

Isolation of the human *ePAB* and *ePABP2* cDNAs and analysis of the expression patterns

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Abstract

Purpose Identification of the unique genes playing critical roles in human embryo cleavage.

Methods Isolation of human *ePAB* cDNA using human ovary cDNA libraries and mouse *ePAB* amino acid sequences, followed by analysis of its expression pattern in various adult tissues and stages during early oocyte development excluding *ePABP2*.

Results Human *ePAB* encodes a 330-aa protein and is located on chromosome 20q12-q13.1. The amino acid sequence is 72% homologous with that of mouse *ePab*. Human *ePAB* has only three RRM and lacks a PABP domain; the expression pattern is nonspecific in adult tissues and detected in all stages, from oocyte to blastocyst. Human *ePABP2* encodes a 282-aa protein and is located on chromosome 16q24.3. The amino acid sequence is 68% homologous with mouse *ePabp2*.

Conclusions We identified human *ePAB* and *ePABP2* cDNA. Human *ePAB* cDNA is not expressed specific to the ovary. Biological discrepancies exist between the human and the mouse.

Keywords Cleavage · Embryo · *ePAB* · *ePABP2*

Introduction

Infertility is a common problem faced by the clinician; approximately 10% of couples have difficulty conceiving a child [1]. Regardless of the cause of infertility, the procedure that leads to the highest pregnancy rate per cycle is in vitro fertilization (IVF). Since its inception in 1978 [2], there has been a remarkable world-wide increase in the number of IVF procedures. For 2003 in the United States, the clinical pregnancy rate per cycle for IVF cycles with fresh, non-donor eggs was 34% [1]. However, there are several risk factors associated with IVF; the most common complication of IVF is multiple gestation. In general, the transfer of more than one embryo results in a higher pregnancy rate than single-embryo transfer; however, this technique is also associated with a high risk of multiple gestation. An obvious way to reduce multiple gestations is to transfer a single embryo. Normally, the transferred embryo is at the cleavage stage (2-day embryo). However, recent studies indicate that transfer of a single blastocyst (5-day embryo) may result in an even higher rate of pregnancy while dramatically lowering the rate of multiple gestations [3, 4]. However, not all embryos grow to the blastocyst-stage. Moreover, no eggs grow to the blastocyst-stage in some patients.

It has been demonstrated that several genes may play critical roles in cleavage in both mice and humans [5]. However, most genes that play a role in cleavage are still unknown, especially in humans. Oocyte maturation is accompanied by a complex network of translational activation and repression of dormant maternal mRNAs [6–9]. These maternal mRNAs drive the oocyte's reentry into meiosis and control the rate of mitosis during the early cleavage divisions of the embryo [9–12]. The first step in translational activation of stored maternal mRNAs is cyto-

Capsule We successfully isolated the human *ePAB* and *ePABP2* cDNAs and analyzed the expression patterns in humans.

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plasmic extension of their poly(A) tails. This process, called cytoplasmic polyadenylation, was initially thought to be confined to gametes and embryos [13]. It has been reported that a poly(A)-binding protein (PABP) can bind to the newly elongated poly(A) tail [14]. Two structurally distinct groups of PABPs have been identified in vertebrates. *PABP1* is the prototype of the first group and contains four RNA recognition motifs (RRMs) at its N terminus and a unique C-terminal PABP domain [15–17]. *PABPN1* is the prototype of the second group of PABPs. It is smaller, with only one RRM, and is present in all cells of organisms, including oocytes and embryos [18–20]. An embryonic poly(A)-binding protein (ePAB) was identified in *Xenopus* and belongs to the first group of PABP [21]. As the predominant PABP during *Xenopus* early development, *ePab* most likely regulates unmasking/translation of maternal mRNAs and may influence poly(A) tail length as well. Therefore, it is suggested that *ePab* may play an important role during oocyte maturation and early embryonic development. Dysregulation of *ePab* may cause problems during oocyte and early embryonic development. Recently, the mouse *ePab* gene was identified and its expression pattern was characterized during mammalian oogenesis as well as preimplantation embryo development in the mouse [22]. Subsequently, an embryonic poly(A)-binding protein 2 (ePABP2) was identified in *Xenopus* and mouse, and was similar to the second group of PABP in the structure [20]. The mouse *ePabp2* expression pattern was also characterized in the early development and adult ovarian tissue. In this study, we isolated the human *ePAB* and *ePABP2* cDNAs and analyzed the expression patterns in various adult human tissues, oocytes, and preimplantation embryos.

