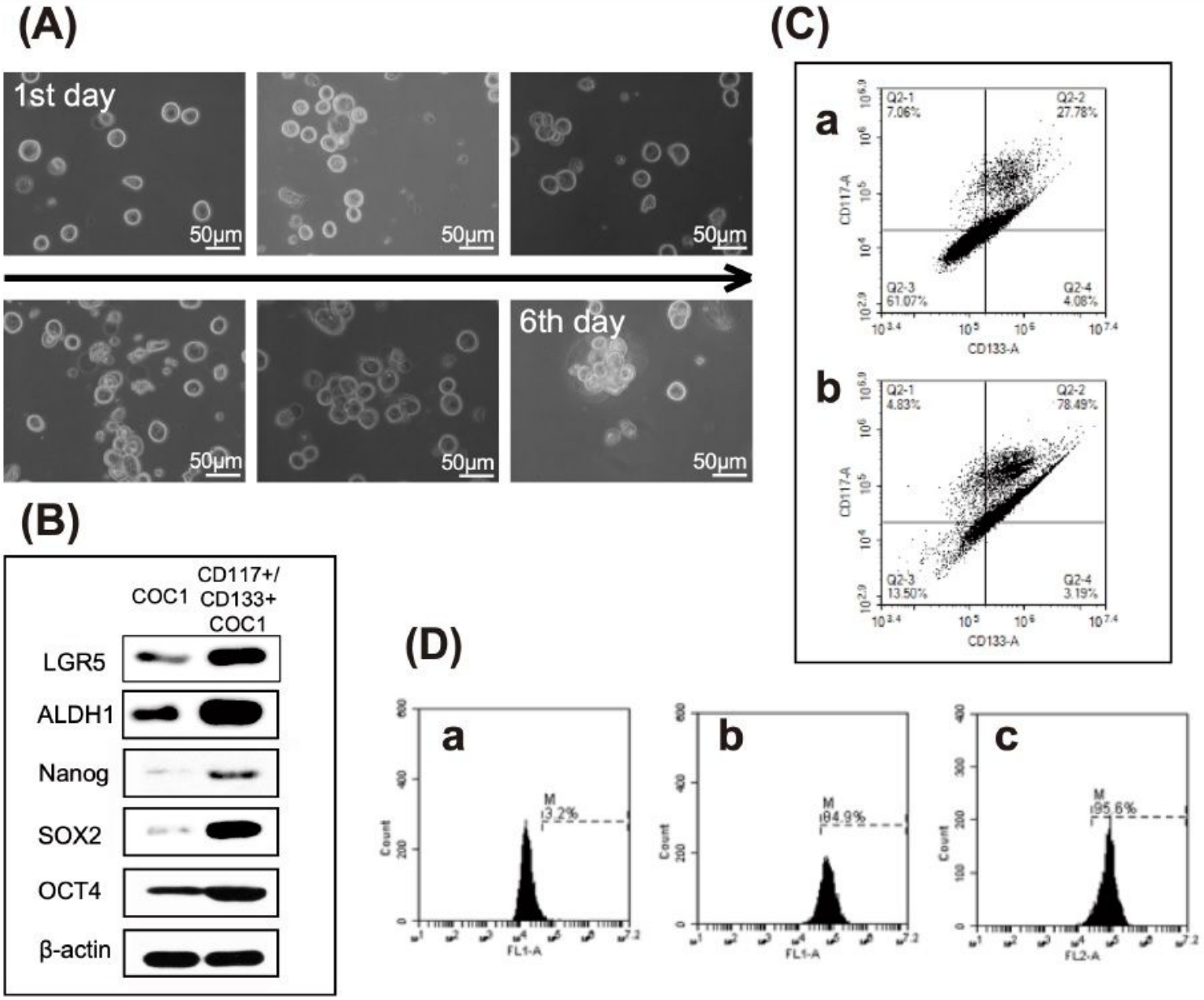


Figure 1

The sorting of ovarian cancer stem cell-like cells (CSCs) and the detection results of stem cell markers: (A) sphere formation in COC1, (B) detection of differences in expression of stem cell markers in COC1 after treatment by Western blot. Detection of the percentage of CD117+/CD133+ cells in COC1 after treatment by flow cytometry: (C) a, COC1, b, CD117+/CD133+ cells; (D) a, COC1, b, CD117+ cells, c, CD133+ cells.



