XLOC-040580, a Novel lncRNA, Modulates Porcine Zygote Activation Progress

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Research

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Abstract

Background: Long noncoding RNAs (lncRNAs) play an important role in porcine preimplantation embryonic development, but the regulatory mechanism of lncRNAs during zygote activation (ZGA) remains unclear.

Methods: We analyzed the transcriptome data of porcine fetal fibroblasts (PEF), porcine induced pluripotent stem cells (piPSCs) and embryos at different developmental stages before implantation, and found that lncRNAs specifically expressed at ZGA stage including *XLOC-040580*, and further predicted its target genes *TPRA1* and *BCL2L1*. *XLOC-040580* was knocked down by microinjection and the expression of ZGA related genes was detected by qRT-PCR. Microinjection of siRNA corresponding to target genes at the 1-cells, the blastocyst development rate was counted, and single cell sequencing was performed to analyze the regulatory network of target genes and its related mechanisms.

Results: By analyzing the transcriptome data of porcine preimplantation embryos, we found that *XLOC-040580* was specifically expressed during zygote activation. Knockdown of *XLOC-040580* resulted in decreased blastocyst development rate, total blastocyst cell number and TE cell number. *XLOC-040580* target genes *TPRA1* and *BCL2L1* are also specifically expressed in ZGA stage. The knockdown of *XLOC-040580* target genes also blocked porcine embryonic development and affected the gene expression of ZGA-related pathways such as cell cycle, histone modification and trophoblast formation during porcine embryonic development.

Conclusion: We found and confirmed that lncRNA *XLOC-040580* played a key role in the ZGA process, and its target genes (*TPRA1*, *BCL2L1*) also coordinated early embryonic development.

Background

During early embryonic development, zygote activation (ZGA), cell polarization, asymmetric division, and the dynamic balance of pluripotency and differentiation-related genes are essential for embryonic development, any developmental defects will have a profound impact on individual embryos[1, 2]. In the process of embryonic development, the first key process is ZGA, in which embryos undergo DNA replication and modification, transcription factor and enhancer activation, protein modification and response to external signals. The normal progress of ZGA is crucial to embryo cleavage. Any level of error will lead to the termination of embryonic development[3, 4]. Previous studies have shown that mouse ZGA occurs in late 1-cell and late 2-cell stages[5]. ZGAs in pigs and humans be similar to, both occurring from 4-cell to 8-cell stage[6].

Pigs share many similarities with humans in anatomical structure, physiological metabolism and pathogenesis of diseases, and are considered as ideal for development and disease models. Therefore, in-depth study of early porcine embryos helps to deepen the understanding of human embryonic development and related diseases. lncRNAs have tissue-specific expression and spatio-temporal-specific expression, there are a large number of lncRNAs in early porcine embryos, and they are specifically

expressed at different stages[7]. Several novel porcine lncRNAs have been identified in previous studies. Its unique expression pattern and structure make it play an important role in fetal development[8]. Similarly, abnormal expression of Xist may be associated with midterm abortion in porcine nuclear transfer embryos[9]. Other lncRNAs were also identified in specific tissues, such as lung[10], endometrium[11] and frontal cortex[12].

Studies have shown that lncRNA-*IL17D* in mouse 1-cells embryos are knocked out during mouse ZGA, leading to embryonic death, which is rescued by *IL17D* protein at the 4-cell stage *in vitro*[13]. *LincGET* related to cleavage marker *MERVL* can form RNA-protein complexes with *hnRNP U*, *FUBP1* and *ILF2* to regulate the transcription of ZGA[14]. Interestingly, *lincGET* can also form complexes with *CARM1* to regulate the expression of *Sox2*, *Nanog* and *Sox21* necessary for early embryonic development in mice[15]. lncRNA-*37* was also found to target *TTLL12* and *NDST1* to mediate ZGA in goat embryos[16]. Therefore, previous studies have revealed that functional lncRNA could affect the ZGA process of animals, according to our assumption, we choose to employ scRNA-seq and microinjection technology to study the important role of regulatory lncRNAs during the ZGA period of pigs.

Materials And Methods

Porcine oocyte collection, Parthenogenetic (PA) activation and embryos culture

Porcine ovaries were collected at the slaughterhouse and transferred to the laboratory within 3 h in sterile physiological saline at 37 ℃ with 100 IU/L penicillin and 100 g/mL streptomycin sulfate. The ovaries were rinsed with normal saline (38 ℃) for three times. The cumulus oocyte complexes (COCs) were extracted from normal follicles with a diameter of 2–8 mm on the surface of the ovaries by a syringe and placed in a 9 cm culture dish to select COCs of more than three layers of intact and dense cumulus cells. The selected oocytes were washed three times in IVM solution and cultured in a 38.5 ℃ incubator for 42–44 h. Mature oocytes were transferred to non-electrolyte solution containing 0.25 mM mannitol, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 0.01 % PVA (w/v), 0.5 mM HEPES (pH 7.0-7.2) and washed three times after removal of cumulus cells by 0.1 % hyaluronidase. Then, the somatic hybridization instrument (Shi-madzu SSH-2, Kyoto, Japan) (pH 7.0-7.2) was used to activate with two DC pulses of 1.56 kV / cm. The activated embryos were cultured in PZM-3 medium[17] at 38.5 ℃.

In vitro mRNA synthesis

Before microinjection, the expression vectors need to be linearized, then extracted with phenol / chloroform and precipitated with ethanol. The linearized DNA was transcribed in vitro according to the MESSAGE T7 mMACHINE® Kit (Ambion). The synthesized product was recovered with lithium chloride and dissolved in nuclease free water.

siRNA injection

siRNA designed and synthesized by Suzhou Jima Pharmaceutical Co, Ltd. Control siRNA without a specific target and siRNA of lncRNA *XLOC-040580* are following: Use Eppendorf for siRNA microinjection. There is 3 ul RNA (50 nM siRNA, 30ng/ul GFP mRNA) in the injection needle.

