Embryo implantation failure and other reproductive defects in *Ube2q1*-deficient female mice

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Abstract

The ubiquitination process is indispensable for proteome regulation. Three classes of ubiquitin (Ub)-related proteins can be distinguished: E1, E2 and E3. Proteins from the E2 class are responsible for the transfer of Ub from E1 to the target protein. For this activity, interaction with class E3 ligases is usually required. Ub-conjugating enzyme E2Q 1 (UBE2Q1) belongs to the E2 class of Ub-related enzymes and is demonstrated to be involved in the regulation of membrane B4GALT1 protein. Here, we demonstrate that human *UBE2Q1* and mouse *Ube2q1* are widely expressed and highly conserved genes. To elucidate the function of UBE2Q1 protein, we generated knockout mouse model. No overt phenotype was detected in UBE2Q1-deficient males, but in mutant females, pleiotropic reproductive defects were observed including altered oestrus cycle, abnormal sexual behaviour and reduced offspring care. Moreover, in the uterus of mutant females, significantly increased embryonic lethality and decreased implantation capacity of homozygous mutant embryos were noticed. We found that *Ube2q1* is not expressed in the uterus of non-pregnant females but its expression is up-regulated during pregnancy. Taken together, *Ube2q1* is involved in different aspects of female fertility.

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Introduction

Ubiquitination is involved in selective protein degradation, which is an important part of cellular proteome regulation. The essential step in this process is covalent binding of ubiquitin (Ub) or Ub-like proteins (UbLs) to the substrates (for review, see Dye & Schulman (2007)). The way Ub and UbLs are processed and attached to the target protein is highly conserved, three-step process, involving the subsequent participation of activating enzymes (E1), conjugating enzymes (E2) and ligase activity (E3) (Pickart & Eddins 2004). The central role in this cascade is assured by members of the E2 family, which consists of dozens of proteins identified in higher eukaryotes (Hofmann & Pickart 2001, Burroughs *et al.* 2008). E2 enzymes drive the specificity of Ub or UbLs conjugation and thus determine the fate of targeted proteins (reviewed in van Wijk & Timmers (2010)). A characteristic feature of this family is the presence of a 150–200 amino acid Ub-conjugating domain that serves as a binding site for E1s, E3s and Ub/UbLs (Hofmann & Pickart 2001, Burroughs *et al.* 2008, van Wijk & Timmers 2010).

Increased ubiquitination was reported in the uterus during embryo implantation (Bebington *et al.* 1999a, 1999b, 2000, 2001); thus, the proper control and turnover of the key signalling protein activity seems to play critical role for initiation of pregnancy. This might also be emphasized by the observation that Ub is accumulated in trophoblast of expanding bovine and mouse blastocysts (Sutovsky *et al.* 2001). The 26S proteasome, which regulates the proteolytic activity of the matrix metalloproteinases (MMPs)-2 and -9, is required during implantation process (Wang *et al.* 2004). The important function of other ubiquitination-related proteins such as the Ub-conjugating enzyme UBCM4 (Harbers *et al.* 1996), the proteasome activator PSME3 (PA28γ; Murata *et al.* 1999), Ub ligase CUL7 (Huber *et al.* 2005) and FBXW8 (Tsutsumi *et al.* 2008) was demonstrated by the phenotype of mouse models with placenta and intrauterine embryonic growth retardation. The *Smurf2* gene, encoding E3 class protein, is involved in trophoblast cell invasion and embryo implantation (Yang *et al.* 2009). β-Catenin ubiquitination through two E3 ligases, BTRC and CACYBP, plays an important role during decidualization (Herington *et al.* 2007). Also, E2s

were demonstrated to be involved in the implantation process. Rabbit *Ube2q2* (also known as *UBCi*) expression is up-regulated in the endometrial epithelial cells by the embryo implantation event (Melner *et al.* 2004). The up-regulation of E2 conjugating enzymes from the 1 to 8 protein families was detected in the uterus of pregnant bovine (Pru *et al.* 2001).

Human *UBE2Q1* was identified among other genes related to epidermal differentiation complex (EDC) and epidermis maturation by screening of keratinocyte cDNA library (Marenholz *et al.* 2001). Human and mouse *UBE2Q1* proteins share 98% identity and both contain a consensus sequence for Ub binding at its C-terminus. It has been demonstrated that mouse *UBE2Q1* is an active E2 class Ub-conjugating enzyme. The N-terminal end of this protein is critical for the binding and internalization of UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 1 (*B4galt1*, also known as *GalT1*) (Wassler *et al.* 2008). The authors detected the expression of *Ube2q1* in the inner cell mass (ICM) and in the trophectoderm cells of early blastocyst. To elucidate the role of this gene in mammals, we generated a knockout mouse model in which the *Ube2q1* was deleted by homologous recombination. We discovered that this gene plays a role in female fertility and embryo implantation process.

