

cDNA cloning of porcine CDH1 and its expression profile in porcine early parthenotes*

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The study aimed at cloning the complete cDNA sequence of porcine CDH1 gene and detecting its expression in different developmental stages of early parthenotes. After cloning the CDH1 gene from porcine oviduct using rapid amplification of cDNA ends (RACE) method, the sequence analysis revealed that porcine CDH1 gene complete cDNA nucleotide sequence amounted to 4283 bp including 2652 bp of open reading frame (ORF), 105 bp of 5' untranslated region (UTR) and 1526 bp of 3' UTR. The ORF encoded a deduced protein precursor of 97 kD with 883 amino acid residues. The precursor protein including signal peptide, extracellular region, membrane-spanning region and cytoplasmic region had a single transmembrane structure, and its extracellular region had HAV motif and some Ca²⁺ binding regions. The porcine CDH1 protein showed high homology with cattle (89%), horse (87%), dog (86%), human (84%), chimpanzee (83%) and mouse (83%) CDH1. The results from RT-PCR and real-time PCR indicated that CDH1 gene could be expressed in both immature and mature oocytes, as well as early parthenotes (2-, 4-, 8-cell embryos, morula, blastocysts). The data of real-time PCR showed that the expression of CDH1 was the highest at 2-cell parthenogenetic embryos stage, and then it decreased significantly till 8-cell embryos stage; the second increased expression level occurred at morulas stage, and then also showed a diminishing trend in the following developmental stage. This is the first report of cloning and analysing the porcine CDH1 cDNA that provides critical information for further research of its functions in pig embryo development.

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E-cadherin, also known as CDH1 (Cadherin 1) or uvomorulin [Hyafil *et al.* 1981, Peyrieras *et al.* 1983], is essential for normal compaction and blastulation in mammals. Functional depression or deletion of CDH1 may result in abnormal embryo compaction and blastocyst formation. Embryos injected with antisense RNA showed markedly diminished CDH1 level and delayed compaction compared to control embryos injected with sense RNA [Ao and Erickson 1992]. Moreover, when the CDH1 encoding gene was inactivated by homologous recombination, the CDH1-negative embryos failed to form a trophectodermal epithelium or a blastocyst cavity, and the embryo died around the time of implantation due to the presence of residual maternal CDH1 [Larue *et al.* 1994]. In cattle, microinjection of CDH1 dsRNA resulted in the reduction of E-cadherin mRNA and protein levels at the morula and blastocyst stage, and the blastocyst rate was reduced [Nganvongpanit *et al.* 2006].

Embryonic engineering technology of pig is attracting attention because of its importance as livestock [Jiang and Rothschild 2007] as well as model animal for human diseases [Lunney 2007]. As already mentioned, porcine embryonic compaction and blastulation which are critical events in embryonic development, cannot be ignored. However, compared to human, mice, cattle and some other mammals, relevant research data in pigs are still very few. In the present study, The classic RACE (rapid amplification of cDNA ends) method [Frohman 1994] has been used to isolate and characterize porcine CDH1 cDNA, while the CDH1 expression profile has been investigated with porcine parthenotes at the different developmental stage as materials using RT-PCR and real-time PCR technology. It is anticipated that the results of this research will provide the molecular basis for further study of CDH1 activity during the development of porcine embryo, and also to enhance our knowledge of the role of CDH1 in porcine early embryos.

Material and methods

Ovum recovery, *in vitro* maturation (IVM) and parthenogenetic activation of porcine oocytes

Porcine ovaries were collected from a local slaughterhouse and transported to the laboratory at 30-37°C in 0.9% (w/v) saline (0.85% NaCl, 0.02% KCl, 0.02% CaCl₂·2H₂O, 0.01% MgCl₂·6H₂O) supplemented with 75 µg/ml potassium penicillin G and 50 µg/ml streptomycin sulfate. Cumulus-oocyte complexes (COCs) were obtained from follicles with a diameter of 2 to 8 mm, using a 12-gauge needle connected to a 10 ml disposable syringe. The medium used for oocyte maturation was TCM-199 (GIBCO) supplemented with 10% (v/v) porcine follicular fluid, 0.57 mM cysteine, 3.05 mM D-glucose, 0.91 mM Na pyruvate, 75 µg/ml penicillin, 50 µg/ml streptomycin, 10 ng/ml epidermal growth factor (E-4127), 1.0 µg/ml FSH and 1.0 µg/ml LH. The COCs were washed three times with Tyrode's lactate-(TL)-HEPES-PVA medium. From 60 to 100 COCs were cultured in 200 µl of maturation medium droplets