Control-siRNA	**Forward**	**UUCUCCGAACGUGUCACGUTT**
	Reverse	**ACGUGACACGUUCGGAGAATT**
XLOC-040580-siRNA-1	Forward	GCAACUUUCCUCCUCCUGUTT
	Reverse	ACAGGAGGAGGAAAGUUGCTT
XLOC-040580-siRNA-2	Forward	GGUAAGUCGAAGAAACCCUTT
	Reverse	AGGGUUUCUUAGACUUACCTT
XLOC-040580-siRNA-3	Forward	GAUCAGAUGCCCUCAGGAUTT
	Reverse	AUCCUGAGGGCAUCUGAUCTT
TPRA1-siRNA-1	Forward	GCUGCUGUACGAAGACAUUTT
	Reverse	AAUGUCUUCGUACAGCAGCTT
TPRA1-siRNA-2	Forward	GCCACCACCUUCCUCUACUTT
	Reverse	AGUAGAGGAAGGUGGUGGCTT
TPRA1-siRNA-3	Forward	GCGCUGGAAGGCCAUCAAUTT
	Reverse	AUUGAUGGCCUUCCAGCGCTT
BCL2L1-siRNA-1	Forward	GCCAUCAAUGGCAACCCAUTT
	Reverse	AUGGGUUGCCAUUGAUGGCTT
BCL2L1-siRNA-2	Forward	GGCGGGCGAUGAGUUUGAATT
	Reverse	UUCAAACUCAUCGCCCGCCTT
BCL2L1-siRNA-3	Forward	GCGUGGAGAGCGUAGACAATT
	Reverse	UUGUCUACGCUCUCCACGCTT

Plasmid electro transfection

siRNAs were transformed into shRNAs, and primers for PCR amplification were designed with porcine 4–8 cell embryo cDNA as the template. The fragment were digested with Bgl II and Hind III and inserted into pSuper (Addgene) plasmid by T4 ligase Plasmids were extracted using EndoFree Plasmid Maxi Kit (QIAGEN, 12391). Then 4 μg of shRNAs plasmid were transfected to 5×104 ES cells using Lonza Amaxa Nucleofector-2b program A023. After electric transformation, cells were back into a 37 °C incubator for culture. About 24 h, transfected cells were selected with puromycin (0.3 μg/mL) for 48 h.

Immunocytochemistry

For immunocytochemical analysis, embryos were fixed with 4 % paraformaldehyde (PFA) in DPBS for 20 min at room temperature. Fixed embryos were washed three times with DPBS, incubated in 0.2 % Triton X-100 buffer for 15 min, and washed three times with DPBS. After blocking in 2 % BSA blocking buffer for 1 h, embryos were incubated at 4°C overnight in 1 % BSA buffer containing primary antibodies. The following primary antibodies were used: anti-*OCT4* (Santa Cruz), and anti-*CDX2* (Cell Signaling Technology). The embryos were washed in DPBS and incubated for 1 h with secondary antibody. For nuclear staining, the cells were incubated for 2 min with Hoechst 33342 (10 ng/ml) (Life Technologies). The images were captured using Nikon microscope.

RNA Purification and Quantitative Real-Time PCR

Total cellular RNA of 100 embryos was extracted using RNeasy Mini Kit (QIAGEN). Reverse transcription was performed using an oligo-dT primer and M-MLV Reverse Transcriptase (Promega). Quantitative RT-PCR analyses were performed using the LightCycler 480 SYBR Green I Master Kit (Roche) and detected with LightCycler 480II (Roche). The data were analyzed using the comparative CT (2-ΔΔCT) method. The ΔCT was calculated using Actin as an internal control. All experiments were performed with more than three biological replicates. The sequences of the PCR primers are following.

Single-cell RNA-seq and reads mapping.

The two-cell embryos were selected by mouth-suction tube under the stereomicroscope, digested with 0.5 % streptomycin, and washed three times in 1 % PBS, then stored in cold scRNA-seq cell lysate. we used a modified STRT-seq protocol to construct the libraries[18]. Single cell cDNA was obtained by terminal repair, reverse transcription and PCR amplification. The unit bar code obtained by Single-cell RNA-seq matches the end sequence, and reads 1 should be attached when reading the UMI sequence in reads 2. The transcript abundance of each gene was estimated using HTSeq (v0.6.1), and the reads with duplicate UMIs of each gene were excluded[19]. Finally, the expression levels of genes were normalized to the number of TPMs (transcripts per million reads).

Alignment of RNA-seq data and differential expression analysis

Raw reads from scRNA-seq were split by 8 bp cell barcodes located on Read 2 allowing 2 mismatches. Additionally, the 8 bp unique molecular identifiers (UMIs) located on Read 2 were switched to the identifier line of paired Read 1, then Read 1 was processed to remove the template switch oligo (TSO) primer, low quality bases and polyA sequence. The trimmed reads were aligned against the Ensembl Sus scrofa reference genome (Sscrofa11.1) using STAR software with default parameters. the gene expression levels were estimated by counting the UMIs assigned to the genes using using featureCounts, yielding an expression matrix consisting of UMI counts for each cell and gene.Differential expression genes were identified by DESeq2 package, and functional enrichment for Gene Ontology (GO) and KEGG were performing with GOstats package[20]. lncRNA targets were identified by co-expression modules in

previous study, then we use LncTar[21] to verify the potential of lncRNA-mRNA interaction, which included promoter, CDS and 5'-UTR region of each gene, and protential targets were filtered with the minimum normalized binding free energy(ndG). lncRNA regulatory network was visualized by Cytoscape.

Statistical analysis.

Each experiment was repeated three times, and the results are presented as the mean standard error. Data were analyzed by one-way analysis of variance (ANOVA) by LSD tests and $\chi 2$ using SPSS software. $P < 0.05$ was considered to be statistically significant.

Results

lncRNA XLOC-040580 is specifically expressed during porcine ZGA

We analyzed the transcriptome data of porcine oocytes, 1-, 2-, 4-, 8-cell embryos, morula, blastocyst intracellular cell mass (ICM), trophoblast cells, piPSCs, and PEF cells[7], and organized the results into 24 modules according to the expression levels of lncRNAs in embryos and cells. Nine of these modules are associated with early embryonic development in pigs: modules 1, 3, 5, 6, 8, 11, 17, 22, 24 (Fig. 1A). ZGA occurs in the 4–8 cell stage of porcine embryos, in this stage, lncRNAs related to embryos are mainly concentrated in module 17, and then the expression profile is drawn according to the differential expression of lncRNAs in module 17, lncRNA *XLOC-040580* is the most significant gene in the 4–8 cell stage (Fig. 1B). By analyzing RNA-seq data, *XLOC-040580* had the highest expression in 8-cell stage (Fig. 1C). In addition, qRT-PCR was used to verify the expression levels of early embryos, piPSCs and PEF at different stages (Fig. 1D). We found that the expression pattern of *XLOC-040580* was consistent in porcine embryos and PA embryos, and the expression level was the highest in the ZGA period.