Results

Expression pattern and evolutionary conservation of human *UBE2Q1* and mouse *Ube2q1*

In order to study the expression of human *UBE2Q1* gene, total RNA isolated from different tissues was hybridized with ³²P-labelled 1 kb cDNA fragment containing exons 5–13. Two transcripts, 2.4 and 4 kb, were detected in all the analysed tissues. RNA quality was proven by membrane rehybridization with human *ACTB* probe (Fig. 1A). It was published that the *Ube2q1* gene is highly conserved among many species (Schulze *et al.* 2003, Wassler *et al.* 2008). Therefore, we used human *UBE2Q1* cDNA probe to hybridize with RNA isolated from testis or brain from different mammals and one bird species. The human probe detected single transcript in RNA isolated from bovine, pig, goat, rabbit (2.4 kb) and pigeon (2.2 kb) and two transcripts in mouse, rat (2.4 and 4 kb) and rhesus (2.2 and 2.4 kb) (Fig. 1B). Next, we have analysed the expression of *Ube2q1* in mouse tissues by RT-PCR. Using AltRTPCRF and AltPCRRP primers, a 467 bp product was amplified in all the tissues analysed except the uterus of non-pregnant female. To prove primer specificity, negative control without reverse transcriptase was used. The cDNA quality was verified using *Hprt*-specific primers (Fig. 1C). These results indicated that human and mouse *UBE2Q1/Ube2q1*-genes are expressed in many tissues and are highly evolutionary conserved.

Figure 1 Analysis of ubiquitin-conjugating enzyme E2Q family member 1 (*Ube2q1*) expression. The expression of human *UBE2Q1* was analysed in different tissues by northern blot with ³²P-labelled 1 kb cDNA fragment containing exons 5–13 as a probe (A). 2.4 and 4 kb transcripts were detected in all tissues analysed (upper panel). The RNA quality was proven by membrane rehybridization with human *ACTB* probe (lower panel). (B) The same cDNA probe was used to detect the *Ube2q1* transcript in RNA isolated from brain or testis of different species. One transcript was observed in RNA isolated from bovine, pig, goat, rabbit (2.4 kb) and pigeon (2.2 kb), and two transcripts were present in mouse, rat (2.4 and 4 kb) and rhesus (2.2 and 2.4 kb) RNAs. (C) The expression of mouse *Ube2q1* was analysed using RT-PCR. The 467 bp PCR product was amplified in all tissues analysed except for the uterus of non-pregnant female. cDNA quality was demonstrated using the *Hprt*-specific primers.

Generation of the *Ube2q1*-deficient mouse

To determine the function of *Ube2q1*, we used a gene targeting approach to generate homozygous *Ube2q1*^{−/−} null mice. Mouse *Ube2q1* gene was deleted by homologous recombination with the knockout vector containing neomycin (neo) and thymidine kinase cassettes. After electroporation of the knockout vector into ES cells, exons 5–11 of *Ube2q1* were replaced by the neo cassette (Fig. 2A). ES clones harbouring recombinant allele were identified using Southern blot analysis (Fig. 2B). The 5' external probe detected a 13 kb EcoRI fragment of the recombinant allele and an 8 kb EcoRI fragment of the wild-type allele. Chimeric mice were generated by aggregating 10–15 compact ES cells,

Figure 2 The strategy of targeted disruption of the mouse *Ube2q1* gene. (A) Schematic structure of the wild-type and recombinant *Ube2q1* alleles is shown. Relevant restriction sites (Xh-XhoI; E-EcoRI and Xb-XbaI) and applied primers (gM7F, gM8R and Pgk3) are given. The knockout strategy was designed to replace exons 5–11 with the *Pgk1/neo* cassette. The 5.0 kb long genomic XhoI fragment and 4 kb long XbaI fragment of mouse *Ube2q1* gene were used as 5' and 3' homologue sequence in the knockout vector respectively. (B) Genomic DNA from electroporated ES cell clones was digested with EcoRI enzyme and probed with 32 P-labelled 5' external probe. Southern blot analysis shows an 8 kb band representing the wild-type *Ube2q1* allele (*Ube2q1* WT) and a 13 kb band for the recombinant allele (*Ube2q1* rec.). (C) The F2 generation was genotyped by Southern blot and PCR using genomic DNA isolated from mouse tails. In the Southern blot, WT and recombinant allele were detected as describe earlier. In the PCR assay, a product of 498 bp was amplified from the wild-type *Ube2q1* allele and a 308 bp product from mutated *Ube2q1* allele. +/+ : wild type, +/- : heterozygous, -/- : mutant homozygous. (D) The expression of *Ube2q1* was analysed in the knockout mice using northern blot and cDNA fragment containing exons 4–9 of *Ube2q1* as a probe. In testis of *Ube2q1*^{-/-} mutants, no expression of *Ube2q1* gene was detected, and in *Ube2q1*^{+/-} animals, the signal was weaker than that in *Ube2q1*^{+/+} (upper panel). RNA integrity and quantity were monitored by ethidium bromide staining (lower panel).

carrying the homologous recombinant allele, with two 2.5-day-old embryos of the CD1 mouse strain as described previously (Nagy & Rossant 1993). Male chimaeras were mated with 129/Sv females to establish the knockout line on 129/Sv genetic background. Heterozygous animals were identified by PCR using gM7F, gM8R and Pgk3 primers (Fig. 2A) and crossed to obtain homozygous mutants. The genotype of the first pups from the heterozygous breeding was determined by Southern blot and PCR (Fig. 2C), while the genotype of all the following offspring was determined by PCR only. To confirm the null mutation in the *Ube2q1* locus, northern blot with testicular RNA from wild-type (*Ube2q1*^{+/+}), heterozygous (*Ube2q1*^{+/-}) and homozygous (*Ube2q1*^{-/-}) animals was hybridized with mouse cDNA probe containing exons 4–9 of the *Ube2q1*. No expression of *Ube2q1* gene was detected in RNA from *Ube2q1*^{-/-} mutants, while in *Ube2q1*^{+/-} animals, the expression level was weaker than that in *Ube2q1*^{+/+} testis. The quality and integrity of the RNA samples were visualized by ethidium bromide staining (Fig. 2D). Furthermore, the expression of *Ube2q1* was also analysed in many other tissues of *Ube2q1*^{-/-} male and female, but no signals were detected (data not shown).