for 20-22 h and then transferred to 200 µl of maturation medium droplets without LH and FSH for another 20-22 h at 39°C in humidified air containing 5% CO₂. Oocytes were electrically activated with three 100 V/mm DC pulses of 30 µs, after which the COCs were treated with hyaluronidase to complete removal the granulosa cells, and then washed three times. The electrically activated oocytes were cultured in PZM-3 medium at 39°C in humidified air containing 5% CO₂. Granulosa cells, immature oocytes, mature MII oocytes, Day 1 two-cell embryos, Day 2 four-cell embryos, Day 3 8-cell embryos, Day 5 morulas and Day 7 blastocysts were collected.

RNA extraction, reverse transcription and PCR reaction

The total RNAs were extracted with TRIZOL (INVITROGEN) and the RT-PCR was carried out with RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa, China). PCR was performed with 1.0 U Taq (TaKaRa, China) in 25 µl of reaction mixture containing 1 µl template DNA, 10×PCR buffer 2.5 µl, 2.5 mM of each dNTP and 0.2 µM of each primer.

Rapid amplification of cDNA ends

The primers (BS1 and BS2) of conserved sequence were based on the alignment of results from cattle, dog, mouse, human and horse CDH1 sequence in GenBank (Tab. 1). With the porcine oviduct total RNA as template, the amplification of conserved sequence was performed following the programme shown in Table 1.

Table 1. Primers for amplifying the CDH1 gene complete cDNA

Primer	Sequence	Remarks
		Conserved sequence amplification
BS1	up: ATTTATCGTCGTCACAGACCC low: GCCTTCGTAATCAAACACCAG	BS1 amplified condition: 94°C 4 min; 40 cycles of 94°C 30 s and 55.3°C 30 s; 72°C extension for 7 min
BS2	up: TTTCTGATGACACCCGATTC low: GTGCCATTCACATCTATC	BS2 amplified condition: 94°C 4 min; 40 cycles of 94°C 30 s, 53°C 30 s; 72°C 1.5 min, 72°C extension for 7 min
		3' RACE amplification
GSP1	GAAGTGACTCGTAATGATGTG	The first round PCR reaction: 94°C 4 min; 40 cycles of 94°C 30 s, 55°C 30 s; 72°C 2 min; 72°C extension for 7 min
3RAAP	GTTTTCCAGTCACGACTTTT	The second round PCR reaction: 94°C for 4 min; 40 cycles of 94°C 30 s, 55.6°C 30 s; 72°C 2 min; 72°C extension for 7 min
		5' RACE amplification
GSP4	CAAATCCGATACGTGATCTTCTG	The first round PCR reaction: 94°C 4 min; 30 cycles of 94°C 30 s, 53°C 30 s; 72°C 2 min; 72°C extension for 7 min
5RAAP	GAGCGTACTACAGGGCACTAC(G) ₁₀	The second round PCR reaction: 94°C 4 min, 15 cycles of 94°C 30 s, 54°C 30 s; 72°C 1.5 min; 72°C extension for 7 min
AUAP	GAGCGTACTACAGGGCACTAC	
GSP2	CGGGGGTCATTACATCT	The third round PCR reaction: 94°C 4 min; 25 cycles of 94°C 30 s, 54°C 30 s; 72°C 1.5 min; 72°C extension for 7 min
GSP3	ATAGTCCCAGGCGTGATGAG	The fourth round PCR reaction: 94°C 4 min; 40 cycles of 94°C 30 s, 56.4°C 30 s; 72°C 30 s; 72°C extension for 7 min

The 3' RACE upstream primer GSP1 and adaptor primer 3RAAP were designed according to the conserved sequence of CDH1 and the OligodT-Adaptor Primer sequence, respectively. With the porcine oviduct, total RNA as template and OligodT-Adaptor Primer as primer, the cDNA first strand was synthesized by RT-PCR as mentioned above. Then, with the cDNA first strand products as template and GSP1 as primer, the 3' RACE first round PCR reaction was performed; finally, with the 3' RACE first round PCR products as template and DNA, GSP1 and 3RAAP as primers, the second round PCR reaction was performed with the programme shown in Table 1.