XLOC-040580 knockdown influences ZGA-related gene expressions

We first designed siRNA and investigated the knockdown efficiency of *XLOC-040580* on piPSC, the results showed that the two targets of si-*XLOC-040580* significantly decreased their expression (Fig. 2A). Then, porcine 1-cell embryos were injected with lncRNA- siRNAs at a concentration of 25 μM to study the effect of *XLOC-040580* on embryonic ZGA. The control group was the control-siRNA (si-control group) that had no knockdown effect on any gene. In order to verify the successful injection of siRNAs, we mixed GFP mRNA into siRNA solution, and only GFP-positive embryos were selected for subsequent experiments (Fig. 2B). qRT-PCR was used to detect the efficiency of siRNAs at the 4–8 cell stage, and the results showed that the knockdown efficiency of *XLOC-040580* was about 70 % (Fig. 2C). To test the effect of *XLOC-040580* on embryonic development, we observed and counted the embryonic development. We found that there was no significant difference in 4–8 cell embryo formation rate between the two groups

(n = 82.2 ± 2.22%, P = 0.0636), while the blastocyst formation rate of the si-*XLOC-040580* group was significantly lower than that of the si-control group (n = 6.8 ± 1.39%, P = 0.0008) (Table. 1, Fig. 2D).

Knockdown XLOC-040580 reduces the formation rate of blastocyst and the number of TE cells

We continue to explore the influence of *XLOC-040580* in ZGA. Interestingly, we found that the number of trophectodermn (Cdx2 positive) cells in E6.5 blastocysts as well as the total number of blastocysts in the si-*XLOC-040580* group was significantly reduced (P < 0.0001 and P = 0.0245 respectively) (Fig. 3A and 3B). The results showed that *XLOC-040580* might affect normal embryonic development by interfering with TE cells. At the same time, we found that the expression levels of ZGA-related genes such as *eIF1a*, *SMG*, *ACADL* and *HSP70.2* were down-regulated after knocking out *XLOC-040580* (Fig. 3C), which further proved that knocking down *XLOC-040580* might block embryonic development by affecting ZGA-related genes, which supported the correlation between *XLOC-040580* and ZGA.

The knock-down of XLOC-040580 target genes caused the decrease of early embryonic development rate

In order to clarify the regulatory mechanism of *XLOC-040580* on porcine ZGA stage, lncRNA targets were identified by co-expression modules, then we use lncTar[21] to verify the potential of lncRNA-mRNA interaction, which included promoter, CDS and 5'-UTR region of each gene, and protential targets were filtered with the minimum normalized binding free energy (ndG). ZGA occurs in 4–8 cell stage, and the expression changes of candidate genes should be consistent with ZGA, including *TPRA1*, *BCL2L1*, *MED27*, *BABAM1*, *VPS72* and *UBE2A*. It was previously reported that *TPRA1* was closely related to cell growth and embryonic differentiation pathways, and *BCL2L1* was involved in apoptosis. Therefore, we screened *XLOC-040580* target genes: *TPRA1* and *BCL2L1* (Fig. 4A). Similarly, TPRA1 and BCL2L1 siRNAs were designed to verify the knockdown efficiency on pESC, and the optimal siRNA was selected for subsequent experiments (Fig. 4B). We continued to knockdown the target genes at the 1-cell stage of porcine embryos, and counted the development of embryos. The experimental results showed that the 4–8 cell embryonic development rate and blastocyst formation rate of si-*TPRA1* group and si-*BCL2L1* group were significantly lower than si-control group (Table. 2, Fig. 3C). These data showed that the wrong expression of *XLOC-040580* target genes (*TPRA1*, *BCL2L1*) inhibited early embryonic development.

Knockdown of TPRA1 and BCL2L1 disrupts early embryonic development regulatory networks by single cell transcriptome analysis

In order to explore the potential causation of embryonic development failure after knocking down the target genes, we used RNA-seq to analysis differentially expressional profile in porcine embryos before

and after knocking down the target genes (Fig. 5A). The results showed that 100 related genes were down-regulated and 233 related genes were up-regulated after knocking down *TPRA1*. After knocking down *BCL2L1*, 153 genes were down-regulated and 179 genes were up-regulated (log2 [fold-change ≥ 1]; adjusted p-value ≤ 0.05) (Fig. 5B and 5C). We found that the related gene *AGO2* of *TPRA1* was involved in oocyte development, and the related genes SNRNP and *PRDX5* of *BCL2L1* were involved in oocyte meiosis and maturation by GO enrichment analysis (log2 [fold-change ≥ 1]) (Fig. 5D and 5E). In order to understand the key pathways that *TPRA1* and *BCL2L1* can affect the development of porcine embryos, we performed KEGG enrichment analysis, and both target genes were involved in MAPK signaling pathway. In addition, *TPRA1* is also involved in progesterone-mediated oocyte maturation, oocyte meiosis and other signaling pathways related to early embryonic development (adjusted p-value < 0.05) (Fig. 5F and 5G). Finally, we generated the *XLOC-040580* gene network with potential targets based on co-expression data and protein interaction database (Fig. 5H).

Discussion

ZGA is essential for early embryonic development with a series of physiological and biochemical reactions, including transcription factor activation, DNA methylation, protein modification, RNA binding, metabolic changes, and maternal factor degradation[22, 23]. However, there are few reports on lncRNA in pig early embryos. In this study, we used transcriptome data at different stages of early porcine embryos to identify a novel lncRNA specifically expressed at ZGA stage: *XLOC-040580*. We found that the expression of ZGA related genes changed after knocking down *XLOC-040580*, which proved that it was indispensable in this process. After knocking down *XLOC-040580* in pig embryos, it was found that the blastocyst formation rate was significantly reduced, the number of TE cells were significantly reduced, and the total number of blastocysts were also significantly reduced. In addition, we found that two target genes of *XLOC-040580* were also involved in the regulatory network of early embryonic development in pigs. The above results support that *XLOC-040580* plays a key role in the ZGA process, and provide a reference for further study of pig ZGA-related lncRNAs.