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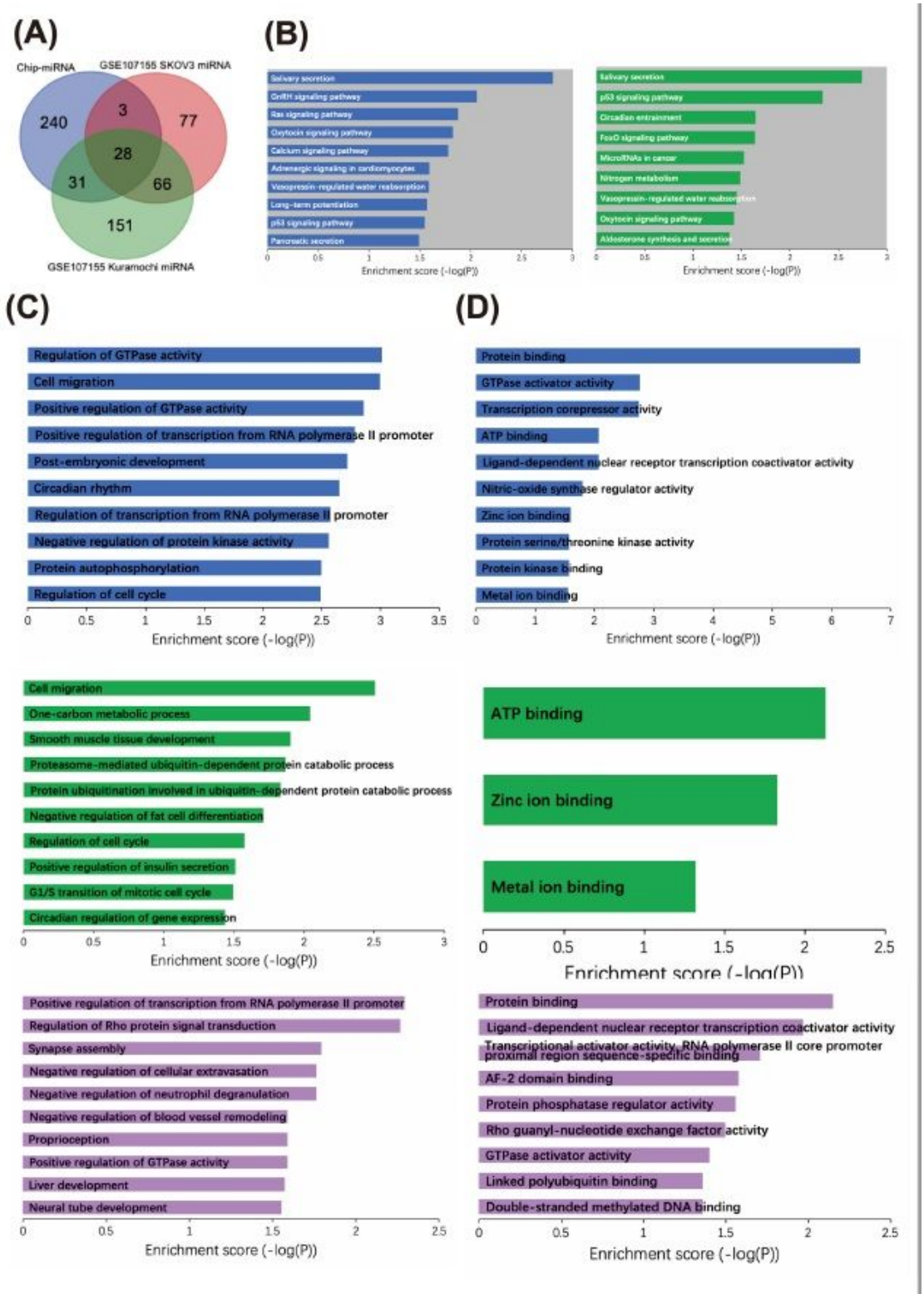
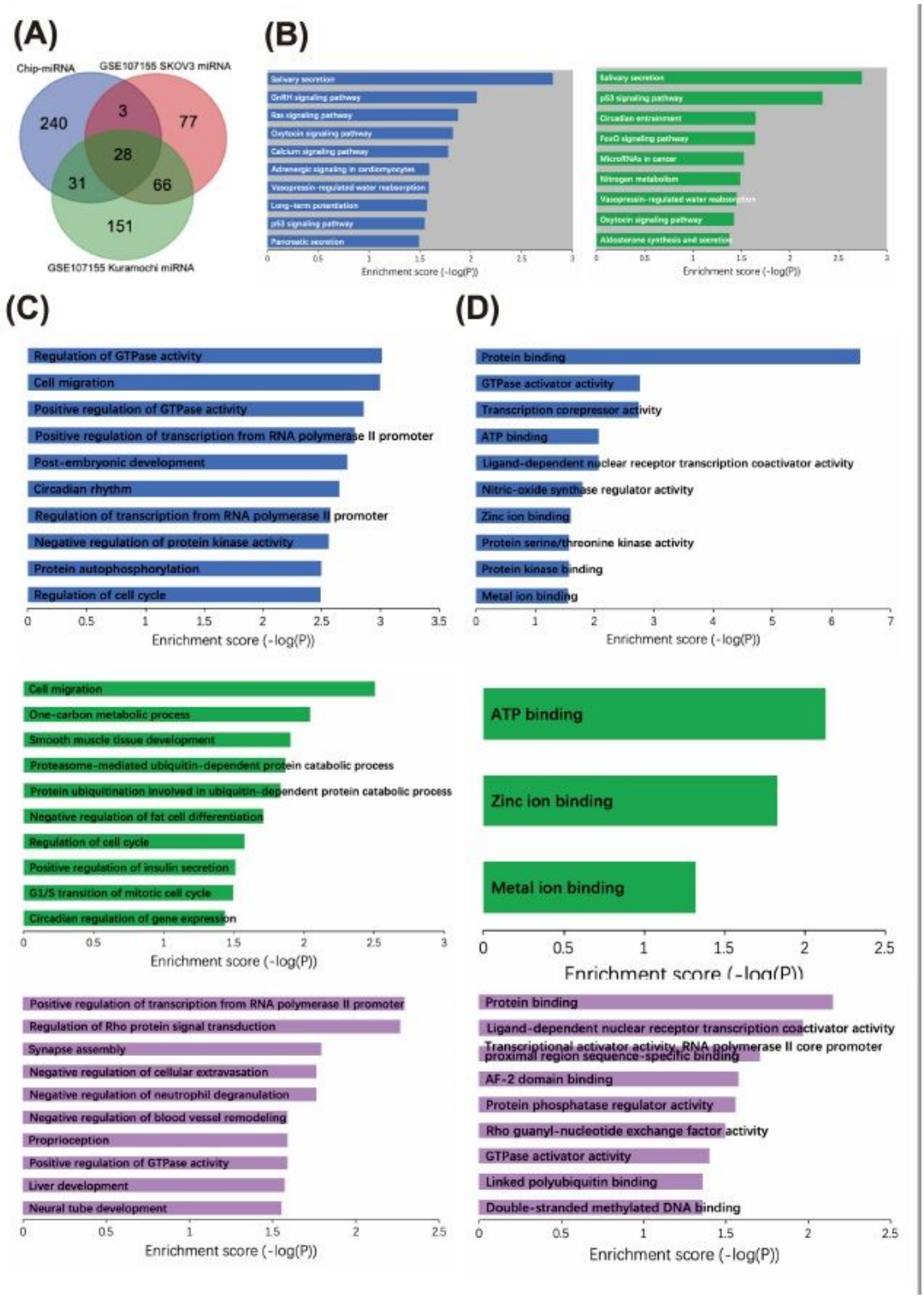


Figure 2

(A) Identification of DEMs in miRNA microarray, GSE107155-SKOV3 and GSE107155-Kuramochi datasets. The enrichment analysis of corresponding target DEGs in GO and KEGG pathways: (B) KEGG pathway enrichment analysis of corresponding target DEGs, (C) biological process enrichment analysis of corresponding target DEGs, (D) molecular function enrichment analysis of corresponding target DEGs.

