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Loss of preimplantation embryo resulting from a *Pum1* gene trap mutation

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ABSTRACT

Pumilio is a member of the highly conserved PUF family of RNA-binding proteins that function as a developmental regulator in diverse animal species. Two *Pumilio* genes, *Pum1* and *Pum2*, have been identified in mammals and are found to be involved in sperm development, neuron development as well as human diseases such as neurodegeneration. Generation of animal models disrupting different parts of Pum protein could help to further dissect their physiological function. Here we described characterization and analysis of a mouse line possessing a gene trap mutation of the *Pumilio1* (*Pum1*) gene. Mice homozygous for the mutation ($Pum1^{XE002}$) cannot be recovered in the adult offspring, at birth or at different time points of embryonic development (E18, E14, E12). Careful analysis of preimplantation embryos showed that no homozygous blastocysts could be detected on day 3.5 of gestation. 96-hr in vitro culture of 1-cell embryos either by natural mating or in vitro fertilization between heterozygotes failed to uncover any homozygous blastocysts, suggesting an early loss of homozygous preimplantation embryos. The lack of *Pum1* gene trap homozygotes suggests a role of *Pum1* in very early embryonic development or fertilization. This novel animal model affecting the beginning of embryonic development could help to understand not only the genetic mechanism underlying preimplantation embryonic development but also the translational regulation in development and diseases.

1. Introduction

RNA-binding proteins (RBP) play particularly important roles in a variety of RNA-related cellular processes during development and human diseases [1]. Drosophila Pumilio (Pum) and *Caenorhabditis elegans* FBF (fem-3 mRNA binding factor) proteins are founder members of the evolutionarily conserved family of RNA-binding proteins, known as the PUF (Pumilio and FBF) family of proteins [2]. PUF proteins are found in many eukaryotic organisms, from yeasts to animals and plants [3]. A salient feature of PUF proteins is the presence of a conserved RNA-binding domain called Pum-homology domain (PUM-HD), composed of eight repeats of 36 amino acids [2,4].

Two *Pumilio* related genes are present in the human and mouse genomes, *Pumilio1* and *Pumilio2* [3,5,6]. Both mouse *Pum1* and *Pum2* are involved in sperm development and required for normal male fertility [7,8]. Besides their roles in germ cell development, Pum proteins have also been shown to be involved in dendrite morphogenesis during neuronal development as well as regulation of embryonic stem cells and adipose stem cells [9–12]. Furthermore, Pum proteins are also found to act as critical translational regulators in animal models of neuronal degeneration, Parkinson diseases as well as cancer cell lines, suggesting important roles in diverse human diseases [13–15]. Since loss-of-function mouse model of *Pum1* and *Pum2* have been generated, it will be advantageous to study physiological roles of PUM protein domains by generating mouse mutants disrupting different regions of PUM protein. Gene trap mutation offers an added advantage to allow an easy visualization of endogenous expression profile of the disrupted gene. A *Pum2* gene trap line has been reported but none is established for *Pum1* [8]. Thus we report here the characterization of *Pum1* mutant mice ($Pum1^{XE002}$) generated by a gene trap insertion in N terminal region of the *Pum1* protein.

Abbreviations: Pum, Pumilio; PUF, Pumilio and FBF; hr, hour.

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2. Materials and methods

2.1. Generation of the Pum1 gene trap mouse

Mouse embryonic stem cell (ESC) line, XE002 (strain 129/ola) containing an insertional mutation in *Pum1* was identified from the BayGenomics database [16]. This ESC line contains an insertion of a gene trap vector (pGT1Lxf) that contains a splice-acceptor sequence upstream of the reporter gene, beta-geo (a fusion gene of beta-galactosidase and neomycin). The ESC clone was used to generate chimeric mice as described previously [8]. The *Pum1*^{XE002} mutant mice were then backcrossed for six generation into C57B1/6 inbred background. All the analyses were done on the N5 and N6 mice unless indicated otherwise. All experiments were approved by the Animal Care and Use Committee (ACUC) of Nanjing Medical University and of Northwestern University respectively.