In this study, we selected PA embryos similar to fertilization activation mechanism. PA embryos can develop to blastocyst stage, and because the offspring of PA embryos are not affected by sperm, it is more conducive to the study of single controllable variable. At the early stage of embryonic development, the transcription of embryonic genome is completely regulated by maternal mRNA and protein during embryonic development, but maternal mRNA is gradually degraded during ZGA[24, 25]. Previous studies have shown that there is no significant difference in the expression between IVF and PA during early embryonic development[26]. Similarly, eIF1A associated with differential expression can be used as a marker gene for IVF and PA in ZGA, *KDM7A*[26], *PDHA1*[27], *KDM5B*, *KDM5C*[28] and other ZGA-related transcription factors have also been shown that share the same expression pattern in IVF and PA embryos. In this study, the expression trend of *XLOC-040580* was consistent with the qRT-PCR results of RNA-Seq and PA embryos. So PA embryos can be used for subsequent experiments.

In previous reports, 252 testis-related lncRNAs and 159 placenta-related lncRNAs were analyzed by sequencing of porcine placenta and testis tissues[29]. It has also been reported that lncRNAs *TCONS_01729386* and *TCONS_01325501* may play an important role before embryo implantation[11], providing ideas for the study of lncRNAs in early porcine embryos. We further explored early embryos and found a novel lncRNA specifically expressed in 4–8 cell stages of early embryonic development, *XLOC-040580*. When we knocked down *XLOC-040580* by siRNA, the blastocyst formation r ate decreased significantly. More importantly, the expression levels of ZGA-related genes were affected, including *eIF1a*[30], *Smg*[31], *ACADL*[32] and *Hsp70*[33]. These genes were considered to be the marker genes in the ZGA period, which proved that *XLOC-040580* played a more extensive role in the ZGA period. More interestingly, knockdown of *XLOC-040580* significantly reduced the number of embryonic TE cells, suggesting that *XLOC-040580* may play a role in late ZGA and affect the normal development of embryonic TE cells. This is consistent with recent studies, transcription factors generally enriched in cis-regulatory elements of ICM and TE, and the expression of transcription factors in ZGA is the highest consistent, indicating that ICM and TE programs first started in ZGA[34].

In order to better test the regulatory network of lncRNA, we focused on two *XLOC-040580* target genes: *TPRA1* and *BCL2L1*. We found that the expression abundance of *TPRA1* and *BCL2L1* increased from 4-cell stage to 8-cell stage, and peaked at 8-cell stage, which were consistent with ZGA expression pattern. *TPRA1* is generally believed to be closely related to cell growth and embryonic differentiation. It has been reported that it mediates intracellular cAMP level, mainly affects cell growth and differentiation by regulating Hedgehog (Hh) signaling pathway, and has a positive regulatory effect on embryonic formation and tissue homeostasis[35]. BCL2 protein family is a key regulator of apoptosis and participates in a variety of signaling pathways related to cell development[36]. We knocked down these two target genes, resulting in early embryonic development blocked. Interestingly, we used single-cell transcriptome sequencing technology to detect the effect of knockdown target genes on the whole gene of porcine embryos, and found that *TPRA1* was related to oocyte gene inhibitor *AGO2*[37], male reproductive factor *TDRP*[38] and *WDR19*[39]. *BCL2L1* is related to the 2-cell cleavage and pretreatment mRNA factor *SNRNP*[40], the *PRDXs* that affect mitochondrial activity throughout embryonic developmen[41], and the apoptosis and senescence-related gene *GADD45B*[42]. We further drew the regulatory network diagrams of *XLOC-040580*, *TPRA1* and *BCL2L1*. *TPRA1* is associated with many genes in the G protein-coupled receptor (GPR) family, and *GPR107* is the key gene for embryonic lethality[43]. However, it was found that *AGO2* and *TDRP* were not directly related to *TPRA1*. We speculated that the knockdown of *TPRA1* might affect the expression level of *XLOC-040580*, thereby changing the expression of *TDRP* and *AGO2*. More interestingly, we found that *BCL2L1* could be associated with mediator complex subunit (MED) family, and MED family was also associated with ZGA marker genes *eIF1a* and *HSP70.2*. *MED31* can promote embryonic growth and cell proliferation[44], and excessive expression of *MED28* can also disturb cell cycle and lead to genomic instability[45]. We continued to carry out KEGG enrichment analysis, which found that *TPRA1* and *BCL2L1* jointly participate in MAPK signaling pathway. In addition, *TPRA1* is involved in signaling pathways such as oocyte maturation, gonadotropin-releasing hormone signal and oocyte meiosis, and MAPK factor is also

involved in these pathways. It is well known that MAPK signaling pathway plays a key role in spindle connection, microtubule organization and asymmetric division during oocyte maturation. It is well known that MAPK signaling pathway plays a key role in spindle connection, microtubule organization and asymmetric division during oocyte maturation[46], and is closely related to many intracellular events such as cell proliferation, cell differentiation and apoptosis. In addition, *MAPK14* induces BCL2 phosphorylation in apoptotic germ cells[47]. The above results showed that the target genes *TPRA1* and *BCL2L1* of *XLOC-040580* played an important role in the regulatory network of oocyte maturation and preimplantation embryo development.

Conclusions

In summary, in this study, a novel lncRNA *XLOC-040580* and its target genes *TPRA1* and *BCL2L1* were specifically expressed at ZGA stage in pigs by analyzing the transcriptome data of early embryos. It was proved that *XLOC-040580* affected blastocyst formation rate and TE cell generation in porcine embryos, and its target genes were also involved in the regulation network of early embryonic development in pigs. Therefore, this novel lncRNA *XLOC-040580* plays a key role in the ZGA process of early porcine embryos.