The blue bars represented all DEGs, the green ones represented up-regulated DEGs, and the purple ones represented down-regulated DEGs. (P<0.05)



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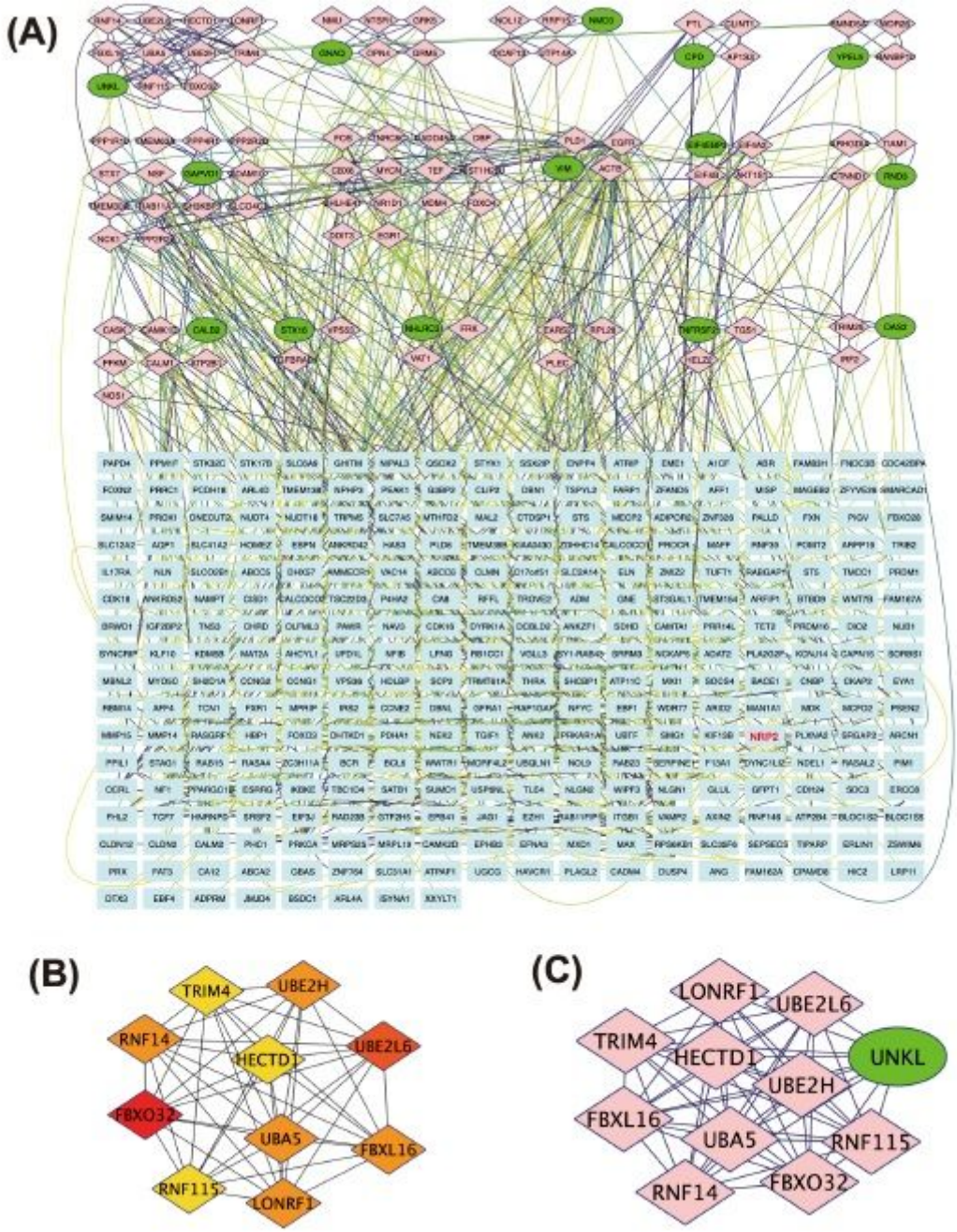
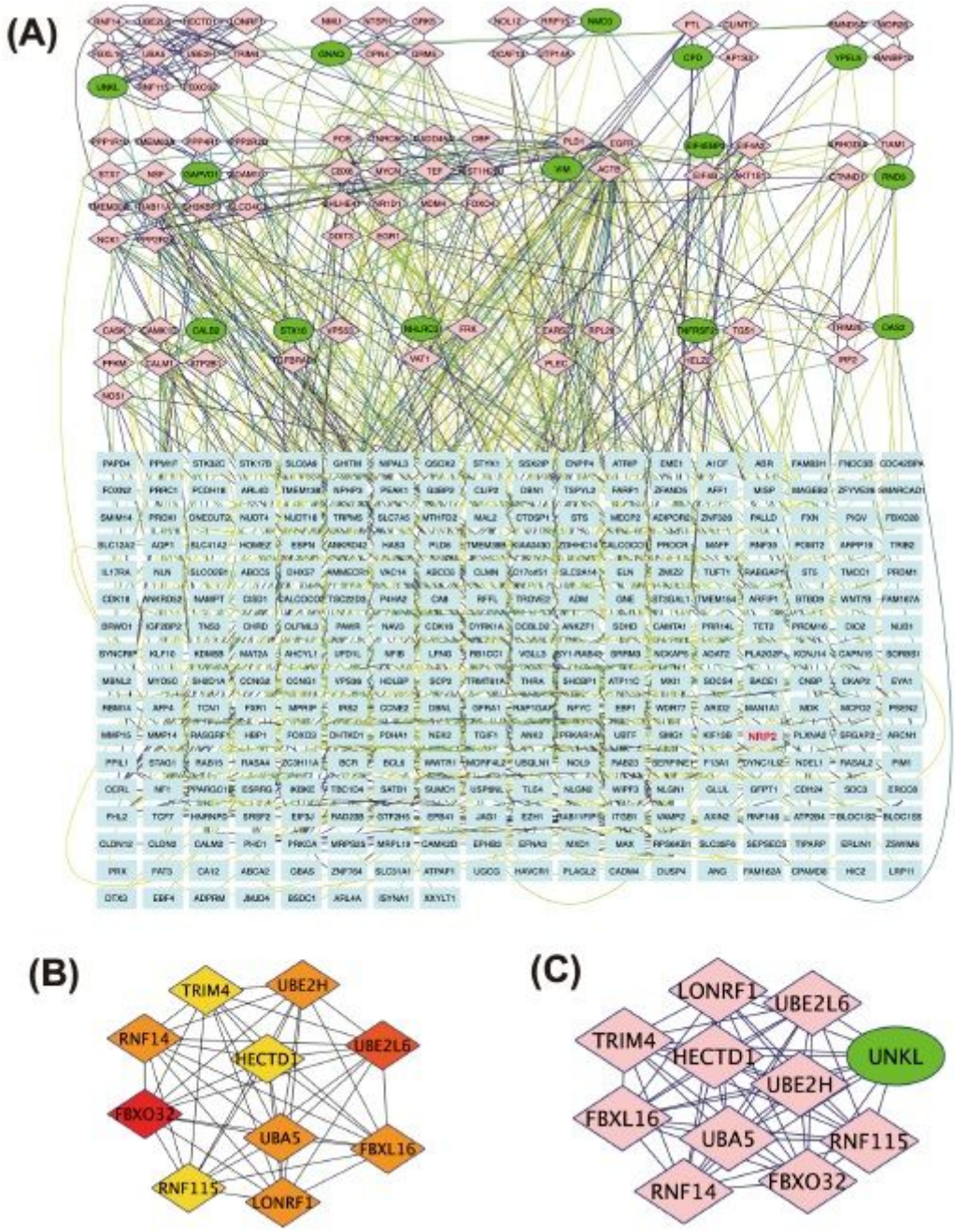