2.2. Mapping of the insertion site and genotyping assays

The genomic insertion site of pGT1Lxf into the *Pum1* gene was determined using a long range PCR method. The insertion site was successfully amplified as part of a 1.2 kbp (kilo base pair) fragment with a forward primer from the 3′end of the intron (GGCAGCTGCTTTCCTAAACA) and a reverse primer from the vector (AACAGAAGAACCCGTTGTGG). The insertion mapped 10433 bp downstream of exon 2 of *Pum1*, and there is a 193 bp 5′end deletion of the vector. And the insertion was verified by Southern blot. Genomic DNA was digested with SacI or SphI and hybridized to both *Pum1* gene specific probe and β-geo probe. We use a common forward primer alongside reverse primers specific to each allele to genotype all offspring. The sequences were as follows: forward, CACCACCATCCAGCTTCTTT; wild type reverse, GGAAGGGGGCTAGAGAGATG; mutant reverse, GCACGCCATACAGTCCTCTT. The product size is 580 and 274 bp respectively. To assay preimplantation embryonic development, we only genotyped blastocysts due to their genotyping reproducibility.

2.3. RT-PCR

Total RNA extracted from adult mouse tissues with Trizol reagent (Invitrogen) was reverse-transcribed using Superscript III First-Strand System for RT-PCR (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instruction. PCR was carried out with the following primers: *Pum1* Exon 2 forward: AGCAAACATCGATGGCCTAC and *Pum1* Exon 3 reverse: TGCTCTGGAAGGGAGAGCTA for wild-type transcripts; *Pum1* Exon 2 forward: AGCAAACATCGATGGCCTAC and pGT1Lxf LacZ1: GATCCCGTCGTTTTACAACGTCG or pGT1Lxf Opt459: CCAACGTAACCTATCCCATTA for chimeric transcripts. β-actin was used as a control.

2.4. Embryo manipulation

Time of fertilization was determined by observation of copulation plugs, and noon of that day was defined as E0.5. Embryos were collected at different stages by tearing the ampulla of oviduct or flushing uteri with M2 medium (Sigma), followed by culturing in M16 medium (Sigma) for the appropriate times.

Individually isolated embryos were then digested for 16 h at 55 °C in 20 μL of lysis buffer (50 mM Tris–HCl, 0.5% Triton X-100, 200 μL/mL proteinase K, pH 8.0), followed by 10 min at 95 °C to inactivate the proteinase K. Lysates were then used for a nested PCR genotyping approach with GGCAGCTGCTTTCCTAAACA and GGAAGGGGGCTAGAGAGATG (forward primer and reverse wild-type primers), or CACCACCATCCAGCTTCTTT and AACAGAAGAACCCGTTGTGG (forward and reverse neomycin resistance gene primers) for the first PCR performed at 95 °C for 2 min followed by 25 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Primers CACCACCATCCAGCTTCTTT and GGAAGGGGGCTAGAGAGATG (forward primer and reverse wild-type primers), or CACCACCATCCAGCTTCTTT and GCACGCCATACAGTCCTCTT (forward and reverse neomycin resistance gene primers) were used for the second amplification using the same condition. β-galactosidase expression was visualized by staining with 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-Gal) as described.

2.5. Western blotting

Protein extracts from the testis were fractioned by SDS-PAGE and immunoblotted onto nitrocellulose. The blots were incubated in a 1:2000 dilution of the Primary anti-Pum1 antibody (Bethyl Lab) at 4 °C overnight. Horseradish peroxidase-conjugated Rabbit anti-goat secondary antibodies (Bio Rad) were used at a 1:2000 dilution and the blots were developed using the Supersignal West Pico Kit (Pierce, Rockford, IL, USA).

3. Results

3.1. Generation of a mouse gene trap mutation (*Pum1*^{XE002}) disrupting Pum1 gene

In order to establish a gene trap line for mouse *Pum1*, we searched BayGenomics gene trap ES cell database and identified ESC line XE002 containing an insertion in *Pum1* gene [16]. We generated several chimera via blastocyst injection of the gene trap ES cell line XE002 and achieved germline transmission from multiple chimera mice.