Abbreviations

PZM-3: PZM-3medium; PBS: Phosphate buffered saline; DPBS: Dulbecco's phosphate-buffered saline; PVA: Ployvinyl alcohol; PFA: Paraformaldehyde; FPKM: Fragments per kilobase of exon model per million mapped fragments; TPM: Transcripts per million reads; GFP: Green fluorescence protein; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; qRT-PCR: Quantitative real-time PCR; RPKM: Reads per kilobase of exon model per million mapped reads; UMI: unique molecular identifiers; SPSS: Statistical Product and Service Solutiona; ICM: Inner cell mass; TE: Trophectoderm; TPM: Transcripts per kilobase of exon model per million mapped reads; piPSCs: Porcine induced pluripotent stem cells; ESC: Embryonic stem cell; lncRNA: Long non-coding RNA; ZGA: Zygotic genome activation; siRNA: Small interfering RNA; shRNA: Short hairpin RNA; PA: Parthenogenetic.

Declarations

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Author contributions

M.L., H.E. performed main experiments. Z.Z., Z.L. established an in vitro culture platform for porcine embryos. L.Z., D.G. provides transcriptome data.H.M. designed siRNA and shRNA. J.G. was electrotransfected into cells. M.L. wrote the manuscript. S.C., L.Z., Z.H. conceived the study. All authors read and approved the final manuscript.

Founding

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Availability of data and materials

All data generated or analyzed in this study are included in this published article.

Ethics approval and consent to participate

All experiments involving animal embryos were approved and conducted according to the guidelines of the Institutional Animal Care and Use Committee of China Agricultural University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Table 1. Development of porcine embryos after injection with *XLOC-040580* siRNA in the 1-cell stage embryos

Type of siRNA	NO. of 2-cell	NO. of 4-8cell	NO. of Blastocyst
si-control	72	65 (89.8±2.75%) [a]	14 (16.6±1.25%) [a]
si-*XLOC-040580*	72	60 (82.2±2.22%) [a]	2 (6.8±1.39%) [b]

Values in the same column with different letters (a, b) differ significantly (P< 0.05)

Table 2. Development of porcine embryos after injection of *TPRA1* siRNA and *BCL2L1* siRNA into 1-cell embryos

Type of siRNA	NO. of 2-cell	NO. of 4-8cell	NO. of Blastocyst
si-control	40	33 (81.7%±1.44) [a]	7 (18.3%±3.82) [a]
si-*TPRA1*	40	23 (56.7%±5.77) [b]	3 (8%±0.87) [b]
si-*BCL2L1*	40	23 (56.7%±3.82) [b]	3 (6.7%±2.89) [b]

Values in the same column with different letters (a, b) differ significantly (P< 0.05)

Figures

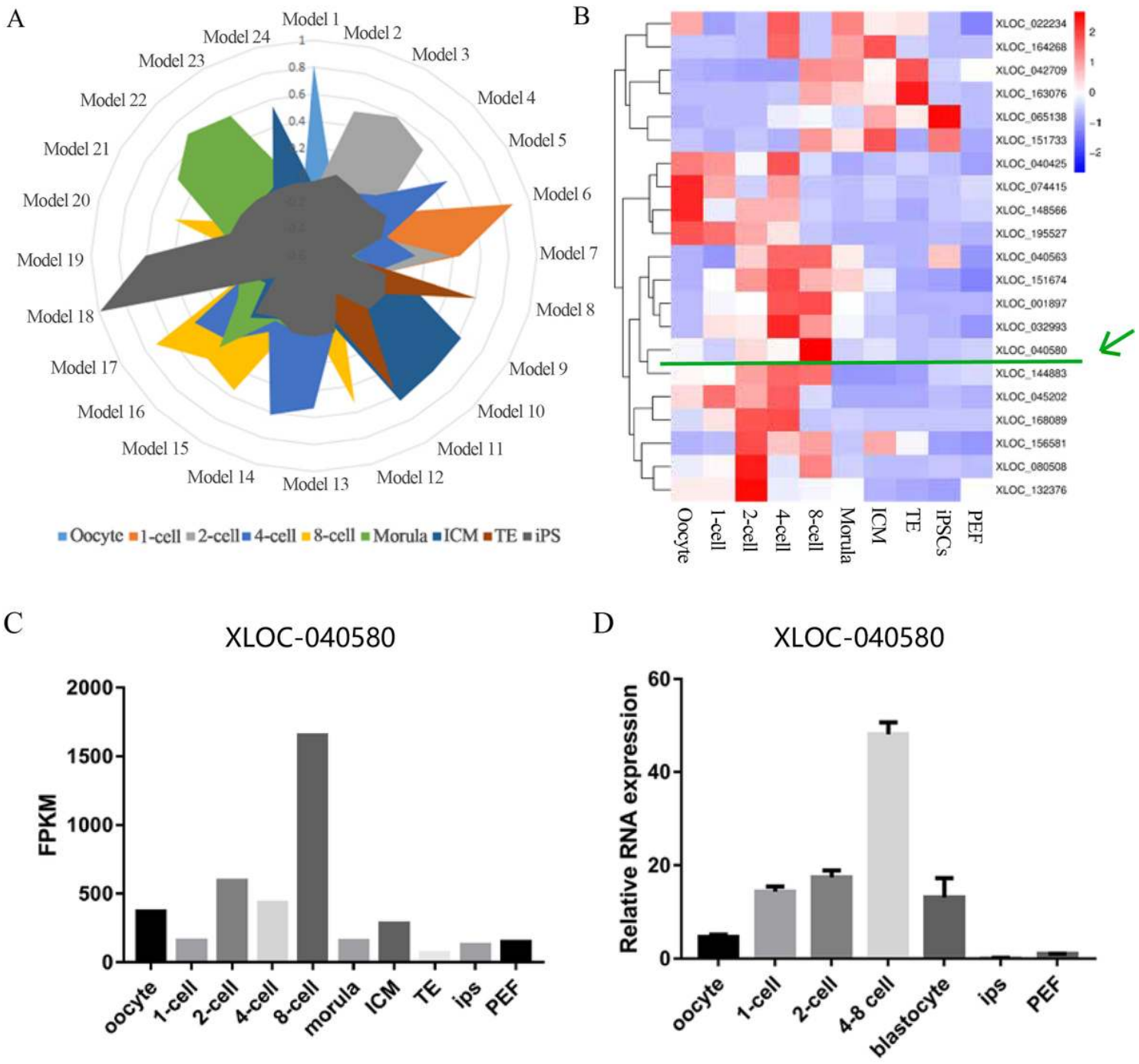

Figure 1

Identification of the specifically expressed lnRNAs in porcine ZGA (A) Function module diagram of lncRNAs. Different colors represent functional blocks in different periods, the closer to the periphery from the center, the higher lncRNAs expression. (B) Heat map of lncRNAs differential expression in module 17. Green arrow as candidate lncRNA. (C) Expression of XLOC-040580 in embryos or cell types at different stages. (D) qRT-PCR analysis of XLOC-040580 differential expression in embryos or cell types at different stages.