Figure 3

Constructed PPI network by STRING and cytoHubba: (A) PPI network was constructed for 452 predicted target DEGs of the 24 DEMs mentioned above by STRING, (B) the top 10 key genes ranked by MCC degree, (C) the most significant cluster including 11 nodes and 55 edges according to MCODE analysis.



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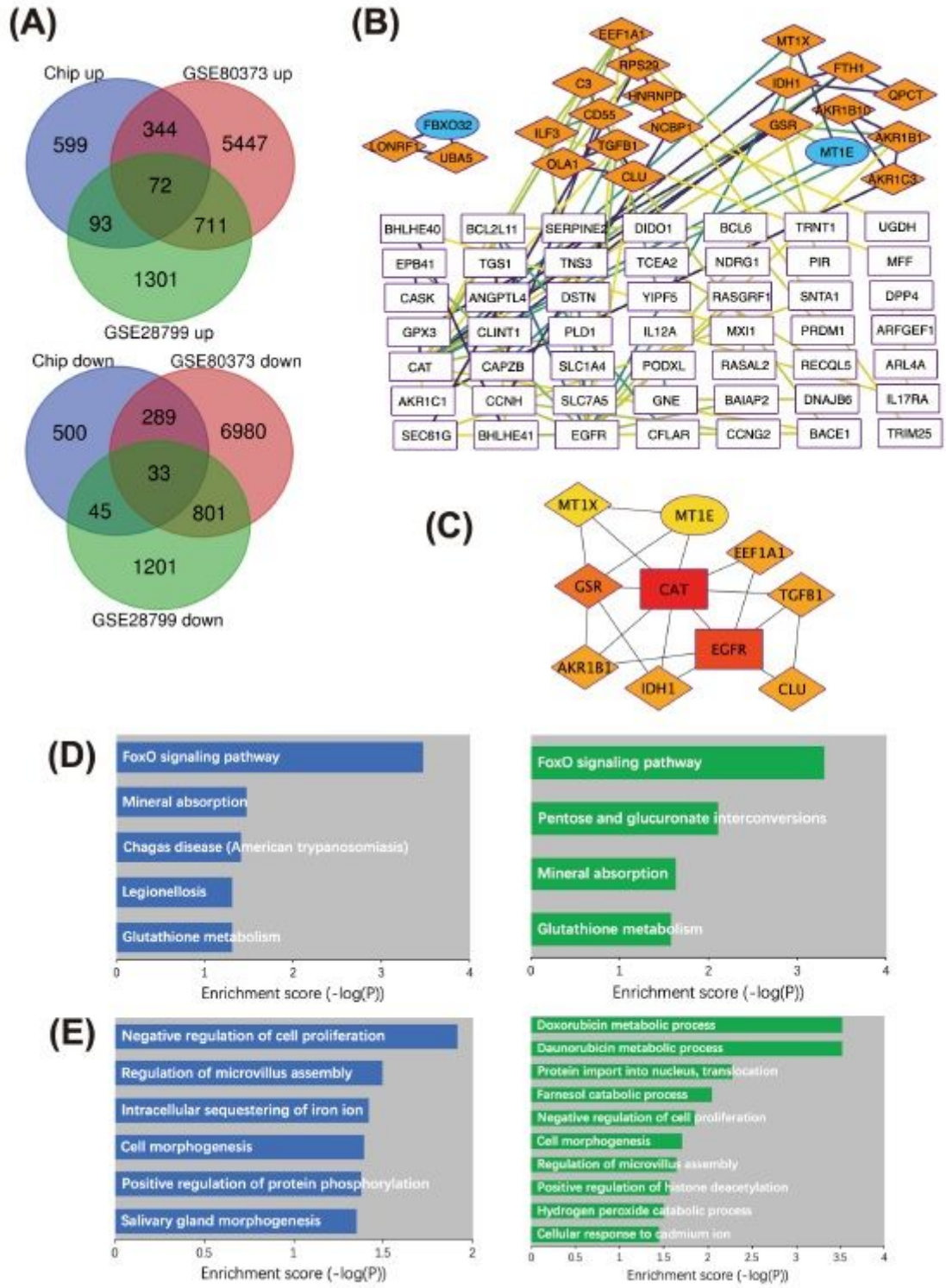

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(A) Identification of DEGs in miRNA microarray, GSE80373 and GSE28799 datasets, and the venn diagram on the top was for up-regulated genes, while the bottom one was for down-regulated gene, (B) constructed PPI network by STRING and cytoHubba, DEGs not participating in PPI network were not shown, (C) the top 10 key genes of the PPI network ranked by MCC degree, (D) KEGG pathway and E.