The insertion site was confirmed inside the intron between exon 2 and exon 3 by Southern blot using probes specific to *Pum1* genomic region (Fig. 1A, B). The precise location of the XE002 genomic insertion was further mapped via long range PCR using primers specific to the exon 2 or 3 and gene trap vector. Sequence analysis showed that the gene trap was inserted about 10-kb downstream of exon 2 of the *Pum1* gene, leading to a new splicing product composed of exon 1, exon 2 and the β-galactosidase gene (Fig. 1A). RT-PCR was used to confirm the presence of a fusion mRNA transcript in heterozygote, validated by sequencing. A forward primer on the *Pum1* exon 2, upstream of the insertion site, and two different reverse primers on the β-geo gene amplified the fusion transcript in heterozygotes only (Fig. 1D). Based on the sequence information, we developed a PCR genotyping strategy to distinguish all three genotypes. The PCR genotyping method was confirmed by sequencing of both wild-type and mutant PCR products and by Southern blot (Fig. 1B, C). We next examined the expression of chimeric fusion protein by western analysis. Because there is only 2 KDa difference between the Pum1 wild-type protein and mutant chimeric protein, we cannot distinguish between wild-type Pum1 protein and mutant Pum1 protein. But we used anti-LacZ antibody to confirm the absence of β-geo protein in the wild-type testis and the presence of Pum1-β-geo fusion protein of about 129 KDa in the heterozygous testis (Fig. 1E). These data clearly established a gene trap mutation disrupting Pum1 protein in the beginning of N-terminal, resulting in a novel chimeric Pum1-β-geo protein. Southern blot using β-geo probe confirmed that the gene trap insertion in the *Pum1* locus is the only β-geo vector present in the entire genome (Fig. 1B). Hence we have established a mouse mutant with a single insertional mutation in *Pum1* locus.