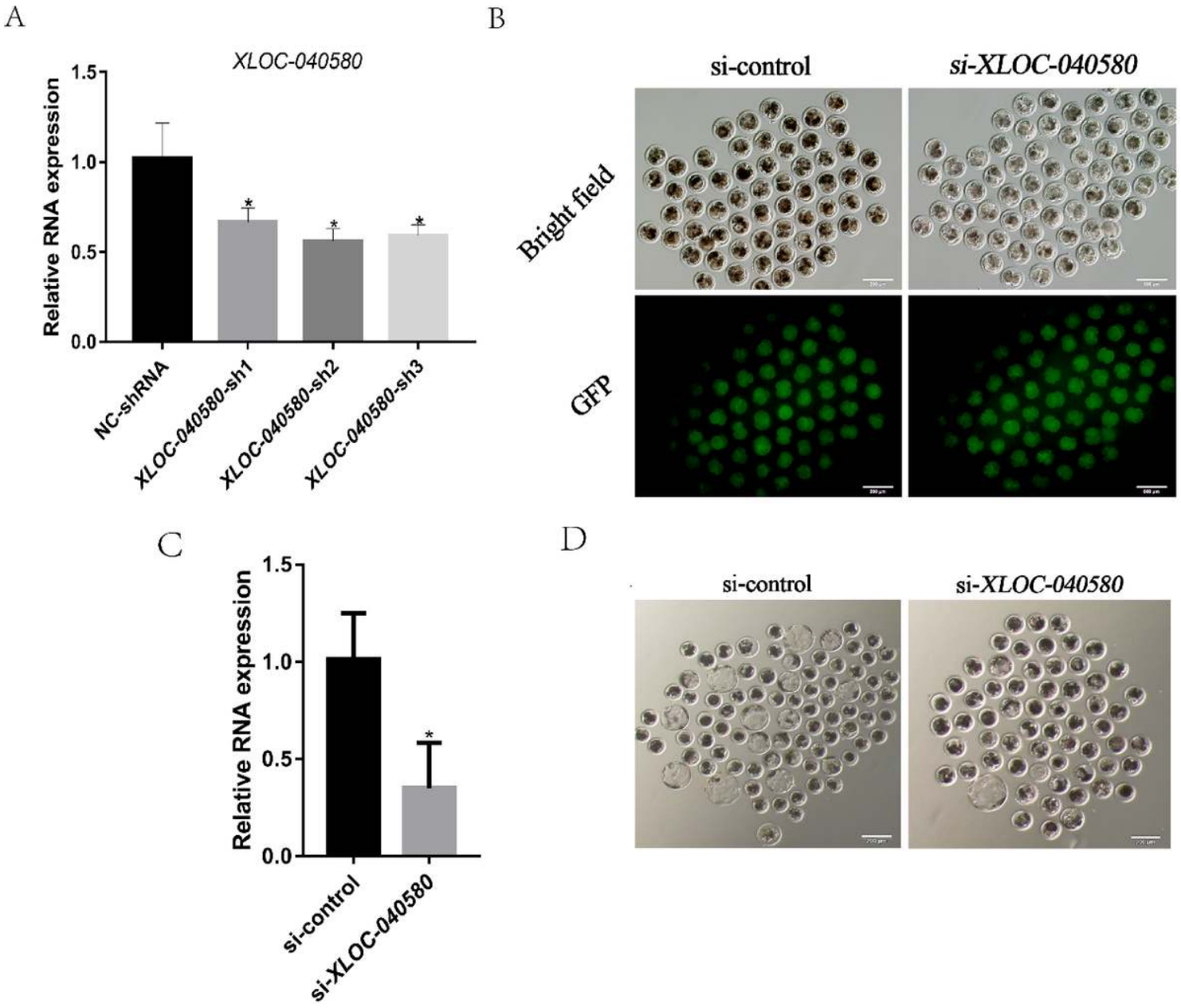

Figure 2

Knockdown of XLOC0 - 40580 resulted in reduced blastocyst development level (A) qRT-PCR analysis of the knockdown efficiency of XLOC-040580 siRNAs. *P < 0.5. (B) 1-cell stage embryos were injected with lncRNA-siRNAs and GFP mRNA (as a positive control of microinjection). Scale bar = 200 μm. (C) qRT-PCR analysis of the knockdown XLOC-040580, the efficiency of siRNAs was tested at the stage of 4-8 cells. *P < 0.5. (D) Representative photos of si-control and si-XLOC-040580 embryos from blastocyst. Scale bar = 200 μm.