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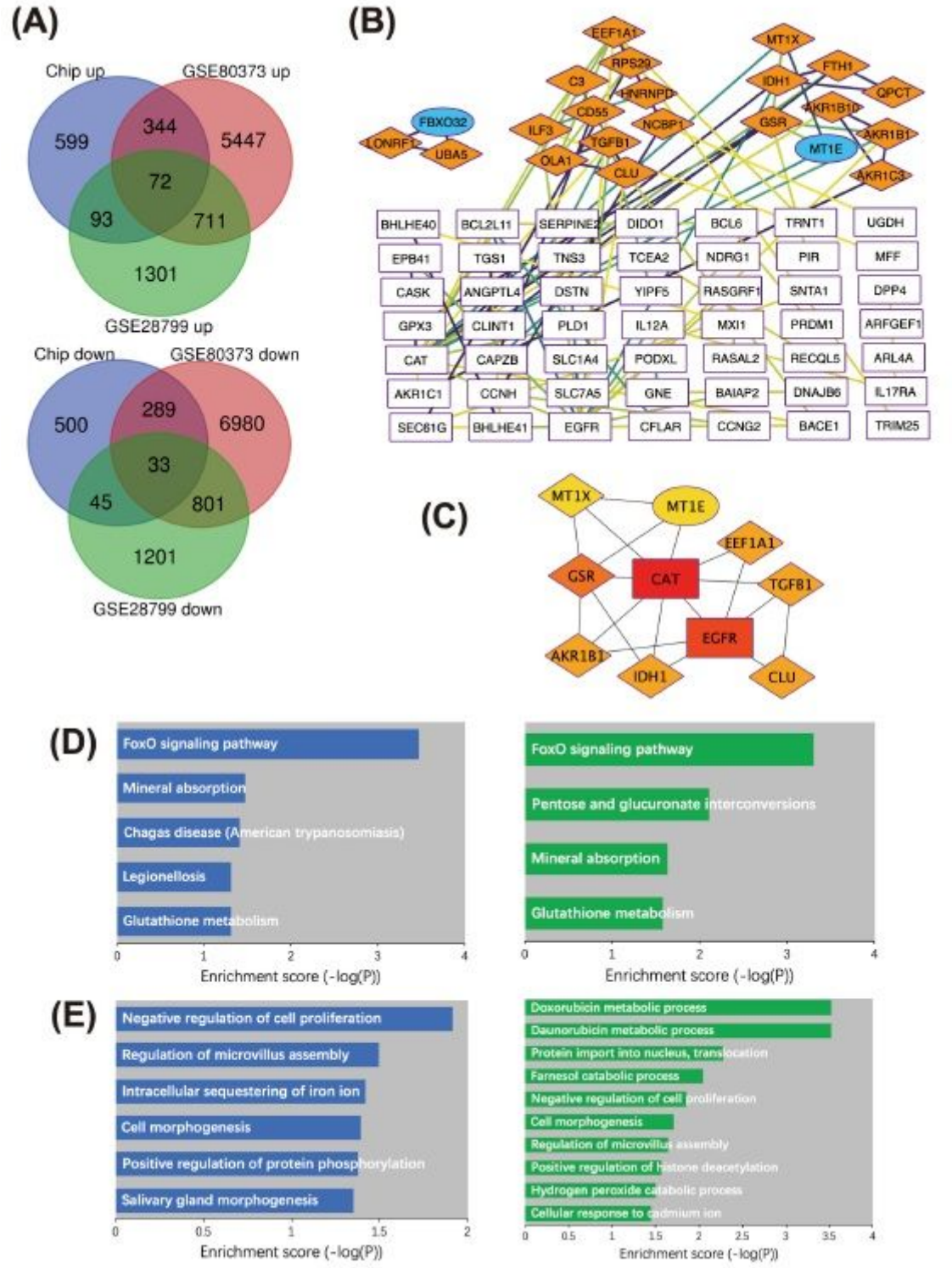