The phenotype of *Ube2q1*^{-/-} mice

Heterozygous males and females did not demonstrate any overt phenotype, the fertility was normal and the litter size was not significantly different from wild-type breeding (Fig. 3A). No significant difference in spermatozoa number, sperm morphology and motility was observed between homozygous mutant and wild-type males, and sperms of *Ube2q1*^{-/-} animals undergo normal acrosome reaction (data not shown). In contrast, homozygous mutant females demonstrated pleiotropic reproductive defects. They needed markedly longer mating time to become pregnant with either wild-type or homozygous mutant males when compared with *Ube2q1*^{+/+} or *Ube2q1*^{+/-} females. While wild-type females gave birth to their first litter in 8th–9th week of age, the majority of *Ube2q1*^{-/-} females had their first litter much later, namely around 10th–12th week of age.

To further elucidate the effect of *Ube2q1* deletion on the fertility, different types of breeding were established and analysed. The litter size of *Ube2q1*^{-/-} females, which were mated with wild-type or homozygous mutant males, was significantly reduced comparing with wild-type breeding ($P < 0.001$, Fig. 3A). When

Figure 3 Phenotype of *Ube2q1*^{-/-} mutants. The litter size was significantly reduced when the *Ube2q1*^{-/-} females were mated with wild-type or homozygous mutant males, when compared with wild-type or heterozygous breeding (A). The litter size of homozygous mutant male mated with wild-type female was significantly lower compared with wild-type breeding ($P=0.03$); however, it was not significantly different from heterozygous or heterozygous and wild-type pairs ($P>0.05$). (B) In the breeding of homozygous females, the number of pups that survived the 30 days *post partum* was significantly reduced when compared with any other type of breeding. (C) Ten *Ube2q1*^{-/-} and three *Ube2q1*^{+/+} females were subjected to vaginal smear examination to determine the oestrus cycle. The filled box marked with X represents the day when oestrus was noticed. On an average, in *Ube2q1*^{+/+} females, oestrus was detected every 5 days, while in *Ube2q1*^{-/-} females, oestrus was detected every 11–12 days. (D) Five *Ube2q1*^{-/-} and five *Ube2q1*^{+/+} females were mated with vasectomized males and vaginal plugs (VP) were controlled daily for a period of 60 days. Regular mating, indicated by the presence of VP (filled, X-marked boxes), was observed in wild-type females. The mutant females demonstrated irregular mating behaviour and no average mating frequency could be estimated. **Significance level with $P<0.001$.

homozygous mutant male was mated with wild-type female, the litter size was slightly but significantly lower than that in the wild-type breeding pairs ($P=0.03$) but was not significantly different from heterozygous or heterozygous and wild-type breeding ($P>0.05$, Fig. 3A, Supplementary Table 1, see section on supplementary data given at the end of this article). The weight of pups born by all kinds of breeding pairs was recorded at the morning, when the litter was noticed in the cage. The weight of newborns was not significantly different between homozygous mutant, heterozygous and wild-type crosses (1.6 ± 0.09 , 1.8 ± 0.25 and 1.8 ± 0.31 g respectively, $P=0.23$). However, most of the pups of homozygous mutant females died within the first 48 h after parturition; thus, the number of animals surviving the first 30 days was reduced (Fig. 3B). Dead pups were dehydrated and no milk was observed in their stomach. This observation indicated that homozygous mutant females were not able to properly feed their pups. Histological analysis of the mammary gland did not

reveal any abnormalities in homozygous mutant females (data not shown). It should be noticed that the weight of pups from homozygous mutant breeding, which survived 30 days after birth, was not significantly different from the weight of pups born to wild-type animals at the same age (data not shown). It is well known that hormones are controlling many aspects of mother-offspring relations and lactation; therefore, we analysed the serum prolactin (PRL) level. Blood of eight *Ube2q1*^{-/-} and five *Ube2q1*^{+/+} females was collected 1 day after parturition (lactation day 1). The PRL concentration was significantly lower in homozygous mutant females when compared with that in wild-type animals (2.2 ± 0.4 vs 11.6 ± 6.9 ng/ml respectively, $P < 0.001$). This finding suggested that the hormonal homeostasis might be impaired in homozygous mutant females; therefore, the oestrus cycle was analysed. Ten *Ube2q1*^{-/-} and three *Ube2q1*^{+/+} females were subjected for vaginal smear examination. The smear was taken daily at 1400 h for a period of 20 days.

As demonstrated in Fig. 3C, oestrus was detected every 5 days in wild-type females. In contrast, mutant females demonstrated prolonged dioestrus phase and oestrus was detected approximately every 11–12 days. To check whether the prolonged oestrus cycle affects the sexual behaviour, five *Ube2q1*^{-/-} females were mated with vasectomized males and vaginal plug (VP) was controlled daily for a period of 60 days. In a control group, wild-type females (*n*=5) mated with vasectomized males regularly, on an average every 11–12 days. In contrast, homozygous mutant females revealed irregular mating (Fig. 3D). The mating frequency of mutant females was highly differentiated between females, and even the same female demonstrated irregular mating frequency during the time of observation. No average value could be estimated from the mating frequency for homozygous mutant females. These results indicate the disturbance of the oestrus cycle and sexual behaviour in *Ube2q1*^{-/-} females.