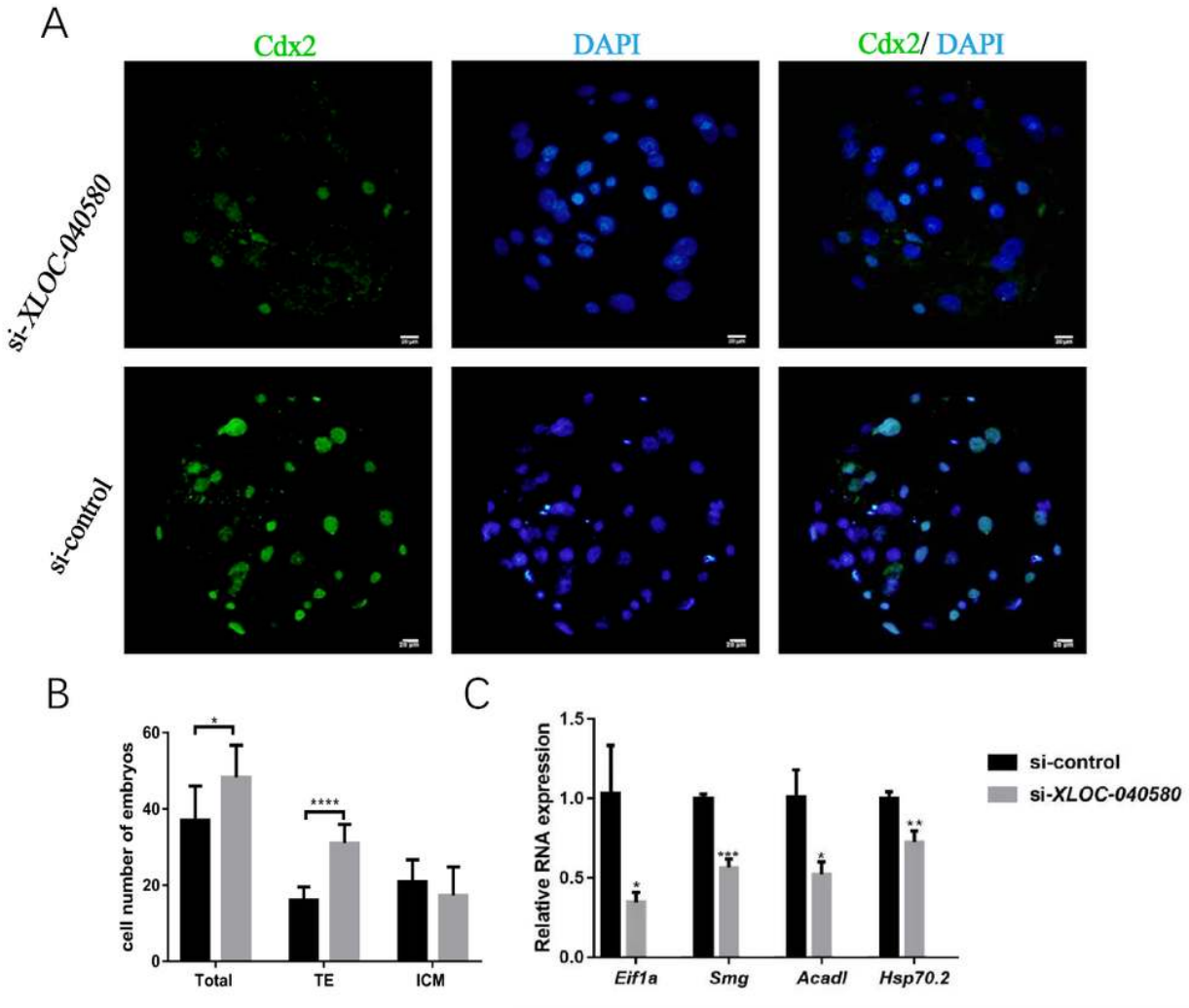

Figure 3

Knockdown of XLOC-040580 affects ZGA (A) Immunofluorescence histochemical staining of CDX2 and DAPI in E6.5 blastocysts. TE, trophectoderm; ICM, inner cell mass. Scale bar, 20 μm. (B) Knockdown of XLOC-040580, blastocyst, TE and ICM formation rate at 6.5d blastocyst cell count. *P < 0.5, ****P < 0.001. (C) qRT-PCR analysis of the knockdown XLOC-040580, expression levels of genes associated with ZGA. *P < 0.5, **P < 0.01, ***P < 0.001, ****P < 0.0001.