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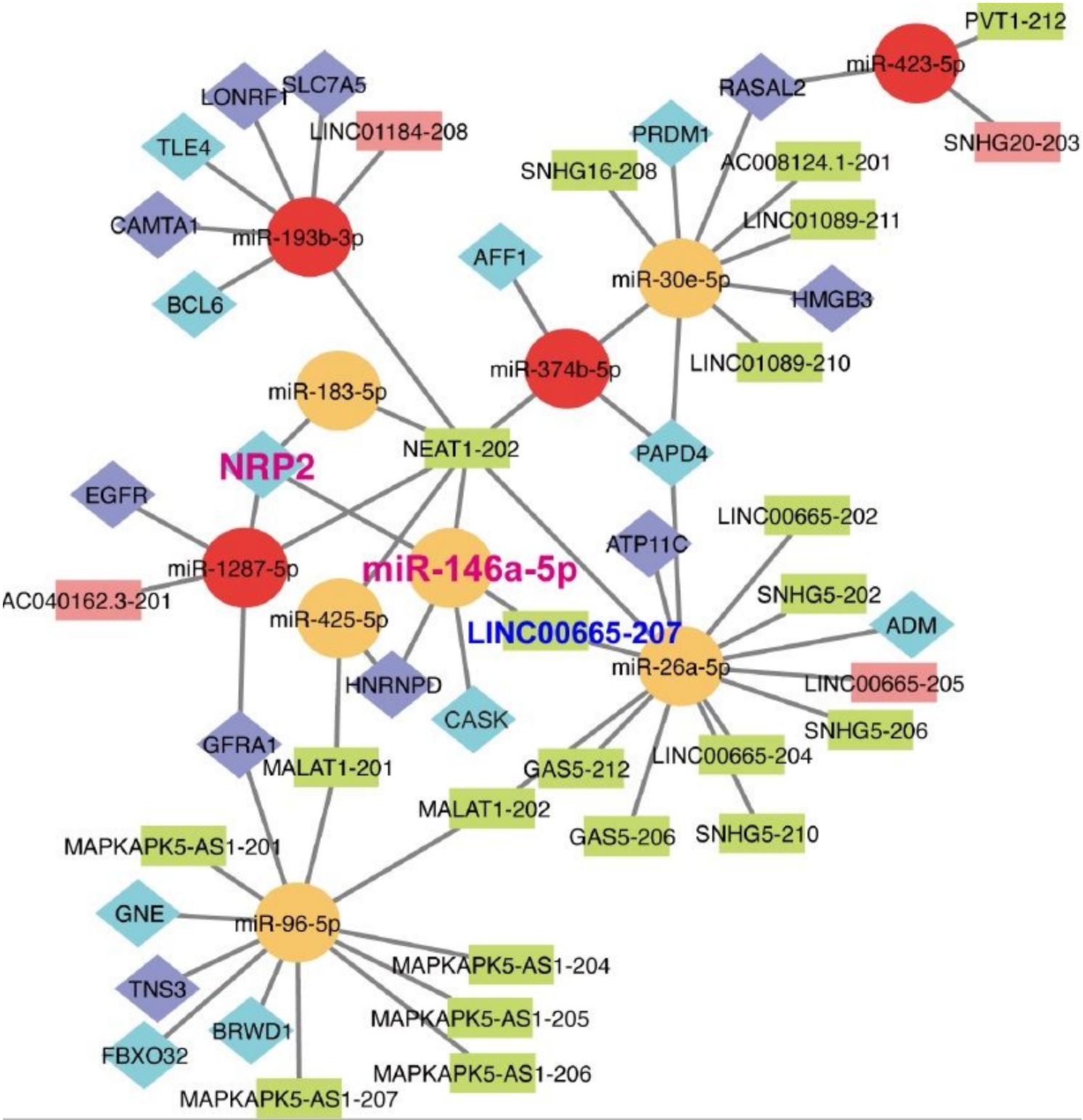
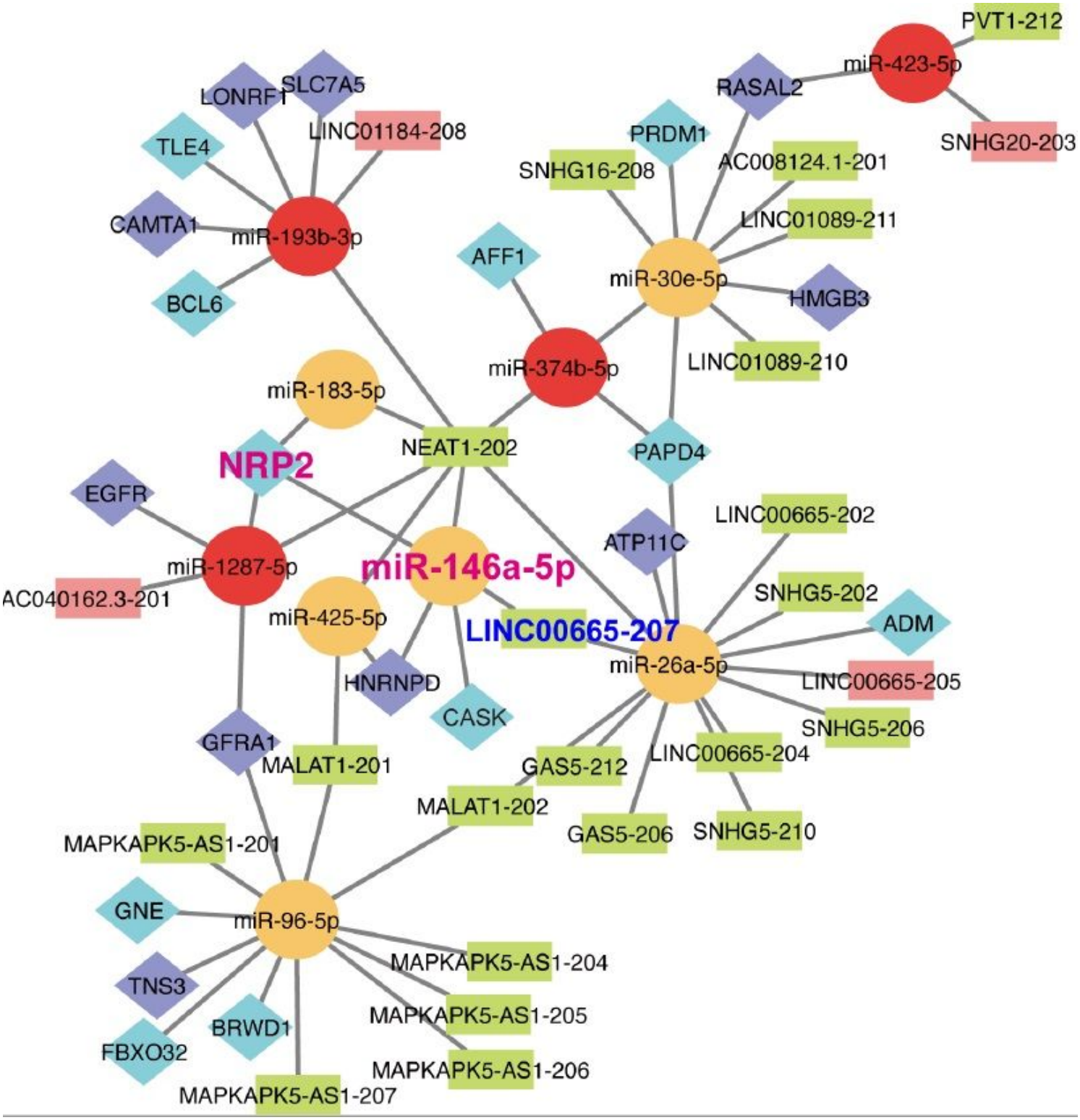


Figure 5

The final ceRNA regulatory network in OCSCs.



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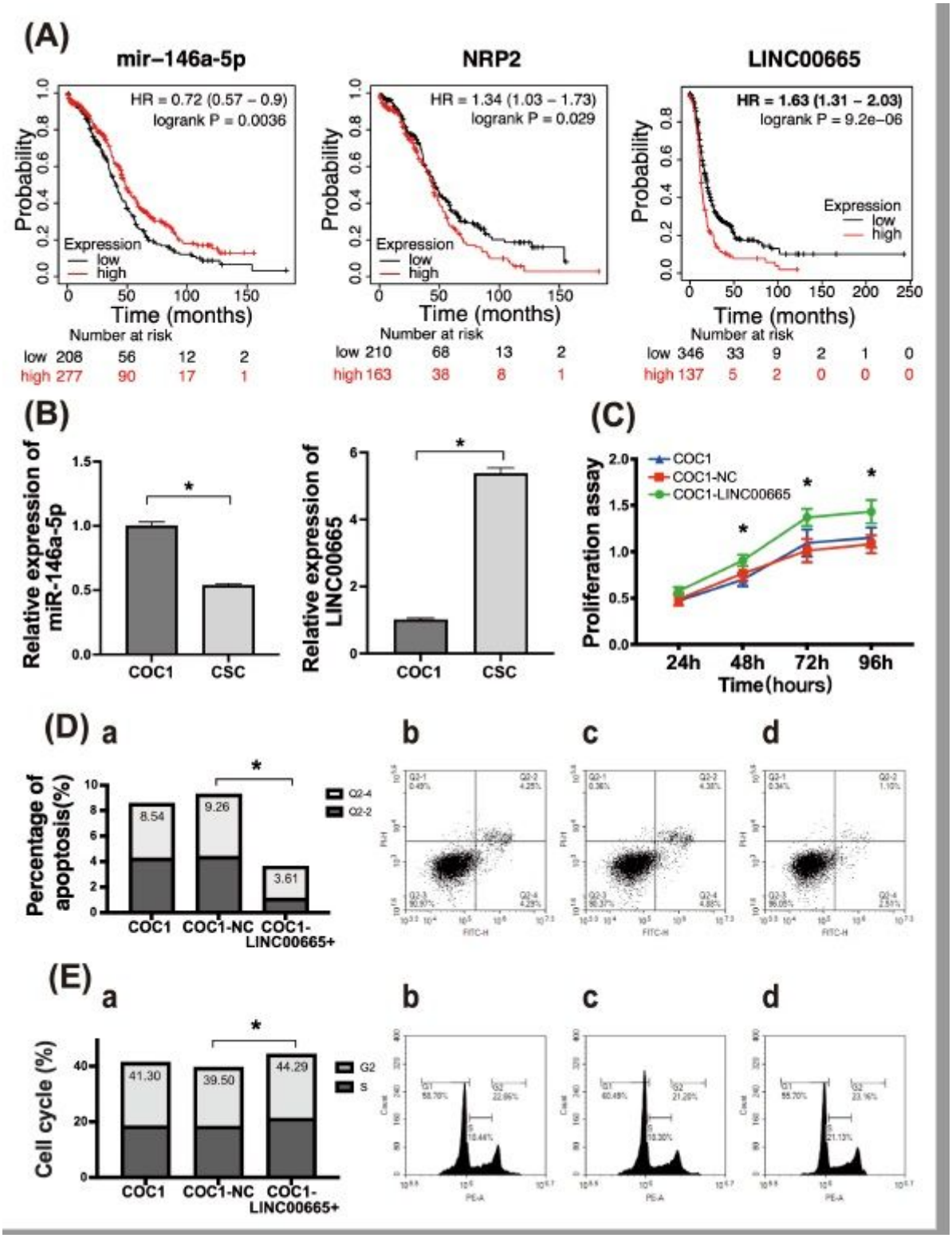