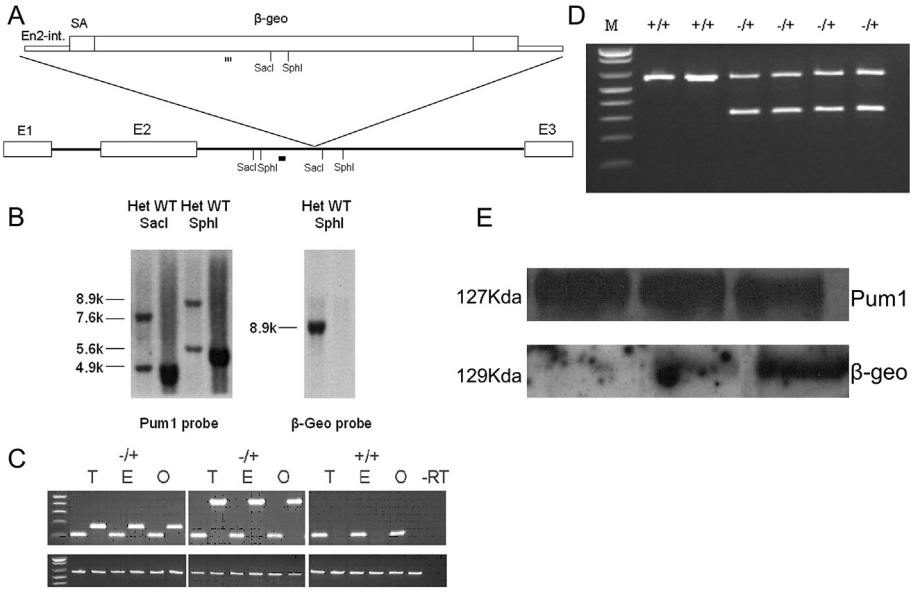


Fig. 1. Generation of a murine *Pum1* gene trap mutation. (A) Schematic representation of the gene trap insertion into the *Pum1* gene between exon 2 and exon 3. Short linear bar and black bar show the β-geo probe and Pum1 probe used for Southern blot. Exons are represented as boxes. SA, splice acceptor; β-geo, β-galactosidase–neomycin resistance cassette. (B) Southern blot containing DNA isolated from postnatal pups of heterozygous parents. DNA was digested with SacI or SphI and hybridized with the probes shown in A (black bar). The expected fragments generated by SacI are 4.9 kb for the wild-type allele, and 7.6 kb for the mutant allele. For SphI digestion, the expected fragments are 5.6 kb for the wild-type, and 8.9 kb for the mutant allele. β-geo probe recognized a 8.9 kbp fragment when digested with SphI. (C) Characterization of Pum1 mRNA transcript. Total RNA was extracted from adult testis (T), epididymis (E) and ovary (O) of wild-type and *Pum1* heterozygous mice. Two different pairs of primers were used to amplify the mutant transcript. Wild-type and mutant RTPCR products were run side by side for each sample. β-actin was used as a control. (D) PCR genotyping analysis to distinguish wild-type allele (580 bp) and mutant allele (274 bp). (E) Confirmation of Pum1- βgeo fusion protein. Testis lysates from wild-type and heterozygous mice were first blotted with anti-Pum1 antibody, and then reprobed with β-geo antibody. The Pum1 antibody can only detect one band because the 127 kDa Pum1 protein co-migrates with the 129 kDa fusion protein.

3.2. Absence of preimplantation embryos of $Pum1^{XE002}$ homozygous mutation

Heterozygous $Pum1^{XE002/+}$ mice were apparently normal, healthy and fertile with no detectable developmental abnormalities over a 2-year observation period. Both males and females can transmit the mutant allele to their progeny (Table 1). In contrast, no viable homozygous $Pum1^{XE002/XE002}$ mice were recovered out of 364 offspring from heterozygous intercrosses (Table 1), indicating that homozygosity of the $Pum1^{XE002}$ mutation may result in embryonic lethality. However an analysis of embryos from different time points of pregnancy (E18.5, E14.5 and E12.5) revealed that none of the embryos were $Pum1^{XE002/XE002}$. To determine the developmental stage at which $Pum1^{XE002/XE002}$ may cause death, blastocysts were isolated from heterozygous crosses and genotyped by PCR. We developed a nested PCR method to genotype blastocyst embryos and confirmed the PCR products by sequencing. Fig. 2A showed blastocyst genotyping result from one litter of embryos from heterozygote mating and one from wild-type mating respectively. Of 83 embryos (E3.5) examined in total, none are homozygotes (Table 1). We also noticed the underrepresentation of wild-type embryos in the litters from heterozygote mating (Table 1).

To exclude the possibility of PCR genotyping error, we performed LacZ staining, which will identify heterozygous or homozygous embryos but not wild-type embryos. We examined 23 blastocysts by LacZ staining followed by PCR genotyping (Fig. 2B). The PCR genotyping result is consistent with LacZ staining; all LacZ positive embryos are heterozygotes by PCR genotyping. No homozygote was found by PCR. Though there is an intensity difference in LacZ staining among LacZ positive heterozygous embryos, such difference may reflect *Pum1* expression difference resulting from a small difference in developing time of the embryos. These data indicate that $Pum1^{XE002/XE002}$ embryos are absent at the blastocyst stage.

Intriguingly we found that the ratio of $Pum1^{+/+}$ to $Pum1^{XE002/+}$ animals from heterozygous intercrosses was substantially less than

Table 1
Mouse breeding and genotyping data.

Cross	Age	No. of litter/total no. of mice	No. of mice with indicated genotype			P value*
			WT (+/+)	Het (−/+)	Hmz (−/−)	
(+/−) ♀x (+/−) ♂	Postnatal	53/364	59	305	0	0.000
	E18.5	1/7	1	6	0	0.286
	E14.5	2/12	1	11	0	0.066
	E12.5	1/9	0	9	0	0.034
	E3.5	12/83	1	82	0	0.000
	E3.5 LacZ staining	4/23	1	22	0	0.003
(+/−) ♀x (+/+) ♂	Postnatal	10/63	25	38	0	0.101
(+/+) ♀x (+/−) ♂	Postnatal	16/119	42	77	0	0.001

WT, wild-type; Het, heterozygote; Hmz, homozygote. Probability of a high ratio of heterozygote to wild-type determined by a χ^2 test. *, $P < 0.05$.