The 5' RACE RT-PCR primer (GSP4), downstream primer (GSP2 and GSP3) and adaptor primer (5RAAP and AUAP) were designed based on the conserved sequence of CDH1. With the porcine oviduct the total RNA as template and GSP4 Primer as primer, the cDNA first strand was synthesized by RT-PCR as mentioned above. The RNA strands in the cDNA first strand products were digested with RNase H (MBI FERMENTAS, Lithuania) according to the manufacturer's instructions. The digested products were saturated with TRIS, extracted with phenol and the cDNA and then precipitated with 4M sodium acetate and ethanol [Dickgiesser *et al.* 1982]. For tailing, 5×tailing buffer solution (4 µl), 2 mM dCTP (2 µl), and 1 µl terminal deoxynucleotidyl transferase (MBI FERMENTAS, Lithuania) were added, then the volume of the mixture adjusted to 25 µl. This was incubated for 15 min at 37°C, then heated for 10 min at 70°C, after which the products were purified as described above. With the purified cDNA first strand as template DNA, the 5' RACE first round PCR programme (5RAAP as primer), second round PCR (AUAP and GSP2 as primer), third round PCR (AUAP and GSP2 as primer) and fourth round PCR programme (AUAP and GSP3 as primer) were performed following the programme shown in Table 1. The relationship of primers used in RACE is shown in Figure 1.

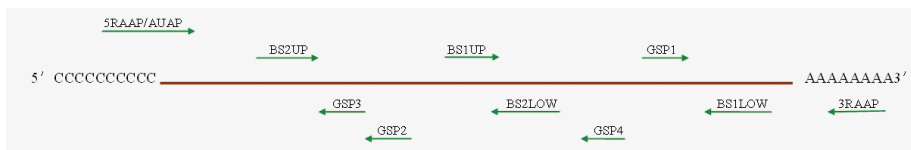


Fig.1 The position of RACE primers.

Cloning and sequencing the cDNAs

RACE products were recovered and purified with Biospin Gel Extraction Kit (BIOER TECHNOLOGY, China) according to the manufacturer's instructions, and the purified products then ligated with the p^{BS-T} vector (TIANGEN, China), and introduced into competent *Escherichia coli* DH5α (stored in our lab). After culturing the *Escherichia coli* DH5α in Amp+LB medium, the vectors were extracted with a plasmidPrep Mini Spin Kit (NEWLIFEGENE, China), and the purified vectors sequenced by the BEEIJING SUNBIOTECH Company (China).

cDNA sequence assembly and analysis

DNA sequence assembly was carried out with CAP3 sequence assembly programme [Huang and Madan 1999] available in <http://pbil.univ-lyon1.fr/cap3.php>. ORF was found in <http://www.ncbi.nlm.nih.gov/gorf/gorf.html>; protein isoelectric point, molecular weight and amino acid composition were analysed in <http://www.expasy.ch/tools/#primary>; signal peptide, N-glycosylation site and protein structure were predicted according to <http://www.cbs.dtu.dk/services/SignalP/>, <http://www.cbs.dtu.dk/services/NetNGlyc/> and <http://www.cbs.dtu.dk/services/TMHMM/>.

The CDH1 expression profile of porcine early parthenotes

For detection of the CDH1 expression in porcine granulosa cells, oocytes and early parthenotes with RT-PCR, approximately 1×10^7 granulosa cells, 100 immature oocytes, 100 MII oocytes and 100 preimplantation parthenogenetic embryos at each developmental stage (2-cell, 4-cell, 8-cell embryos, morulas and blastocysts) were collected. With the total RNA as template and random 9 mers (Provided in RNA PCR Kit (AMV) Ver. 3.0 mentioned above) as primer, cDNA first strands were synthesized as mentioned above. With cDNA first strands as template, BS2UP and BS2LOW (Tab. 1) as primer, the PCR reaction was performed, The PCR products were then detected in 1% agarose gel.