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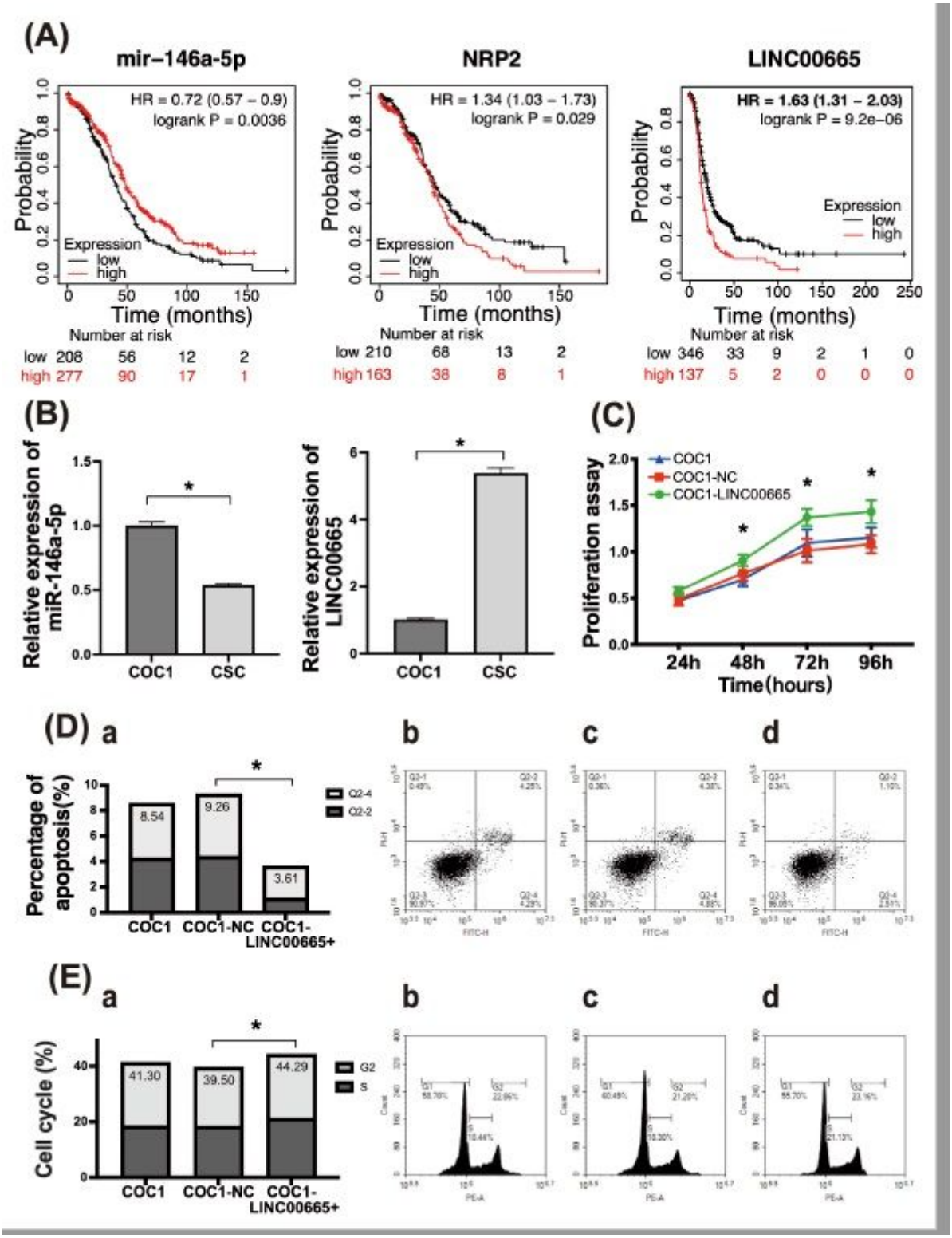