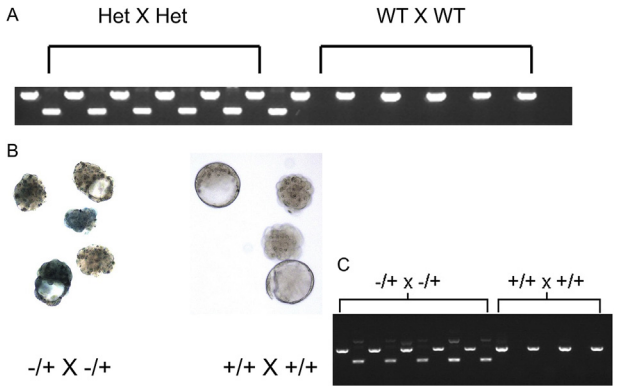
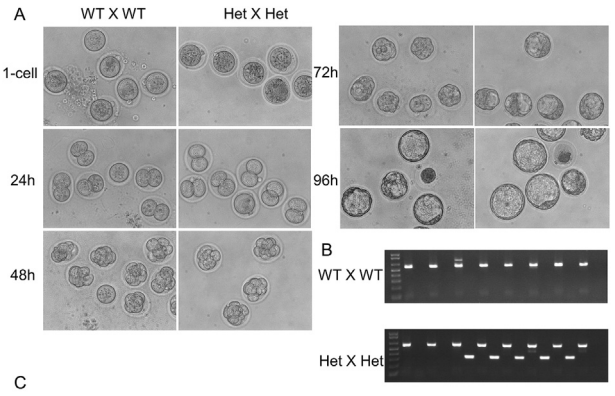


Fig. 2. Blastocyst genotyping by PCR and LacZ staining. (A) Genotyping of blastocysts from both heterozygote intercrosses and wild-type intercrosses. The 580- and 274-bp bands identify the wild-type and mutant alleles, respectively. Blastocysts were used for a nested PCR as described in MM. (B) LacZ staining of blastocysts from heterozygote intercrosses and wild-type intercrosses. (C) Blastocysts were used for genotyping after LacZ staining, Wild-type and mutant genotyping PCR product were run side by side for each sample.



Cross	No. of litter/total no. of embryos	No.of embryos with indicated genotype				P value*
		WT(+/+)	Het (-/+)	Hmz (-/-)	ND*	
(+/-)♀x (+/-)♂	15/110	18	88	0	4	0.000
(+/+)♀x (+/+)♂	4/34	30	0	0	3	-

WT, wildtype; Het, heterozygote; Hmz, homozygote Probability of a high ratio of heterozygote to wildtype determined by a χ^2 test. *, P< 0.05. *ND, not determined.

Fig. 3. In vitro development and PCR genotyping of 1-cell embryos from heterozygote intercrosses and wild-type intercrosses. (A) Microscopic monitoring of 1-cell embryos to blastocysts. Pictures were taken every 24 h. 1-cell embryos were isolated and cultured for up to 96-h. Each litter of embryos was cultured individually and monitored every 24 h. After 96-h in vitro culture, blastocysts were collected for PCR genotyping. (B) The genotypes of each litter of embryos after 96-h culture were determined by nested PCR. Here is the genotyping result of embryos shown in A. Wild-type and mutant genotyping PCR product were run side by side for each sample. (C) Analysis of genotypes of in vitro cultured embryos from both heterozygote intercrosses and wild-type intercrosses.

the predicted 1:2 Mendelian ratio. We first noticed over-representation of heterozygotes among our initial batch of adult animals from heterozygote interbreeding as we recovered 93 heterozygotes (83%) and 19 wildtype (17%) out of 112 offspring. The skewed ratios among males or females were similar to that in the combined population. A larger than expected proportion *Pum1*$^{XE002/+}$ mice was also evident when combining all postnatal pups (a total of 364), post-implantation and pre-implantation embryos (Table 1). Underrepresentation of wild-type is consistent with our LacZ staining of E3.5 blastocyst (only 1 out of 23 is LacZ-negative). We then asked if the transmission of mutant alleles and wild-type alleles was skewed from heterozygotes by examining the genotypes of offspring from heterozygotes and wild-type backcrossing. There indeed appears to be more mutant alleles transmitted from both male and female heterozygotes than wild type, but over-representation of the mutant allele among offspring is only statistically significant for heterozygote males (Table 1).