Ovulation is not affected in *Ube2q1*^{-/-} females

The impaired oestrus cycle in *Ube2q1*^{-/-} females might explain why they needed longer time to become pregnant and why many copulations did not result in pregnancy. However, this phenotype could not be a reason for the reduced litter size of homozygous mutant females. To elucidate the underlying cause of the reduced litter size, *Ube2q1*^{-/-}, *Ube2q1*^{+/-} and *Ube2q1*^{+/+} females were mated with vasectomized males and the number of ovulated oocytes was determined at the day of VP. No significant difference was noticed in the number of ovulated oocytes of *Ube2q1*^{-/-} when compared with *Ube2q1*^{+/-} or *Ube2q1*^{+/+} females (Table 1). Next, we analysed the response for the hormonal treatment in mutant females. No significant difference in the number of oocytes isolated from superovulated homozygous mutant and control females was observed (Table 1). These results demonstrate that ovulation is not affected in *Ube2q1*-deficient females.

Table 1 Number of ovulated oocytes of *Ube2q1* knockout mice.

Female genotype	No. of oocytes after mating with vasectomized male		No. of oocytes after superovulation	
	Mean ± S.D.	<i>n</i>	Mean ± S.D.	<i>n</i>
<i>Ube2q1</i> ^{+/+}	9.0 ± 0.8	4	38.7 ± 5.8	3
<i>Ube2q1</i> ^{+/-}	7.7 ± 1.3	4	NA	
<i>Ube2q1</i> ^{-/-}	8.0 ± 1.1	6	42 ± 1.4	3

Oocytes were isolated from VP-positive females after mating with vasectomized males. No significant differences were observed between wild-type, heterozygous and homozygous females (*P*>0.05). Similarly, no significant difference in oocytes number after superovulation was observed between wild-type and homozygous mutant females (*P*>0.05). Data are given as a mean number of oocytes per female ± S.D. NA, not analysed; *n*, number of analysed animals.

Impaired implantation of *Ube2q1*-deficient embryos

We analysed the progress of embryonic development in the uteri of mutant females. Homozygous knockout females were mated with wild-type and homozygous knockout males. As a control, wild-type males and females were mated. After VP examination, females were killed at the gestation day 5.5, 7.5, 10.5 and 17.5, and the number of embryos was recorded. As demonstrated in Fig. 4A, a significant reduction in the number of implanted embryos was observed at E5.5, E7.5 and E10.5 in the uteri of homozygous mutant females mated with homozygous mutant males. No indications of embryonic lethality were observed after morphological and histological examination of the uteri of homozygous mutant female at the gestation day 5.5 (Fig. 4B and C, Supplementary Figure 1, see section on supplementary data given at the end of this article). Moreover, histological examination of implanted homozygous mutant embryos at E7.5 revealed that the development was comparable to the wild-type embryos (Fig. 4D). Therefore, we concluded that reduced number of implanted homozygous mutant embryos is a result of impaired pre-implantation development and/or implantation failure. In contrast, in the breeding of *Ube2q1*^{-/-} female with *Ube2q1*^{+/+} male, the number of developing heterozygous embryos at the E10.5 was not significantly different from the control wild-type breeding (Fig. 4A). However, at E17.5, markedly increased number of degenerating heterozygous embryos in uteri of homozygous mutant females was found and the number of living embryos was significantly reduced. We reasoned that embryonic lethality is the underlying cause for the reduced number of newborns in pairs of *Ube2q1*^{-/-} females and *Ube2q1*^{+/+} males (Fig. 3A).

To analyse pre-implantation development of homozygous mutant embryos, the *Ube2q1*^{-/-} females were superovulated and mated with *Ube2q1*^{-/-} males. Control pairs consisted of superovulated wild-type females mated with wild-type males. Embryos at two-cell stage were flushed out from oviducts of plugged females at E1.5 and cultured till E3.5. Embryos were examined and scored each day. Three *Ube2q1*^{-/-} and four *Ube2q1*^{+/+} females possessed fertilized oocytes and were considered for the analysis. The results are given in Table 2 as the average number of embryos per female. As demonstrated, until the blastocyst stage, no significant difference could be observed in the development of *Ube2q1*^{-/-} and *Ube2q1*^{+/+} embryos.

As *Ube2q1*^{-/-} pre-implantation embryonic development was normal and the number of implanted embryos at the early post-implantation stage (E5.5) was already reduced, we concluded that the underlying cause of reduced litter size was the implantation failure of *Ube2q1*^{-/-} embryos. To further validate this hypothesis, we analysed the ratio of genotypes of newborns obtained from heterozygous animals. From such a breeding pairs,

Figure 4 Analysis of embryo development in the uterus of *Ube2q1*^{-/-} females. (A) The number of post-implantation embryos per female was recorded. Compared with wild-type breeding, significant reduction of implanted embryos was observed in homozygous mutant pairs at E5.5, E7.5 and E17.5. In the pairs of homozygous mutant female and wild-type male, the number of embryos at E10.5 was not different from wild-type breeding but was significantly reduced at E17.5. (B) Implantation sites in uteri of female at E5.5 are indicated by arrows. A significant reduction in the number of implanted embryos was observed in homozygous mutant breeding pairs when compared with wild-type breeding, but no indication of degenerating embryos was noticed in the uterus. (C) Histological analysis of the uteri at E5.5 of pregnant homozygous mutant females mated with homozygous mutant males also revealed no marks of embryonic lethality, but reduced number of implanted embryos was noted. As a control, uteri of wild-type females mated with wild-type males were analysed. (D) Normal development of implanted embryos was observed at E7.5 in both homozygous mutant and wild-type females. *Significance level with $P < 0.05$, **significance level with $P < 0.001$, U, uterine epithelium; D, decidua. The magnification is given in lower right, the stage of embryonic development in upper right and the type of breeding in upper left corner.

wild-type, heterozygous and homozygous mutant pups are expected in the 1:2:1 proportion. Among 109 animals that were born, only 15, instead of expected 27, were homozygous. The χ^2 analysis revealed that observed deviation from the expected number of homozygous mutant pups was significant ($P = 0.02$).