Materials and methods

Isolation of the human ePAB cDNA and analysis of its expression pattern in various tissues

The mouse *ePab* cDNA has been previously isolated [22]. Using the mouse ePAB amino acid sequence [22], we found its homologous region in the human genome sequences (XM_001131956 in Genbank). A pair of primers (PABF1 and PABR1) was made using the human homologous region encompassing the putative introns. PCR was carried out with a human ovary cDNA library (BD Biosciences Clontech, Tokyo, Japan) as a template. Nested PCR was performed with the primers, PABF2/PABR2 and a 10-fold dilution of the first PCR product as a template. The oligonucleotides that were employed were: PABF1; 5'-TT ACGTGGGCGATCTGCACC-3', PABR1; 5'-GGGTC CTGTGGTCTGAGTAC-3', PABF2; 5'-CCGAGGCCA TGCTCTATGAG-3' and PABR2; 5'-CTACTCTCTGGG

TATGAGGC-3'. The resultant nested PCR product was subcloned into a T-Easy vector (Promega, Madison, WI, USA), and several representative clones were sequenced in both directions. The 5'RACE and 3'RACE were done with the primers 5RAPAB1, 5RAPAB4, 3RAPAB1, 3RAPAB5, AP1 (Clontech), and AP2 (Clontech). Their sequences of oligonucleotides were as follows: 5RAPAB1; 5'-GACT TGCGAAGTCCTGGGTCTCGCTGGG-3', 5RAPAB4; 5'-AGTTGATGTAGGCGTAGCCCAGCGAGCG-3', 3RAPAB1; 5'-AGAGGCGACAAAGGCCGTGACAGAG ATG-3', 3RAPAB5; 5'-TGAGCAACCCCTCCTGGGCT CCTTTC-3'.

Both RACE products were also subcloned, and several representative clones were sequenced in both directions. The isolated full-length cDNA sequences were compared with human genomic sequences. All of the first PCRs were carried out using an Advantage 2 PCR kit (Clontech) under the following conditions and according to the manufacturer's instructions: initial denaturation at 95°C for 150 s; 32 denaturation cycles at 95°C for 15 s; and annealing and extension at 68°C for 180 s. All of the nested PCRs were performed under the same conditions except for the 20 cycles.

For the expression analysis of the human *ePAB*, PCR was done with the primers PABRTF2 and PABRTR2. The analyzed human cDNAs were: spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, brain, heart, kidney, liver, lung, skeletal muscle, pancreas, and placenta (Clontech). The sequences of the primers were: PABRTF2; 5'-ATCCTGTCCATCCGCGTGTG-3' and PABRTR2; 5'-AGATGTTGGTGA ACTCCAGG-3'. PCR conditions were initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 15 s, annealing and extension at 68°C for 90 s using an Advantage 2 PCR Kit.

RT-PCR of oocytes and preimplantation embryos

Oocytes and embryos that were either unfertilized or were clinically not required for IVF were used. All the patients gave their written informed consent to undergo a molecular analysis of their oocytes and embryos, and the study was approved by ethics committee of Asahikawa Medical College. Total RNA from oocytes or embryos was obtained by using a RNAqueous Microkit (Ambion, Austin, TX, USA), according to the manufacturer's instructions and kept at -80°C until use. First, reverse transcription (RT) reactions with oligo d(T) primers and the RETROscript kit (Ambion) were performed on the total RNA from 70 oocytes or embryos, according to the manufacturer's instructions. Next, PCR was carried out on cDNA from the equivalent of five oocytes or embryos with the primers GF1 and GR2. Nested PCR was done with the primers GF2 and GR4. The sequences of the oligonucleotides used were:

Fig. 1 Comparison of amino acid sequences between human (*upper sequence*) and mouse ePAB (*lower sequence*). An amino acid identity of 72% was observed between the two sequences. *Vertical lines* indicate identical amino acids and asterisks represent stop codons. The four RRM s are *underlined*

human	1	MNASGSGYPLASLYVGD LHPDVTEAMLYEKFSPAGPILSIRVCRDVATR R	50
mouse	1	MDTGGHGCSLSSLYVGD LHPDVTEAMLYEKFSPAGPILSIRVCRDVATR R	50
		<u>RRM1</u>	
human	51	SLGYAYIN FQOPADAERALDTMNFEM LKGOPIRIMWSQRDPGLR KSGVGN	100
mouse	51	SLGYAYIN FQOPADAERALDTMNF EVIKGOPIRIMWSHRQPGLR KSGMGN	100
human	101	I F I K N L E D S I D N K A L Y D T F S T F G N I L S C K V A C D E H G S R G F G F V H F E T H E A	150
mouse	101	I F I K N L E N S I D N K A L Y D T F S T F G S I L S S K V V Y N E H G S R G F G F V H F E T H E A	150
		<u>RRM2</u>	
human	151	AQQAINTMNGMLLNDRKVFVGHFKSRREAE L GARALEFTNIYVKNL PV	200
mouse	151	AQKAINTMNGMLLNDRKVFVGHFKSRQKREAE L GARALGFNTIYVKNLHA	200
		<u>RRM3</u>	
human	201	DVDEOGLQDLFSQFGKMLSVKVMRDNS - GHSRCFGFVNFEKHEEAQAVV	250
mouse	201	NVDEOQLQDLFSQFGNMQSVKVMRD - SNGQSRGFGFVNFEKHEEAQAVD	250
human	251	HMNGKEVSGRLLYAGRAQKRVERONELKRRFEQMKODRLRRYQGVNLYVK	300
mouse	251	HMNGKEVSGQLLYVGRAQKRAERQSELKRRFEQMKQERQNR YQGVNLYVK	300
		<u>RRM4</u>	
human	301	NLDDSIDDDKLRKE - FSPYGVITSAK - - - - - K - - - - -	350
mouse	301	NLDDSI NDERL - KEVFSTYGVITSAKVMTES SHSKGFGFVCFSSPEEATK	350
human	351	- - - - - R - - - - - R - - - - - O - R - - P * - - - - -	400
mouse	351	AVTEMNGRIVG TKPLYVALAQRKEERKAILTNOYRRRPSHPVLSS FQOPT	400
mouse	401	SYLLPAVPQSTAQAVYSSGSITPMQPDPRWTAQPHGPPSTCPPAASV VQ	450
mouse	451	PLSTTQHPCIH LR GASQVSSQVPHTQRV VNIQTTPGPGGESSIPGQLL	500
mouse	501	VPHRGTSAVHSAHG VQESAVYVPGHQPLTVSMLAAAPLHEQKQMIGERLY	550
mouse	551	SLIH DACAPLTGKITGMLLELDNLE LLLLLLESPE SLHAKIEEAVAVLQVH	600
mouse	601	REMESTDRN*	
	650		

GF1; 5'-CAGCACATAGCACCTATCGG-3', GR2; 5'-GGTAATAGGCTGGATGGGTG-3', GF2; 5'-CTGTGCA CATCCCAGGACAG-3', GR4; 5'-AGAGTTTGCAGG CCTTAGG-3'. PCR for G3PDH was performed using the same RT product as a positive control. PCR conditions were: initial denaturation at 95°C for 150 s; 32 cycles of denaturation at 95°C for 15 s; and annealing and extension at 68°C for 90 s, using an Advantage 2 PCR Kit. All of the nested PCRs were performed under the same conditions except for the 20 cycles.