For detection of the CDH1 expression of early parthenotes with quantitative real-time PCR, 40 preimplantation parthenogenetic embryos in each stage (2-cell embryos, 4-cell embryos, 8-cell embryos, morulas and blastocysts) were collected. After that the cDNA first strand was synthesized, Real-time PCR was then performed on an MJ Opticon 2 system (MJ RESEARCH) with GAPDH as reference gene. The product length of GAPDH primers (sense primer: 5'-ACACTCACTCTTCTACCTTTG-3', antisense primer: 5'-CAAATTCATTGTCGTACCAG-3') was 90 bp [Nygaard, *et al.*, 2007]; the product length of CDH1 primers (sense primer: 5'-AACCACAGACAAGAACTT-3', antisense primer: 5'-CTACACAGAACTACATTAGAG-3') was 133 bp. Samples were run in quadruplicate and amplified in a total volume of 20 μ l containing 9 μ l RealMasterMix (SYBR Green I, TIANGEN, China) and 0.4 mM of each primer. The programme used for the CDH1 gene consisted of a denaturing cycle of 2 min at 95°C; 50 cycles of PCR (95°C for 10 s, 57°C for 10 s, and 68°C for 12 s). A melting cycle consisted of 78°C for 1 s, 81°C for 1 s, 84°C for 1 s, and a step cycle starting at 65°C with a 0.1°C/s transition rate to 95°C. The programme used for the GAPDH gene consisted of a denaturing cycle of 2 min at 95°C, 50 cycles of PCR (95°C for 10 s, 57°C for 10 s, and 68°C for 12 s); a melting cycle consisting of 78°C for 1s, 81°C for 1 s, 84°C for 1 s, and a step cycle starting at 65°C with a 0.1°C/s transition rate to 95°C.

Statistical

The relative quantification (RQ) of transcript level of quantitative real-time PCR was calculated by the $2^{-\Delta\Delta C_t}$ method [Livak and Schmittgen 2001] using the following formula:

$$RQ = 2^{-\Delta\Delta Ct},$$

where:

$\Delta\Delta Ct = \Delta Ct (\text{test}) - \Delta Ct (\text{calibrator});$

$\Delta Ct (\text{test}) = t (\text{target, test}) - Ct (\text{ref, test});$

$\Delta Ct (\text{calibrator}) = Ct (\text{target, calibrator}) - Ct (\text{ref, calibrator});$

Ct – cycle at which threshold level of amplification is reached.

Data were tested using the One-way ANOVA method, followed by the Tukey test.

Results and discussion

RACE and sequence analysis

In this study, two conserved fragments, 5' RACE and 3' RACE products of porcine CDH1 gene (Fig. 2 and 3) were successfully isolated from pig oviduct tissue. Collectively, the removal overlapping sequence of conserved fragments 3' RACE and 5' RACE products amounted to 4283 bp cDNA complete sequence. The nucleotide sequence analysis using the BLAST software at the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>) revealed that the gene was not homologous with any of the known porcine genes except for a 121 bp short sequence (AF033019). It was then deposited in the GenBank database (accession number Eu805482).