Mouse *Ube2q1* is necessary for embryo implantation and maintenance of pregnancy

To verify our hypothesis, that the *Ube2q1* might play a role in embryonic implantation and pregnancy maintaining, we analysed the expression pattern of the *Ube2q1* gene during mouse embryonic development. First, we analysed the expression in the uterus of pregnant female at E10.5. The RNA was subjected to cDNA synthesis followed by PCR with AltPCRFP and AltPCRPR primers. The expression of mouse *Ube2q1* was clearly detectable in uterus at the gestation day 10.5

but not in the uterus of non-pregnant female (Fig. 5A). Next, RNA isolated from embryos at different developmental stages was analysed by RT-PCR with AltRTPCRFP and AltRTPCRPR primers or northern blot using cDNA fragment containing exons 4–9 of *Ube2q1* as a probe. The expected 467 bp product representing the *Ube2q1* cDNA was observed in unfertilized oocyte, four-cell stage, blastocyst and E8.5 embryo (Fig. 5B). As a positive control for RT-PCR, testis cDNA was amplified, while in negative control testicular RNA was processed in the absence of reverse transcriptase. The cDNA quality was checked using *Hprt*-specific primers (Fig. 5A and B). In northern blot analysis, both 4 and 2.4 kb *Ube2q1* transcripts were detected in RNA isolated from ES cells and from all analysed stages of embryonic development. RNA quality was tested by rehybridization of the membrane with *EEF2* probe (Fig. 5C). The expression of *Ube2q1* was detected in the placenta of E9.5 and E10.5 with RT-PCR using AltRTPCRFP and AltRTPCRPR

Table 2 The analysis of pre-implantation embryonic development in *Ube2q1* knockout line.

Time of pre-implantation embryonic development (days)	Number of embryos (mean $\pm$ S.D.)					
	Breeding	Not developing eggs	Two-cell	4/8-cell	Morula	Blastocyst
E1.5	+/+x+/+	28.4 $\pm$ 14.2	9.4 $\pm$ 6.4	—	—	—
	-/-x-/-	50.0 $\pm$ 12.7	5.5 $\pm$ 4.9	—	—	—
	P	0.096	0.461	—	—	—
E2.5	+/+x+/+	11.1 $\pm$ 9.6	4.4 $\pm$ 4.8	7.0 $\pm$ 5.8	—	—
	-/-x-/-	21.0 $\pm$ 19.8	1.5 $\pm$ 2.1	5.0 $\pm$ 1.0	—	—
	P	0.326	0.453	0.658	—	—
E3.5	+/+x+/+	10.7 $\pm$ 11.7	4.1 $\pm$ 5.1	2.0 $\pm$ 2.6	4.7 $\pm$ 5.6	2.7 $\pm$ 3.3
	-/-x-/-	14.5 $\pm$ 14.8	5.5 $\pm$ 4.9	2.0 $\pm$ 2.8	3.0 $\pm$ 1.4	2.5 $\pm$ 0.7
	P	0.711	0.747	1.000	0.693	0.934

No significant differences between *Ube2q1*^{-/-} and *Ube2q1*^{+/+} embryo number were noticed at all stages of early embryonic development. Data are given as mean number of embryos per female $\pm$ S.D.

primers. The cDNA quality was verified by RT-PCR with *Hprt*-specific primers (Fig. 5D). The expression analyses clearly indicated that mouse *Ube2q1* is expressed in pre- and post-implantation embryos and might have a role in placenta formation.

Discussion

The expression of *Ubc25* gene, the *Caenorhabditis elegans* homologue of mouse *Ube2q1*, was detected not only in early embryos but also in neurons and muscles of later embryos and adult animals (Schulze et al. 2003). RNAi-mediated down-regulation of the *Ubc25* gene caused late-onset paralysis of muscular functions such as locomotion, egg laying and defecation in *C. elegans*. Mouse and *C. elegans* genes share 42% similarity (Schulze et al. 2003). Here, we generated and analysed an *Ube2q1* knockout mouse model and demonstrated that this widely expressed gene is involved in different aspects of female fertility. *Ube2q1*^{-/-} females showed irregularity of the oestrus cycle,

abnormal mating behaviour, decreased litter size and delayed occurrence of the first pregnancy. The absence of UBE2Q1 protein in the uterus of pregnant female impaired the development of heterozygous embryos. Moreover, we demonstrated that the majority of UBE2Q1-deficient embryos failed to implant in the uterus of homozygous mutant female.