Isolation of the human ePABP2 cDNA

A set of primers, PABP2NF1 and PABP2NR1, in the human homologous region encompassing the putative introns, and PCR was carried out with a human ovary cDNA library as a template. The sequences of the oligonucleotides used were: PABP2NF1; 5'-ATGTGGC CCTTCCCGAGCCG-3' and PABP2NR1; 5'-TCACC GGTCTGCCCTTGTGG-3'. Semi-nested PCR was done with the primers (PABP2NF2/PABP2NR1) and a tenfold

Fig. 2 Schematic representation of the PABP family. RRM s are indicated by *gray boxes* and PABP domains are indicated by *dark boxes*

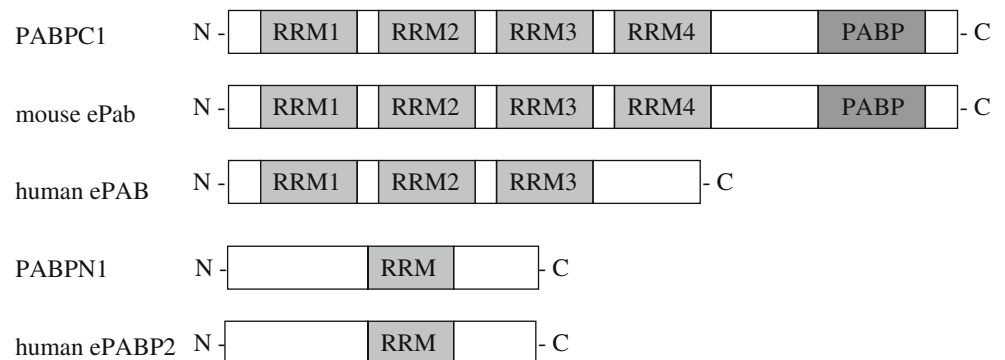
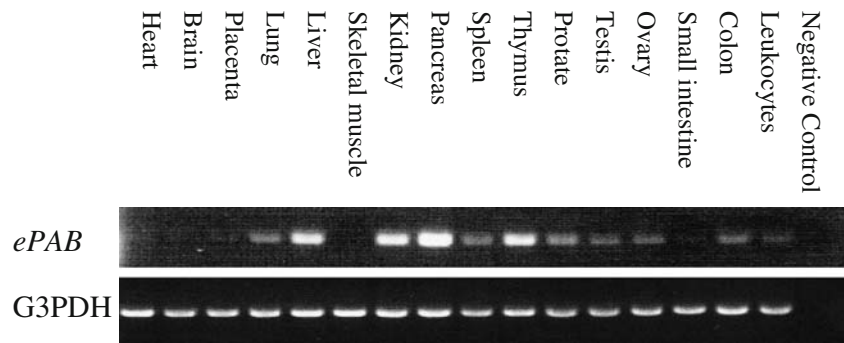


Fig. 3 Expression patterns of *ePAB* in 16 human adult tissues. PCR analysis reveals a non-specific pattern in almost all tissues (*upper panel*). G3PDH was used as a positive control (*lower panel*)



dilution of the first PCR product as a template. The sequences of PABP2NF2 were: 5'-CGACTCAGGCCTG GCTCCAG-3'. The product from the semi-nested PCR was subcloned into a T-Easy vector, and several representative clones were sequenced in both directions. The 5'RACE and 3'RACE were performed with the primers 5RAPABN1, 5RAPABN2, 3RAPABN1, 3RAPABN2, AP1 (Clontech) and AP2 (Clontech). The sequences of the oligonucleotides were: 5RAPABN1; 5'-GGCCTGCTCCATGGCACACA CCTTCATC-3', 5RAPABN2; 5'-CTCCAGCTCCTGGTCA GGCAATGGGCAC-3', 3RAPABN1; 5'-GGGAGGTCCA CCGAGTCACGATCCTGTG-3', 3RAPABN2; 5'-GTT CTCTGGACACCCCAAGGGTTATGCC-3'. Both RACE products were subcloned and sequenced in both directions. The isolated full-length cDNA sequences were compared with human genomic sequences. PCR conditions were initial denaturation at 95°C for 150 s, followed by 32 cycles of denaturation at 95°C for 15 s, annealing and extension at 68°C for 180 s. All of the nested PCRs were performed under the same conditions except for annealing and extension at 68°C for 120 s.