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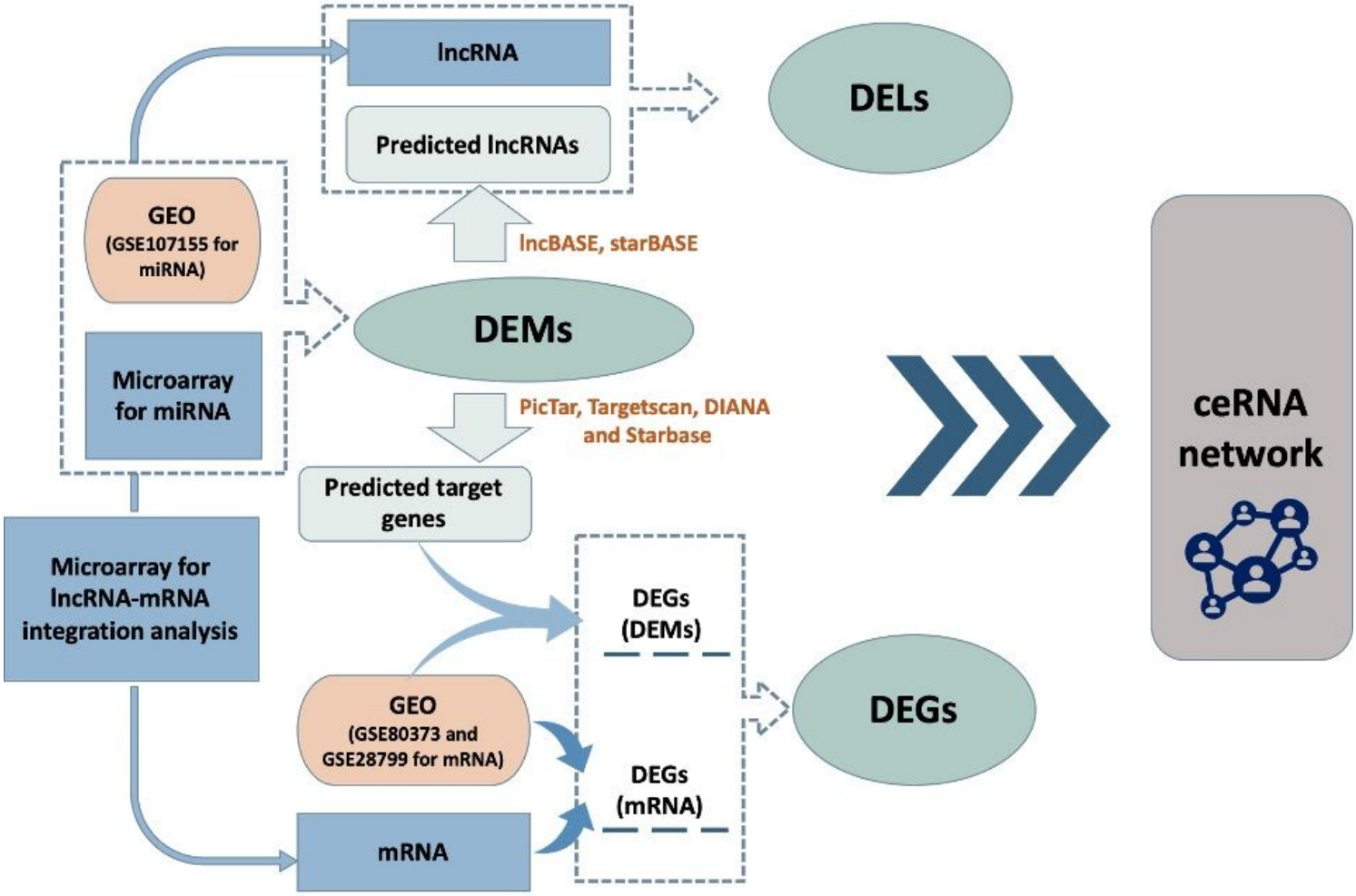
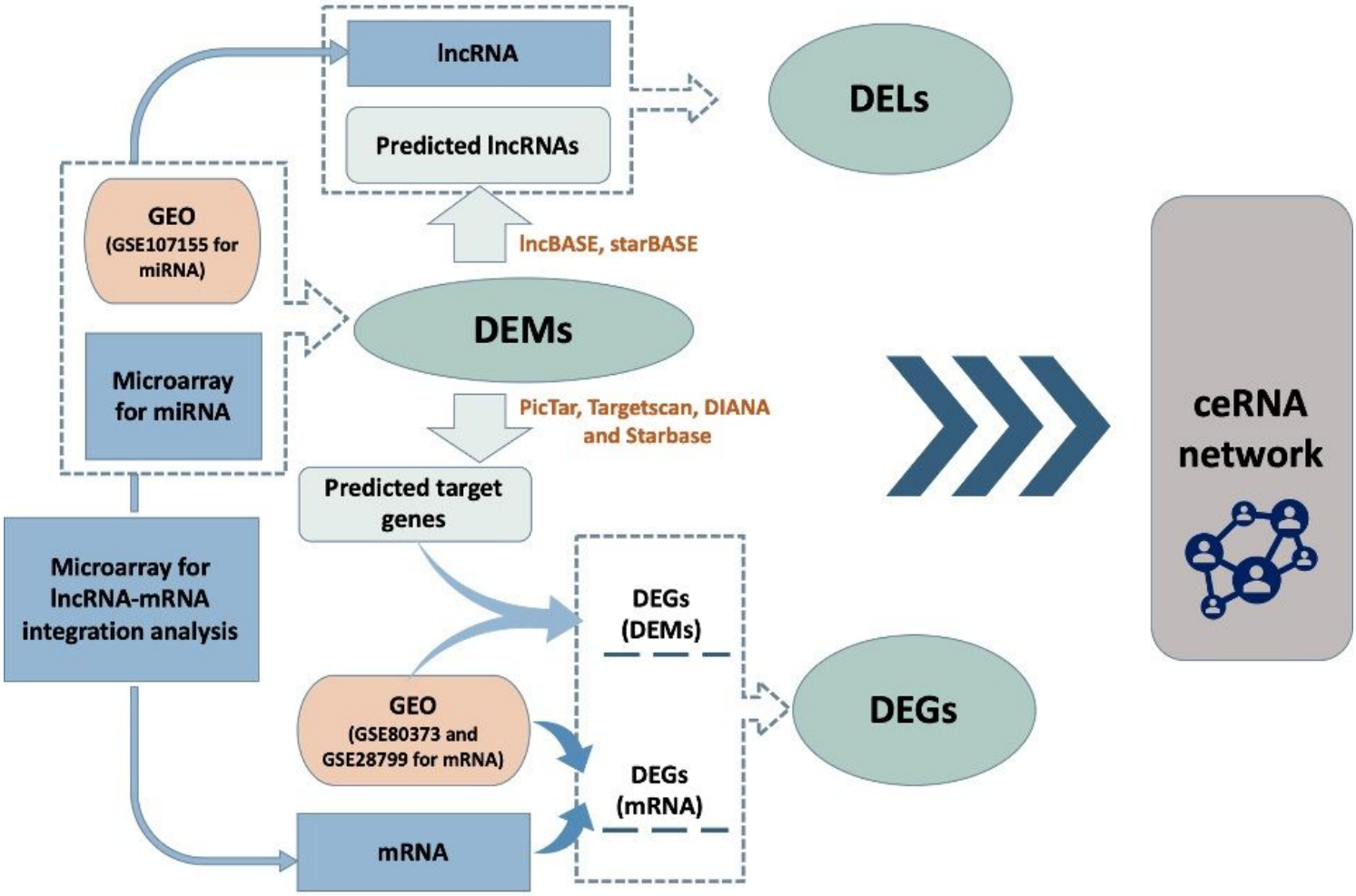


Figure 7

The procedure of the construction of cancer stem cells-associated lncRNA-miRNA-mRNA network for ovarian cancer via microarray and Gene Expression Omnibus database.



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