3.3. Absence of embryos homozygous for the *Pum1*$^{XE002/XE002}$ as early as zygote stage

The failure in recovery of *Pum1* homozygotes suggests that *Pum1* is required for very early embryogenesis. To further define the defects in preimplantation development of *Pum1* homozygous embryos, one-cell embryos from both heterozygote intercrosses and wild-type control were isolated and cultured in vitro to blastocyst stage. To assess the time of preimplantation developmental failure, we monitored embryos at different periods of in vitro culture (Fig. 3A). During 96-h culturing, there is no significant difference in the percentage of abnormal embryos or arrested embryos in both groups. Some of them developed into morula, but most of embryos developed into normal blastocysts, as confirmed by the formation of distinct inner cell mass, blastocoel cavity and trophoblasts. All embryos were then collected individually and used for genotyping. Among 110 embryos genotyped, however, no homozygotes were detected (Fig. 3B, C), which suggested a lack of *Pum1* homozygous embryos from one cell embryo stage. Furthermore, we performed in-vitro fertilization experiment with sperm and egg collected from heterozygotes, and identified 74 embryos, which develop to blastocyst stages. The percentage of embryo developing from 2-cell to blastocyst is similar between heterozygote mating and wild-type mating. Genotyping show none of the blastocysts are *Pum1*$^{XE002/XE002}$, lending further support to the early loss of *Pum1*$^{XE002/XE002}$ preimplantation embryos (Table 2). Further investigation is necessary to pinpoint the exact stage of such embryo loss and to ascertain if *Pum1*^{XE002} sperm and egg may be defective in fertilization.

4. Discussion

In this study, we reported the gene trap disruption of mouse *Pum1* gene and the phenotypic characterization of this mouse model. We found that this new *Pum1* mutant mice homozygous for the *Pum1*^{XE002} mutation cannot be recovered among postnatal offspring or developing embryos. No homozygous blastocysts were detected either on day 3.5 of gestation or after 96-hr in vitro culture of 1-cell embryos.

Failure to recover homozygotes postnatally and at different embryonic development time points led us to investigate when *Pum1*^{XE002} homozygotes die (Table 1). We collected 1-cell embryos from heterozygote mating and followed their development in vitro until blastocyst stage. Again we couldn't detect any homozygotes, nor did we observe any significant increase in arrested embryos (Fig. 3). Furthermore when we use sperm and egg directly from heterozygotes to perform in-vitro fertilization, we observed similar phenomena, prompting us to ask what might cause such an early loss of homozygous preimplantation embryos. First, our PCR genotyping method somehow may miss homozygotes. This PCR genotyping method was developed to distinguish different genotypes. The inner primer pair used in this nested blastocyst PCR genotyping method is the same one as the one used in mice offspring genotyping. And the genotyping result has been validated by Southern blot, LacZ staining and PCR product sequencing (data not shown). Given the potential fluctuation due to small amount of DNA from blastocysts, we performed at least two-time PCR on every blastocyst with appropriate controls to make sure consistency of

Table 2
In vitro fertilization data.