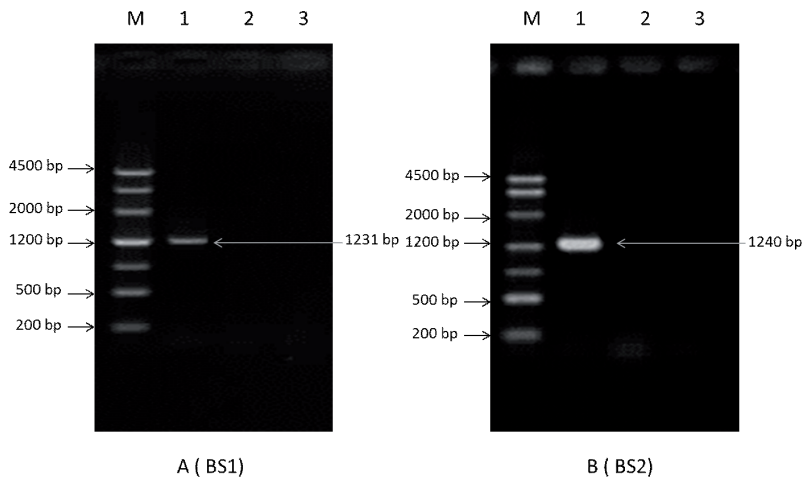


Fig. 2 Electrophoresis results of BS1 and BS2 PCR amplification products with two pairs of primers (BS1 and BS2). Two conserved sequences, 1231 bp (lane 1 in A) and 1240 bp (lane 1 in B), were isolated. RNA control and blank control (lanes 2 and 3 in A and B, respectively) were set in this experiment.

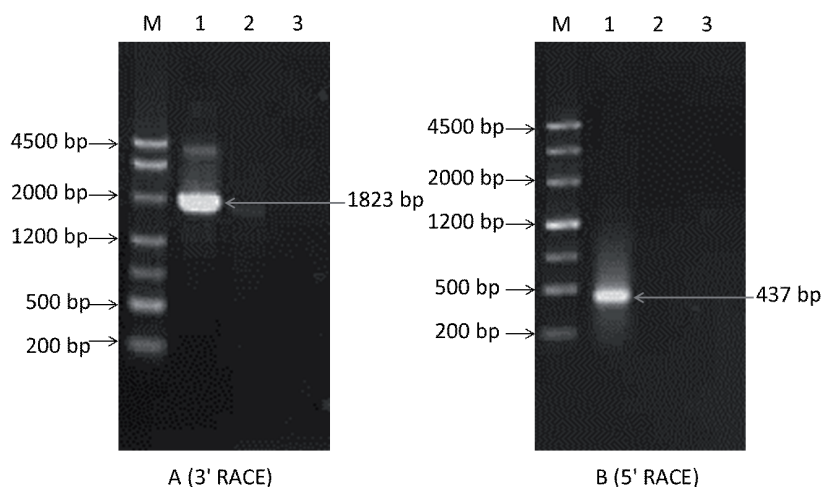


Fig. 3. The electrophoresis results of 3'RACE and 5'RACE amplification products. Two fragments with 1823 bp (lane 1 in A) and 437 bp (lane 1 in B) were isolated. RNA control and blank control (lane 2 and lane 3 in A and B) were set in this experiment.

ATGGGCCCTCCGTGCCGAGTCTCTGCTCTCCTGCTGCTGTTGCAGGTCTCATCGTGGCTCTGCCAA
 ▲▲▲
 M G P P C R S L S A L L L L L Q V S S W L C Q
 AGCTGCCAGAAAATGAGAAAGGCCGTTTCCTAAGGATCTGGTTCAGATCAAATCTAACAGAGAAAA
 S C P E N E K G P F P K D L V Q I K S N R E K
 GAAAGAGAAACAGGGTGGCTGAAGGTGACGGAGCCTCTGGATAGAGAAGCCATTGCCAAGTACATTCT
 E R E T G W L K V T E P I D B E A I A K Y I L
 TACTCTCATGCCGTGTCTTCCAACGGGCAAGCCATCGAGGAGCCCATGGAGATTGTGATCACCGTGACC
 Y S H A V S S N G Q A I E E P M E I V I T V T
 GATCAGAACGACAACAAGCCGAGTTCACCCAGGAGGTCTTCAGGGGCTCCGTATGGAGGGCGCTCTT
 D O N D N K P E F T Q E V F R G S V M E G A L
 ACAGGAGTCATCAGTGTGCTCACCCTGGGCTGGACCGGAGAGTTTTCCACGTACACCCTGGTGATT
 T G V I S V L T T G L D R E S F P T Y T L V I
 AACGACAACCCACCATCTTCAACCCAACCTCGTACCAGGGGTCGGTGCCGAGAACGAGGCCAACGTC
 N D N P P I F N P T S Y Q G S V P E N E A N V
 GCAATCACCACTCACGGTGACCGATGGTGACGTCGCCAATACCCGGGCGTGGGTGGCTGTTACACA
 A I T T L T V T D G D V A N T P A W V A V Y T
 GTGGCCAACAGCTGCCGGAAGGCCGGGCTCTGGCCGAAGCAGGATTGCAAGTTCCTGCCATCCTGGGG
 V A N S C R K A G P L A E A G L Q V P A I L G
 ATTCTGGGAGGCATCCTTGCTTTTCTGATCCTGATTCTGCTGCTCCTGCTCCTATTTCGGAGGAGAAGG
I L G G I L A F L I L I L L L L L I R R R R
 GACATGTACGGAGGCGGTGAGGACGACTAG
 ▼▼▼
 D M Y G G G E D D