For the expression analysis of the *ePABP2*, PCR was carried out with the primers PAB2RTF1 and PAB2RTR1. The analyzed human cDNAs were following: spleen, thymus, prostate, testis, ovary, small intestine, colon, leukocyte, brain, heart, kidney, liver, lung, skeletal muscle, pancreas, and placenta (Clontech). Nested PCR was performed with the primers (PAB2RTF2/PAB2RTR2) and a 10-fold dilution of the first PCR product as a template. The sequences of the primers were: PAB2RTF1; 5'-GCT

TTCTGCTGTCTCTGCTG-3', PAB2RTR1; 5'-GAAG AGGCTCTGGTCCAGCT-3', PAB2RTF2; 5'-CAGGA GCTGGAGGCCATCAA-3' and PAB2RTR2; 5'-CC TTGGTGGCAAACCTCTATG-3'. All conditions of the first PCR and nested PCR were the same as the conditions of the first and nested PCR to isolate *ePABP2* cDNA except for annealing and extension at 68°C for 90 s of the nested PCR.

Results

Based on the amino acid sequence deduced from the mouse *ePab* cDNA, we identified the human *ePAB* cDNA. Its open reading frame (ORF) is from nt 21 to 1013, encoding the proteins of 330 amino acids. The comparison of the isolated cDNA and the corresponding genomic sequence revealed that the human *ePAB* gene is located on chromosome 20 (20q12-q13.1) and consists of 14 exons encompassing over 29.2 kb (annotated as EU190483 in GenBank). There is 72% homology between human and mouse cDNA at the amino acid level (Fig. 1). In addition, human ePAB has a 61% homology with the human *PABP1* at the amino acid level.

In general, the PABP gene family has four RRM that bind RNA and a C-terminal PABP domain. Mouse *ePab* also contains four RRM and a C-terminal PABP domain; however, human ePAB has only three RRM and lacks PABP domain (Fig. 2). The mouse *ePab* gene has two transcripts. It lacks exon11 by alternative splicing. Conversely, the human *ePAB* gene has only one transcript. The

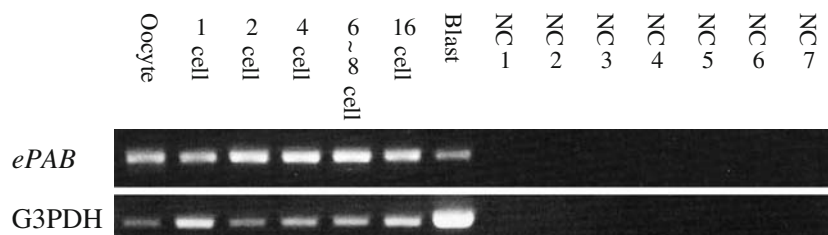


Fig. 4 RT-PCR analysis of human ePAB in human oocytes and preimplantation embryos. NC (negative control) 1: oocyte; NC2: 1 cell embryo; NC3: 2 cell embryo; NC4: 4 cell embryo; NC5: 6–8 cell

embryo; NC6: 16 cell embryo; NC7: blastocyst. G3PDH was used as a positive control (*lower panel*)