By searching the NCBI database (<http://www.ncbi.nlm.nih.gov>) with the sequence of mouse UBE2Q1, we identified similar proteins in a wide variety of different species. The protein is highly conserved within mammals, showing 61–99% similarity of mouse amino acid composition to dog, grey short-tailed opossum, panda, rabbit, marmoset, macaque, human and rat proteins. High similarity of mouse UBE2Q1 was also found with proteins derived from birds (chicken and zebra finch), fish (zebrafish), nematodes (five species), Enteropneusta (Acorn worm), insects (20 species from five orders), Arachnida (deer tick), urchin (sea urchin) and *Anemone* (starlet sea anemone) (Supplementary Table 2, see section on supplementary data given at the end of this

Figure 5 The expression of mouse *Ube2q1* during embryonic development. RT-PCR and northern blot techniques were applied to analyse the *Ube2q1* expression in the uterus of pregnant female and in developing embryos. (A) A 467 bp PCR product of *Ube2q1* cDNA was detected in the uterus of E10.5 pregnant wild-type female but not in the uterus of non-pregnant female. (B) The *Ube2q1* cDNA was detected in unfertilized oocytes and in all stages of embryonic development analysed by RT-PCR (B) and northern blot (C, upper panel) techniques. In the northern blot experiment, the RNA quality was proven by membrane rehybridization with human *EEF2* probe (C, lower panel). (D) Mouse *Ube2q1* is also expressed in the placenta at E9.5 and E10.5. In all RT-PCR, the cDNA quality was verified with *Hprt*-specific primers (A, B, D, lower panels). Testis RNA was used as a positive control (Testis), while testicular RNA processed in the absence of reverse transcriptase served as a negative control (testis – RT).

article). Human cDNA probe detected *Ube2q1* transcript in different species, confirming evolutionary conservatism of this gene. This finding is in agreement with the previous work (Schulze *et al.* 2003) and it is well known that evolutionary conserved genes play an important role in different cellular processes.

Human *UBE2Q1* gene was found to be expressed in all tissues analysed. This is in agreement with previously published data demonstrating *UBE2Q1* transcripts in variety of human tissues by analysis of large cDNA libraries (Strausberg *et al.* 2002, Gerhard *et al.* 2004, Ota *et al.* 2004). In the UniGene database (<http://www.ncbi.nlm.nih.gov/UniGene>), human *UBE2Q1* EST clones were isolated from 37 different tissues and organs. The expression of mouse *Ube2q1* gene was previously reported by Wassler *et al.* (2008), where the authors showed *Ube2q1* transcription in thymus, lung, kidney, testis, ovary and E12.5 embryo but could not detect the *Ube2q1* mRNA in liver, brain, heart and spleen. This might be a result of the low sensitivity of the northern blot technique used in this work. In the UniGene database, EST clones corresponding to the mouse *Ube2q1* were identified in 32 different tissues including liver, brain, heart and spleen. Moreover, except for the uterus of non-pregnant female, we detected the expression of *Ube2q1* in all tissues analysed by RT-PCR, which is more sensitive than northern blot. Therefore, we concluded that both human and mouse *UBE2Q1/Ube2q1* genes are widely expressed.

The targeted disruption of mouse *Ube2q1* was found to affect different aspects of female reproduction. Reduced PRL level in mutant females at the beginning of lactation indicated the altered hormonal regulation. Female mice with the knockout of PRL receptor (*Prlr*) also demonstrated multiple reproductive defects (Ormandy *et al.* 1997), namely cycle irregularities, fertilization problems and complete failure of embryonic implantation. The newborn lethality and lactation problems were noticed in heterozygous *Prlr* knockout females (Ormandy *et al.* 1997). The lower serum PRL concentration 1 day after parturition and the similarities in the phenotype of *Ube2q1* and *Prlr* knockout mice indicated that at least partially similar pathway might be affected in both mutants. Ubiquitination involvement in hormone regulation is well documented in steroid hormone homeostasis. Estrogens and progesterone act through receptor proteins known as the nuclear hormone receptors (NHRs; Lydon *et al.* 1995, Korach *et al.* 1996). These receptors might recruit co-activators that facilitate the assembly of the transcription complex to the target genes (Tsai & O'Malley 1994, Beato *et al.* 1995, Mangelsdorf *et al.* 1995, Kinyamu & Archer 2004). Co-activators are diverse proteins mediating the transcriptional process through a wide array of enzymatic activities such as acetylation, methylation, ubiquitination, phosphorylation and chromatin remodelling (Ogryzko *et al.* 1996, Spencer *et al.* 1997, McKenna

et al. 1999, Chen 2000, Lonard & O'Malley 2007). The Ub protein ligase E3A (*UBE3A*) was reported to act as steroid hormone co-activator (Nawaz *et al.* 1999). Furthermore, the overexpression or knockout of *Ube3a* altered the level of oestrogen (ER- α) and progesterone (PR-B) receptors (Ramamoorthy *et al.* 2010). The authors demonstrated that PR-B level is regulated by the *UBE3A* via the Ub-proteasome pathway. The irregular oestrus cycle and abnormal mating behaviour of the *Ube2q1*^{-/-} females might indicate aberrant steroid hormone signalling.

It was demonstrated that in mouse embryonic stem cells, the expression of *B4galt1* inversely correlated with the expression of *Ube2q1* (Wassler *et al.* 2008). Our findings about the expression of *Ube2q1* in early embryos are in agreement with that finding. It was suggested by the authors that *Ube2q1* might be involved in ubiquitination and degradation of B4GALT1 through the 26S proteasome pathway or that the *UBE2Q1*-B4GALT1 binding can influence the internalization of the B4GALT1 (Wassler *et al.* 2008). The B4GALT1 is involved in cell adhesion (for review see Shur (1991)) and was detected in pre-implantation embryos (Bayna *et al.* 1988), ectoplacental cone cells (Hathaway *et al.* 1989) and uterine epithelial cells (Dutt *et al.* 1987); therefore, proper turnover of this protein might be critical for embryo implantation. Our results strongly suggest that *Ube2q1*, an E2 conjugating enzyme, is important for embryo implantation as most of the *Ube2q1*^{-/-} blastocysts failed to implant in the uterus. But this gene might also be important for further embryonic development because we showed that the expression of mouse *Ube2q1* is induced in the uterus of pregnant mouse. This result is supported by the existence of two *Ube2q1* EST clones, A1157754.1 and A1197002.1, that were generated using RNA isolated from pregnant mouse uterus. The Ub homologue (Ub cross-reactive protein) ISG15 was also induced by pregnancy in the uterus in cow (Austin *et al.* 1996, Hansen *et al.* 1997, Johnson *et al.* 1998, Perry *et al.* 1999, Thatcher *et al.* 2001), mouse (Austin *et al.* 2003) and primates (Bebington *et al.* 1999a, 1999b). It seems that protein turnover is important for the endometrium response and maintaining pregnancy. In the uterus of *Ube2q1*^{-/-} females, normal implantation of heterozygous embryos occurred but they degenerated after E10.5, indicating that *Ube2q1* uterine expression is necessary for the maintenance of pregnancy.