Fig. 4. Whole nucleotide and deduced amino acid sequences of porcine CDH1 gene. A single underline indicates the signal peptide, a double underline the transmembrane region, ○ the Ca²⁺ binding site, ● the HAV motif, Δ the glycosylation sites, ▲ the start codon, ▼ the stop codon.

Further bioinformative analysis demonstrated that the porcine CDH1 gene shared characteristics similar to those of the other CAD proteins. The ORF of pigs' CDH1 gene encoding a deduced protein precursor with 883 amino acid residues was 2652 bp, including 105 bp of 5'UTR and 1526 bp of 3'UTR. The pI of the porcine CDH1 protein was 4.72, and its molecular weight reached 97 kD. The precursor protein has a 22 amino acids signal peptide at its N-terminal whose signal peptidase cutting site is at amino acid 22-23. The precursor protein which includes an extracellular region (1-709), membrane-spanning regions (710-732), and cytoplasmic regions (733-883), is a single transmembrane protein with amino acid 376 a possible glycosylation site. Further BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) analysis of these proteins revealed that the structure and function domain of the deduced protein is similar to the CAD protein in the database, thus indicating that the protein belongs to CAD protein family with a function of mediating cell-cell adhesion [Pertz *et al.* 1999]. The deduced protein, as the other CAD proteins, has some specific sequences in its extracellular region, such as the HAV (His-Ala-Val) motif, and Ca²⁺ binding *loci* – PENE, LDRE, DQNDN *etc.*, (Ringwald *et al.* [1987], Nose *et al.* [1990], Overduin *et al.* [1995, 1996], Shapiro *et al.* [1995], Nagar *et al.* [1996]). More details are shown in Figure 4.

Comparison of the deduced amino acid sequence of porcine CDH1 to its homologues of cattle, horse, dog, human, chimpanzee and mouse shows the identities reaching 89%, 87%, 86%, 84%, 83% and 83%, respectively. The homology among the CDH1 extracellular domain sequence for these species is relatively low (70-85%), whereas it is high between the transmembrane region and cytoplasmic domain (95%-98%). Based on the results of the comparison of different CDH1 proteins structure of species mentioned above, a phylogenetic tree was constructed (Fig. 5). Its structure reveals that the porcine CDH1 gene is genetically related closer to CDH1 gene of cattle than to those of horse, mouse, chimpanzee, human and dog.

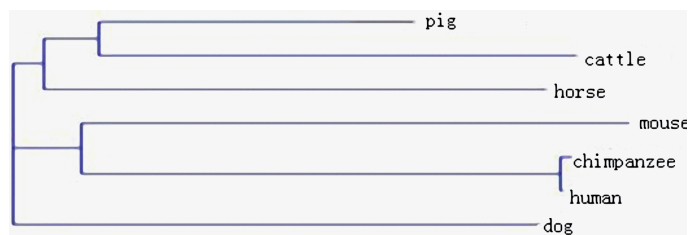


Fig. 5. The phylogenetic tree for seven species on the basis of CDH1 protein sequence.

CDH1 gene expression profile during the development of porcine early parthenotes

Due to the relatively high incidence of polyspermy during the *in vitro* fertilization it is difficult to obtain pig embryos of homogenetic quality; therefore, diploid parthenotes have frequently been used to study the early development in pigs [Van Thuan *et al.* 2002, Hwang *et al.* 2005]. Though the CDH1 of normal embryos much

research has been devoted, the data of CDH1 gene expression in parthenotes have not yet been reported in detail. Thus, in this study, parthenotes were selected as a model to investigate the expression of CDH1 gene in early embryo development. Comparing similarities and differences of CDH1 gene expression patterns among normal embryos and parthenotes will help the further understanding of the mechanism of early embryonic development, and reveal the relationship of maternal and zygotic gene expression of CDH1 during the development of early porcine embryos.