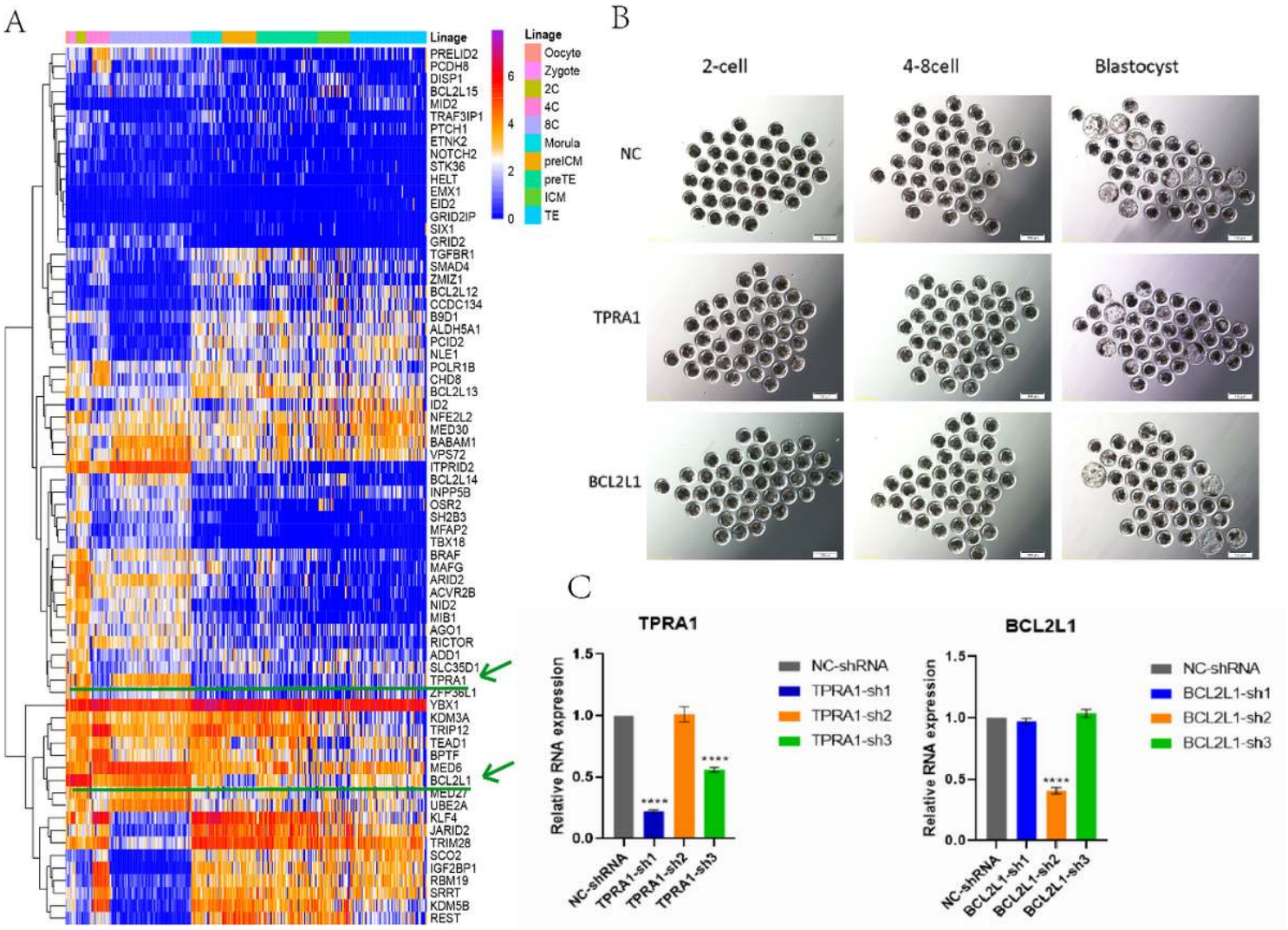

Figure 4

Predicting the target genes of XLOC-040580 during ZGA and verifying their effects on embryonic development. (A) The differential expression of target genes of XLOC-040580 in porcine early embryos was shown by thermal map. ****P < 0.001. Green arrows are candidate target genes. (B) B. qRT-PCR analysis of the knockdown efficiency of TPRA1 siRNAs and BCL2L1 siRNAs. (C) C. Representative photographs of si-control, si-TPRA1 and si-BCL2L1 blastocysts. Scale bar = 200 μm.

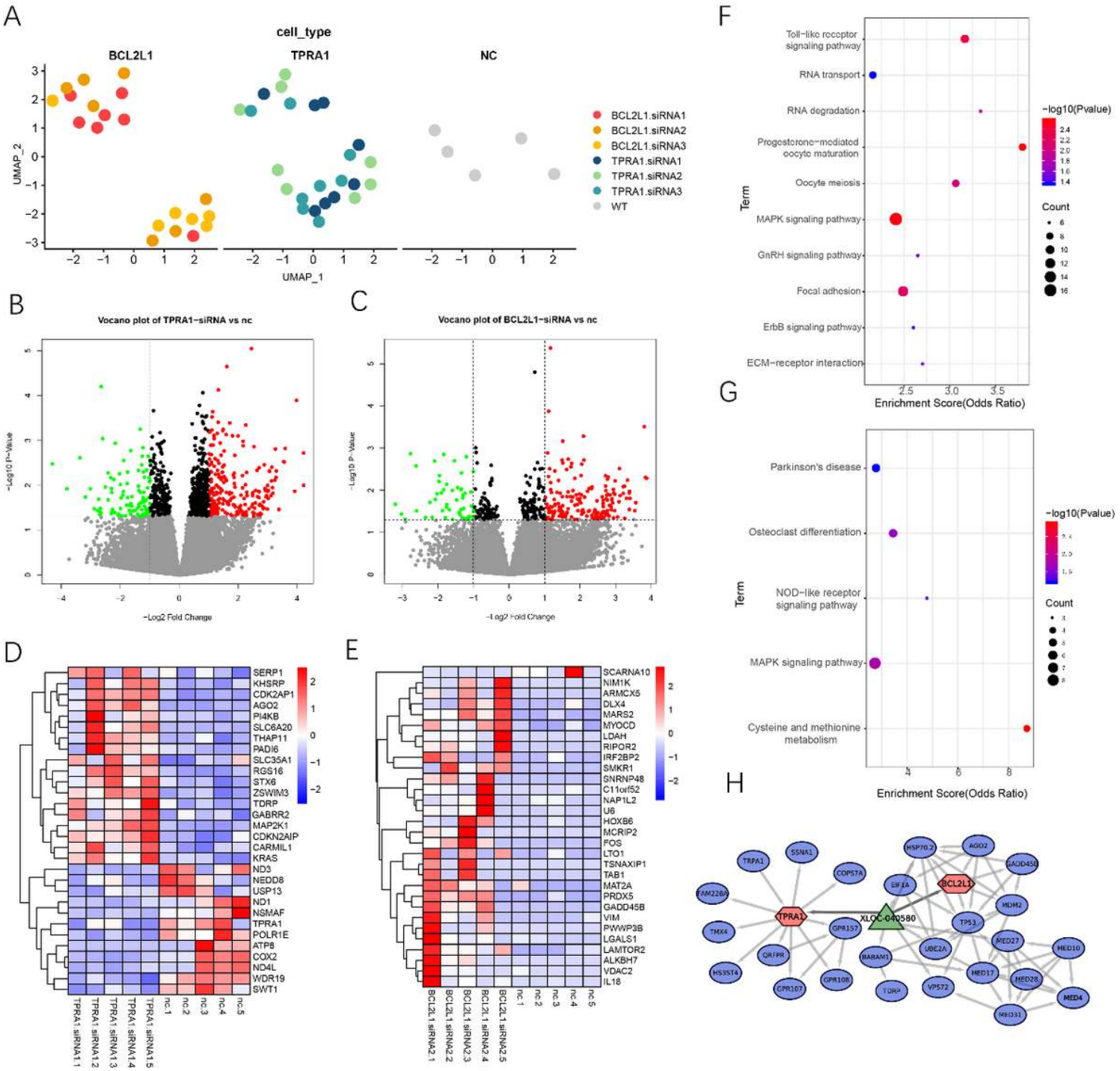

Figure 5

Knockdown of TPRA1 and BCL2L1 disrupts early embryonic development regulatory networks (A) Clustering of 49 single cells that passed quality control metrics using Seurat and displayed using uniform manifold approximation and projection (UMAP) plot. (B) Volcano map showed the differential expression of related genes in porcine embryos after TPRA1 knockdown. (C) Volcano map showed the differential expression of related genes in porcine embryos after BCL2L1 knockdown. Genes represented by gray dots did not meet the criteria for differential expression. (log2 [fold-change ≥1]; adjusted p-value ≤0.05) (D) Heat maps showing differential gene expression in TPRA1 knockdown embryos. (E) Heat maps showing

differential gene expression in BCL2L1 knockdown embryos. (log2 [fold-change ≥1]) (F) KEGG pathway is enriched in porcine embryos knocking down TPRA1. (G) KEGG pathway is enriched in porcine embryos knocking down BCL2L1. (adjusted p-value <0.05) (H) Communication network formed by XLOC-040580 and its target genes.