In humans, 30–50% of conceptions end in spontaneous abortions before the end of the first trimester (Gupta *et al.* 2007), and in general, the spontaneous abortion rate of clinical pregnancies is about 15–20% (Dickey *et al.* 1996). The most common reasons for spontaneous abortions are chromosomal abnormalities, but about 50% of cases remain idiopathic (Cramer & Wise 2000, Gupta *et al.* 2007). The majority of pregnancy losses occur before or during implantation

(Gupta *et al.* 2007, Makrigiannakis *et al.* 2008); therefore, the progress towards improving the success rate of early pregnancies is a very important aim of reproductive medicine. In the review by Makrigiannakis *et al.* (2008), the authors suggested that generation of knockout animals in which early embryonic lethality is observed would enable the analyses of genes controlling implantation and would be helpful to decode fertility defects. According to our results, the *UBE2Q1* may be one of the candidate genes for mutation screening in women with recurrent spontaneous abortions and/or implantation failure.

The importance of better understanding of the ubiquitination pathway is emphasized by the large number of human diseases related to aberrant protein degradation. The searching of Online Mendelian Inheritance of Man (OMIM, <http://www.ncbi.nlm.nih.gov/omim/>) database with the term 'Ub' resulted in 757 hits. The expression of *UBE2Q1* in many tissues suggests that this gene plays a role not only in female fertility. However, the phenotype in different organs of mutant mouse might be not so prominent and thus difficult to detect. This hypothesis is supported by the observation that human *UBE2Q1* gene, which is down-regulated by miR-338, can degrade key enzymes essential for neurite growth and thus negatively control this process (Barik 2008). *UBE2Q1* was also found, among many other genes related to ubiquitination pathway, to be altered in human airway epithelia cells in cystic fibrosis patients (reviewed in Hampton & Stanton (2010)). Recently, the frame shift mutation in *UBE2Q1* gene was identified in gastric carcinoma (Yoo *et al.* 2011). Moreover, the interaction of *UBE2Q1* with STIP1 homology and U-box containing protein 1, E3 Ub protein ligase (STUB1), which is involved in caytaxin ubiquitination, was demonstrated by pull down and yeast two-hybrid techniques (Grelle *et al.* 2006). Mutations in *ATCAY* gene, encoding caytaxin, cause Cayman disease (Bomar *et al.* 2003). Taken together, our mouse model can be utilized to better understand the function of ubiquitination process in proteome regulation in different organs.

Materials and Methods

Expression analysis

Total RNA was extracted from several different mouse tissues; mouse embryos at E13.5, E16.5, E18.5 and from testis or brain of rhesus (*Macaca mulatta*), bovine (*Bos* sp.), pig (*Sus* sp.), rat (*Rattus norvegicus*), goat (*Capra aegagrus hircus*), rabbit (*Oryctolagus cuniculus*) and pigeon (*Columba livia*) using the RNA now kit (Biomol, Hamburg, Germany). In addition, RNA was isolated from the uterus of pregnant female mice at gestation day 10.5, dissected without any fragments of developing embryos. The RNA from oocytes, two-cell and four-cell stage embryos and placentas from embryos at E9.5 and E10.5 was isolated using the peqGOLD TriFast reagent (Peqlab, Erlangen, Germany) according to the manufacturer's recommendations.

For northern blot analyses, 20 µg total RNA was separated in 1.5% MOPS/formaldehyde agarose gel and transferred onto nitrocellulose membrane (Hybond N, Amersham). For the *UBE2Q1* expression analysis in human tissues, multiple tissues northern blots (2 µg polyA RNA for each tissue) (BD Biosciences Clontech, Heidelberg, Germany) were used. Membrane with human RNA was hybridized with radio-active-labelled 1 kb PvuII restriction fragment of human *UBE2Q1* cDNA, containing exons 5–13, and mouse RNA was hybridized with *Ube2q1* cDNA fragment (exons 4–9) amplified with AltRTPCRFP (AGAGCAGTGCACGCAGGAAG) and AltRTPCRRP (TGACGAACGGTGGGTCAAAG) primers. The filters were subsequently rehybridized with ³²P-labelled *ACTB* cDNA (GeneID:60) clone (IMAGp998L23787) or 1.6 kb BamHI/BglII fragment of *EEF2* cDNA (GeneID 1938) probes.