Fig. 5 Comparison of amino acid sequences between human (*upper sequence*) and mouse *ePABP2* (*lower sequence*). An amino acid identity of 68% was observed between the two sequences. Vertical lines indicate identical amino acids and asterisks indicate stop codons. The RRM is underlined

human	1	MWPFPSRSLFPPPTQAWLQTVSSDPEAQGWAWNET-KEILGPEGGE--G	50
mouse	1	MEPYLSNELFPPPTTEAWLQTVSSDPEAQGWAWGRTEKTSLVPRAGSRAG	50
human	51	KEEKEEEEDAEEEDQDGDAGFLLSLLEQENLAECPLPNQELEAIKMKVCAM	100
mouse	51	SD-KEAEEEN-E-D---ASFLLSLLEPENLAKSPVFNQELEAIKCLKWAM	100
human	101	EQAEGTPRPPRVQQAAEEEGTAAG-QLLSPETVGCPLSGTPEEKVEADH	150
mouse	101	EHAEAQPEPPCVQRKATEEER-AEVRQLLSPETVDCFFSRTSKENVEADH	150
human	151	RSVYVGNVDYGGSAEELEAHFSRCGEVHRVTILCDKFSGHPKGYAYIEFA	200
mouse	151	<u>RSVFVGNVDYGGSAAELEAYFSPCGEIHRTVILCDKFSGHPKGYAYIEFA</u>	200
		RRM	
human	201	TKGSVQAAVELDQSLFRGRVIKVLPKRTNFPGISSTDRGGLRGHPGSRGA	250
mouse	201	<u>SHRSVKAAVGLDESTFRGRVIKVLPKRTNFPGISSTDRGGLRTHSGNRAA</u>	250
human	251	PFPHSG-LQGRP-RLRPQGQNRARGKFS-P-WFSPY*	300
mouse	251	-FLH-GSLH-RKARLRAHG--RSRGHGGAPQWFSPY*	300

mouse *ePab* is expressed specifically to the mouse ovary; however, the human *ePAB* is not expressed specifically to the ovary but is present in various adult tissues (Fig. 3).

We evaluated the expression pattern of human *ePAB* mRNA in unfertilized oocytes and preimplantation embryos. Total RNA from 70 oocytes or embryos was used for RT reactions; amplification by PCR was then performed by using cDNA from the equivalent of five oocytes or embryos. Mouse *ePab* mRNA was expressed in oocytes, and in one-cell and two-cell embryos. However, it was not expressed in more advanced embryos. Human *ePAB* mRNA could be detected in at all stages, from oocytes to blastocysts (Fig. 4).

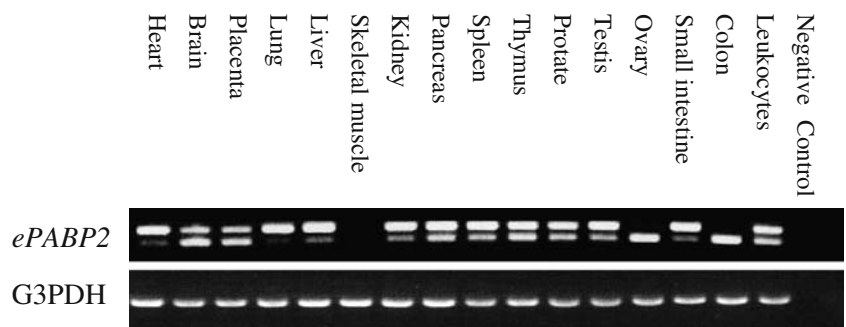
We identified the human *ePABP2* cDNA. The ORF is from nt 13 to 861 and it encodes 282 amino acids (Fig. 5). There is 68% homology between human and mouse cDNA at the amino acid level and the cDNA in the coding region shares a 45% homology with the mouse *ePabp2*. The human *ePABP2* gene is located on chromosome 16q24.3 and consists of seven exons encompassing over 3.2 kb

(annotated as EU190484 in GenBank). This protein has a single RRM similar to that of type II nuclear PABPs. There is 43% homology between human *ePABP2* and human type II nuclear PABP at the amino acid level; however, there is a 75% homology between the RRM. In addition, expression analysis demonstrated that the human *ePABP2* gene is expressed in various adult tissues (Fig. 6).