Cross	No. of experimental times	No. of oocyte	No. of 2-cell	No. of blastocyst	No. of blastocyst genotyped	Genotype		
						−/+	+/+	−/−
(+/−) ♀x (+/−) ♂	16	751	125	74	69	62	7	0
(+/+) ♀x (+/+) ♂	4	123	26	10	10	0	10	0
(+/−) ♀x (+/+) ♂	1	27	5	5	5	3	2	0

genotyping. So we donot believe that the absence of homozygotes could be attributed to our PCR genotyping method. Second, we may miss the homozygous 1-cell embryos in the oviduct. Zygote collection is now a routine procedure. Usually the oviduct was removed soon after fertilization and the zygotes surrounded by cumulus (follicle) cells can be seen in the swollen upper part of the oviduct, the ampulla, and can be released by tearing the ampulla with fine forceps. So it's unlikely we would miss the homozygous embryos unless those homozygous embryos are released from the cumulus cells and reside in the oviduct separately. Because of this concern we also tore open the whole oviduct but never found any dissociated embryos. While we can't completely rule out this possibility, the chance that we missed the homozygous embryos in dissection is very small. Third, homozygote embryos may die during the development from two cell to blastocyst stage. Although we did not detect any significant increase in the number of arrested 2-cell embryos, it remains a strong possibility that homozygotes die from two-cell to four-cell embryos since our genotyping method only allowed reliable genotyping of blastocysts but not of earlier 1-cell or 2-cell embryos. Direct genotyping on two-cell embryos from in vitro culture embryos may provide a definitive answer to this question. Alternatively, a defect in sperm and egg fusion or fertilization process may prevent the formation of *Pum1* homozygous zygotes. At least one copy of wild-type *Pum1* may be needed for the sperm-egg recognition during fertilization. This fertilization defect hypothesis may sound tantalizing when we consider the intriguing departure from Mendelian ratio of wildtype and heterozygote offspring from heterozygote interbreeding. While we could not explain what might cause overrepresentation of heterozygotes among adult offspring and blastocysts, an effect of Pum1 mutant allele on fertilization could be relevant for this phenomena. This, however, is not compatible with our current understanding, as mutant gametes should be similar to wild type gametes from heterozygotes since haploid gametes receive similar cytoplasmic content during maturation regardless of their genetic makeup. Our backcross data showed that both mutant sperm and egg could be transmitted, making detrimental paternal or maternal effect on embryonic development unlikely (Table 1). Hence our current data could not resolve the exact stage when the loss of homozygote takes place, but the loss of homozygotes at the very early embryonic development, likely right after zygotic genome activation, may seem to be a more plausible cause. Indeed *Pum1* gene was recently reported to regulate the differentiation of embryonic stem cells, consistent with a role of Pum in early embryonic development [18].

A loss-of-function of *Pum1* was reported to be viable but produce reduced number of sperm [7], so it is unexpected that this *Pum 1* gene trap mutation, which also should disrupt Pum1 protein, failed to produce any homozygous zygotes. This raises a possibility that $Pum1^{XE002}$ may be a gain-of-function mutation that specifically affects preimplantation embryonic development or fertilization. This was supported by our experiment with another *Pum1* gene trap mutation we generated, $Pum1^{XB063}$. We also failed to recover any postnatal homozygotes for $Pum1^{XB063}$ (unpublished data). It is not surprising that this highly conserved RNA-binding protein can control diverse processes. Previous reports from different species supported *Pumilio* as a conserved translational regulator important for diverse developmental processes. Drosophila Pumilio functions in anterior/posterior patterning of the early embryo, primordial germ cells, mitotic arrest, migration and germline stem cells maintenance [19–24]. *C. elegans* FBF regulates the germline switch from spermatogenesis to oogenesis and germline survival [25,26]. On the one hand, Pumilio can have multiple functions, control different developmental processes directly or indirectly by regulating different mRNAs [2]. On the other hand, Pumilio proteins could interact with additional 3′UTR-binding proteins to assemble distinct protein complexes, which might influence regulation of different mRNA sequences and lead to different biological outcomes [2]. Understanding of such complex regulation required assembly of a RNA-protein and protein–protein network and generation of animal models disrupting various domains of Pum proteins in addition to its RNA binding domain. Our characterization of the newly established *Pum1* gene trap mutant suggests that Pumilio might play a role in mammalian embryonic development, further experiments are needed to illustrate the roles of Pum protein-mediated translational control during embryonic development.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Transparency document

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