For RT-PCR, DNaseI-treated total RNA was primed with oligo-dT primer and reverse transcribed at 42 °C for 1 h using the Superscript reverse transcriptase system (Invitrogen). cDNA (1 µl) was used in a PCR with AltRTPCRFP and AltRTPCRRP primers, generating a 467 bp product. Simultaneously, the reaction without reverse transcriptase was performed to rule out template contamination in reaction components or DNA contamination in RNA samples (negative control). To verify cDNA quality, mHPRT-For-Q (AGCCCCAAAATGGTTAAGGTTGC3) and mHPRT-Rev-Q (TTGCAGATTCAACTTGCCTCAT) primers amplifying a 222 bp fragment of mouse *Hprt* gene (GeneID: 15452) were used.

Generation of *Ube2q1*-deficient mouse

The recombinant phage clone, containing mouse *Ube2q1* gene, was identified by hybridization of the mouse genomic library (kindly provided by Prof. Braun, Bad Nauheim, Germany) with 1 kb PvuII fragment of *UBE2Q1* cDNA. This library contains Sall/BamHI genomic fragments from 129/SvJ mouse cloned in EMBL3 Phage vector. From this recombinant phage clone, a 5 kb XhoI fragment located upstream exon 5 as well as a 4 kb XbaI fragment located downstream exon 11 of *Ube2q1* were isolated and used as a 5' and 3' homologous parts of the knockout vector respectively. Both fragments were subcloned into the pPNT Vector and verified by sequencing. The obtained targeting vector was linearized using NotI and electroporated into R1 ES cells (Wurst & Joyner 1993). Membranes with EcoRI digested DNA, isolated from ES cell colonies resistant to G418 (400 mg/ml) and ganciclovir (2 µM) were hybridized with 2.4 kb EcoRI/XhoI fragment containing part of *Ube2q1* exon 1 and the upstream sequence. This external probe recognized an 8 kb fragment of *Ube2q1* wild-type allele and a 13 kb fragment of homologous recombinant allele. ES cells carrying the disrupted *Ube2q1* allele were used for generation of chimaeras. Male chimaeras were mated with 129Sv females and mice from F1 generation were genotyped by Southern blot (as described for ES cells) and PCR with primers gM7F (TGATAAGTGAAGATAACGATAGAAG), gM8R (TGGCTTGGCGGCTTGCTGAG) and Pkg3 (GGATGTGGAATGTGTGCGAGG). Using these primers, a 498 bp fragment of the wild-type allele and a 308 bp mutant allele were amplified. Heterozygous animals were crossed to obtain homozygous mutants.

Oocytes and embryos analyses

For the analysis of ovulated oocytes, females were mated with vasectomized males and examined daily for the presence of VP. The day of VP was designed as E0.5, and at this day, oocytes were isolated and counted separate for each female. To ascertain the oocyte number after superovulation, females were injected with 7.5 IU pregnant mare serum gonadotrophin (PMS; Intergonan; Intervet, Toennisvorst, Germany) followed 48 h later with 7.5 IU human chorionic gonadotrophin (hCG) (Predalon; Organon, Oberschleissheim, Germany). Thirteen hours after hCG administration, oocytes were isolated and counted. For the analysis of pre-implantation embryonic development, females were superovulated (as described earlier) and mated with males. Embryos at E1.5 were flushed out from oviducts of plugged females and cultivated in M16 medium (Sigma) till E3.5. Embryos were evaluated daily and classified as not developing eggs (degenerating embryos and not fertilized oocytes), two-cell, 4/8-cell, morula and blastocyst stage embryos. For the analysis of post-implantation embryonic development, females were mated with males, VP was inspected daily and positive females were killed at the gestation days 5.5, 7.5 and 10.5. Then, uteri were dissected and embryos were counted from at least five females of each genotype and embryonic developmental stage. Implantation sites at E5.5 were visualized by i.v. injection of 1% Evans blue in PBS (Sigma) 5 min before cervical dislocation (Ye *et al.* 2005). Females without any embryos were considered as non-pregnant and were not included in the statistics.

Oestrus cycle analysis

The oestrus cycle was determined by observation of vaginal smears (Archunan & Dominic 1991). The smears were prepared with PBS and examined directly under light microscopy by trained observer. Four stages were determined: proestrus, oestrus, metoestrus and dioestrus. Females were first trained for 2 weeks to accustom to daily vaginal smear examination, and then oestrus cycle was examined for 20 days. Females that were never in oestrus phase during the time of observation were excluded from the analysis.

PRL RIA

Blood was collected in heparinized tubes, centrifuged (3000 g, 20 min) and the serum was stored at -20°C until further analysis. PRL was measured by RIA using mouse-specific reagents provided by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) National Hormone and Pituitary Program (NHPP; Dr A F Parlow, NHPP, Torrance, CA, USA). Assays were performed using 50 μl serum in duplicate. Results are expressed in terms of mouse PRL RP 3 (RP3). Intra- and interassay coefficients of variation were 7.2 and 12.8% respectively. The results are given as mean $\pm$ s.d.

Histological analysis of post-implantation embryos

Uteri were dissected from pregnant females at E5.5 and E7.5. Probes were fixed in Bouin's solution, embedded in paraffin

and processed routinely for histological examination (haematoxylin and eosin (HE) staining).

Statistical analysis

Data distribution was tested using the Shapiro–Wilk W test with extension described by Royston (1982). All data were not significantly different from normal distribution. For the evaluation of two groups, the t -test was used; when three or more groups were compared, the ANOVA analysis was applied. To test whether genotypes of newborns obtained from heterozygous breeding pairs deviated from the expected Mendelian ratio 1:2:1, the χ^2 test was used. The P value below 0.05 was considered as significant. All statistical analyses were performed using the Statistica 9 software package (StatSoft, Inc., Tulsa, OK, USA).

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-12-0054>.

Declaration of interest

The authors declare no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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