Discussion

In this study, we identified the human ortholog of *Xenopus* and mouse *ePab*. It encodes 330-aa protein with 72% identity compared to mouse *ePab* amino acids. Mouse *ePab* has a spliced form lacking exon11; however, human *ePAB* has no splicing variant and encodes only three RRM and lacks PABP domain. Typically, PABP has four RRM highly conserved in the N terminus, and PABP domain in the C terminus, which is a variable site [23]. The RRM bind poly(A) and eIF4G as a translation initiation factor;

Fig. 6 Expression patterns of *ePABP2* in 16 human adult tissues. PCR analysis shows non-specific pattern in almost all tissues (*upper panel*). G3PDH was used as a positive control (*lower panel*). In expression patterns of human *ePABP2*, one transcript was observed in the ovary; however, two transcripts were observed in the other tissues



the first two RRM domains are specific to poly(A) binding, and the fourth RRM is critical to the nonspecific RNA binding. The C terminus is involved in the interaction between PABP and PABP; it is then necessary for cooperative binding to poly(A). These two parts are conserved in most PABPs but not seen completely in human ePAB ortholog. Lacking the C terminus may reduce the ability of the PABP domain to associate cooperatively to poly(A) and may have an influence because the C terminus is concerned with mRNA stability, as well as the nuclear export and translational repression of PABP mRNA itself [23].

Mouse embryo gene activation begins at the two-cell stage. Mouse *ePab* is expressed in unovulated oocytes and decreases gradually until the two-cell stage; subsequently, it is not present. In contrast, mouse *Pabp1* (cytoplasmic PABP) expression exhibits a low baseline in oocytes through the four-cell stage; it is elevated at the eight-cell, when it becomes more pronounced in blastocysts [22]. It has been suggested that the shift of the expression pattern from *ePab* to *Pabp1* corresponds to the transition from maternal to embryo gene activation; therefore, mouse *ePab* contributes to the control of maternal gene expression.

In the human, zygotic gene activation (ZGA) occurs at the four to eight-cell stage [24]. The human *ePAB* expression presents in oocytes up to the blastocyst stage; it is especially strong from the two- to eight-cell stage and then decays rapidly at the blastocyst stage. The human *ePAB* is expressed in a nonspecific pattern, which is unlike that of the mouse *ePab*; furthermore, human ePAB is not specific to gonadal tissue. Therefore, one should carefully consider the possibility that, in the human, *ePAB* may be involved in translation activation of maternal mRNA and cleavage division. The human ePAB gene is expressed in various adult human tissues. We currently do not know what possible role it plays in other tissues. Thus, further analysis of human ePAB is indicated, such as using human fetal tissues at the various stages.

Recently, *ePabp2* was identified in *Xenopus* and the mouse; it is related to the known *PABP2* [20, 25]. *PABP2* (*PABPN1* in the human) is a nuclear PABP that directs the polymerization and controls the size of the mRNA poly(A) tail during pre-mRNA processing. This nuclear PABP has both a single RRM and an arginine-rich C-terminal domain that is necessary for the recognition of poly(A). The cytoplasmic new RNA binding protein, *ePabp2*, is expressed in oocytes, early development, and in adult ovarian tissues. It has been suggested that *ePABP2* may also contribute to the regulation of maternal translational activation during early development in a similar manner as *ePAB*. The identified human *ePABP2* encodes 278-aa protein, and has a 68% identity with the mouse *ePABP2*, which encodes 273 amino acids. The sequence identity of

single RRM of human *ePAB* is 74% with the *PABP2* RRM domain greater than any other RRM of known PABPs.

Conclusions

This is the first report on human *ePAB* and *ePABP2* that may relate to cleavage division; however, these substances were not expressed specifically to the ovary, and biological discrepancies exist between the human and the mouse. There is a possibility of the presence of a new human PABP family that is predominantly expressed during oocyte and early embryo development. Further analysis is strongly indicated in regard to the genes that play a role in human embryo cleavage.

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