The results of PCR analysis showed that CDH1 gene was expressed in all developmental stages of porcine early parthenotes (2-cell, 4-cell, 8-cell, morula and blastocyst), and also in porcine granulosa cells, immature oocytes and MII oocytes (Fig. 6A).

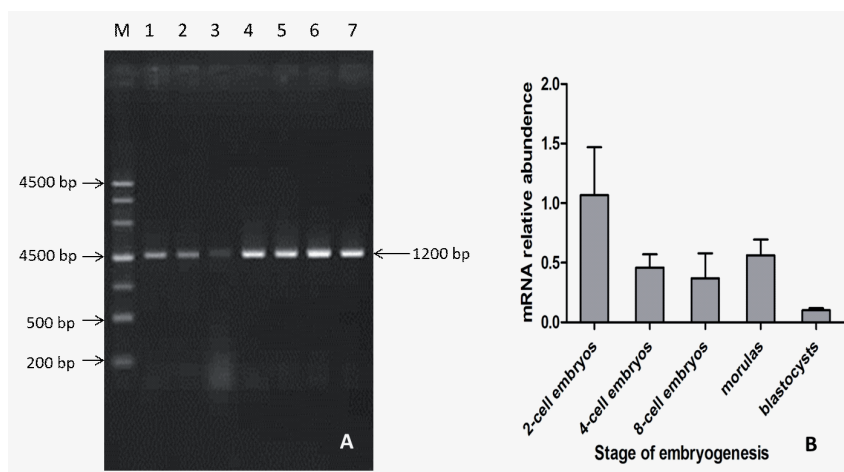


Fig. 6. A. The porcine *CDH1* gene expression profile. M Marker3, 1 premature oocytes, 2 MII oocytes, 3 granulosa cells, 4 2-6-cell-stage embryos, 5 8-cell-stage embryos, 6 morulas, 7 blastocytes. B. Quantitative real-time PCR analysis of porcine *CDH1* gene in samples of in vitro derived porcine early parthenotes at 2-cell-stage, 4-cell-stage, 8-cell-stage, morula and blastocyst stages. Data are shown as mean \pm SD (n=4).

The previous research demonstrated that the relative CDH1 mRNA content greatly increased in a time-dependent manner during early embryonic development from 2-cells stage to blastocyst stage [Kawai *et al.* 2002], and CDH1 was of both maternal and embryonic origin, the maternal CDH1 protein synthesis possibly occurred before oocytes matured, and the maternal protein could continuously exist after zygotic genome activation [Barcroft *et al.* 1998, Wianny and Zernicka-Goetz 2000, Nganvongpanit *et al.* 2006, Modina *et al.* 2010]. In the present study, the CDH1 expression pattern which showed the expression of CDH1 was the highest at 2-cell parthenogenetic embryos stage, after decreasing significantly till 8-cell embryos stage, the second increased expression level occurred at morulas stage, and then also showed a diminishing trend in the following developmental stage, revealed that the CDH1 gene

was the maternal and embryonic original in porcine early parthenotes development. According to our knowledge, the porcine zygotic genome activation usually occurred at 4-8 cells embryonic stage. In this study, the massive amount expression of CDH1 in 2-cells embryonic stage possibly derived from maternal materials; however, in morula stage, the increased expression of CDH1 was probably due to the zygotic genome activation. The expression pattern of CDH1 in porcine early parthenotes, which showed diminishing trend in total, was different from those of early research data based on normal embryos [Kawai *et al.* 2002, Modina *et al.* 2010], implied that the decreased expression of CDH1 of the parthenotes could disturb the embryonic compaction process, and thus could not meet the needs of continuously strengthening intercellular tension during the blastocoel formation and could block the parthenotes subsequent development. Further study of the relationship between CDH1 over-expression and embryo development in porcine early parthenotes could provide more details of the functional mechanism of CDH1 participation in early porcine embryo development.

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