

Homozygous Mutations in *WEE2* Cause Fertilization Failure and Female Infertility

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Fertilization is a fundamental process of development and is a prerequisite for successful human reproduction. In mice, although several receptor proteins have been shown to play important roles in the process of fertilization, only three genes have been shown to cause fertilization failure and infertility when deleted *in vivo*. In clinical practice, some infertility case subjects suffer from recurrent failure of *in vitro* fertilization and intracytoplasmic sperm injection attempts due to fertilization failure, but the genetic basis of fertilization failure in humans remains largely unknown. Wee2 is a key oocyte-specific kinase involved in the control of meiotic arrest in mice, but *WEE2* has not been associated with any diseases in humans. In this study, we identified homozygous mutations in *WEE2* that are responsible for fertilization failure in humans. All four independent affected individuals had homozygous loss-of-function missense mutations or homozygous frameshift protein-truncating mutations, and the phenotype of fertilization failure was shown to follow a Mendelian recessive inheritance pattern. All four mutations significantly decreased the amount of *WEE2* protein *in vitro* and in affected individuals' oocytes *in vivo*, and they all led to abnormal serine phosphorylation of *WEE2* and reduced tyrosine 15 phosphorylation of *Cdc2 in vitro*. In addition, injection of *WEE2* cRNA into affected individuals' oocytes rescued the fertilization failure phenotype and led to the formation of blastocysts *in vitro*. This work presents a novel gene responsible for human fertilization failure and has implications for future therapeutic treatments for infertility cases.

Introduction

In the process of fertilization, the haploid sperm and oocyte fuse to form a diploid zygote that will subsequently develop into a complete individual.¹ Fertilization is therefore a fundamental process of development and a prerequisite for successful human reproduction. In *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatments, mature oocytes are retrieved from affected individuals and fertilized with sperm *in vitro* to start early embryonic development, and the ensuing viable embryos are transplanted into the affected individuals' uterus for establishing pregnancy. However, some affected individuals suffer from recurrent failure of IVF and ICSI attempts. For these affected individuals, although morphologically normal sperm and oocytes can be retrieved, fertilization always fails. Until now, only a single mutation in the *TLE6* (MIM: 612399) gene has been associated with this phenotype,² and the genetic basis of human fertilization failure has remained largely unknown.

Before puberty, oocytes were maintained at a cellular quiescent stage of prophase I termed "dictyate arrest" (also known as germinal vesicle [GV] stage arrest).³ During meiosis, cells undergo two divisions—meiosis I and meiosis II—to create haploid gametes.³ Dictyate-arrested oocytes resume meiosis I upon stimulation by a surge in luteinizing hormone, and meiosis I is completed by

extruding the first polar body. Then, oocytes enter meiosis II and arrest at metaphase II (MII) until fertilization.³ Therefore, defects in the process of meiosis will lead to oocyte maturation arrest and fertilization failure. Wee2, also known as Wee1B, has different roles in mouse oocytes at the GV stage and MII stage. In the GV stage, Wee2 is an important oocyte-specific kinase involved in maintaining meiotic arrest⁴ by inhibiting the maturation-promoting factor (MPF) by inactivating *Cdc2* (MIM: 116940).^{5,6} In the MII stage, Wee2 is essential for MII exit. It has been documented that when Wee2 is downregulated, mouse oocytes have high MPF activity leading to failure of MII exit and the formation of pronuclei, which is an indicator of fertilization failure.⁷ To date, *WEE2* (MIM: 614084; GenBank: NM_001105558.1) has not been associated with any diseases in humans.

In this study, we identified homozygous mutations in *WEE2* that are responsible for human fertilization failure. All four affected individuals had homozygous loss-of-function missense mutations or homozygous frameshift protein-truncating mutations, and the phenotype of fertilization failure in these affected individuals followed a Mendelian recessive inheritance pattern. We investigated the functional mechanism of the corresponding mutations *in vitro* and in live oocytes, and we explored a potential therapeutic treatment in one of the affected individuals by injecting *WEE2* cRNA into her oocytes.

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Subjects and Methods

Clinical Samples

Infertility-affected individuals diagnosed with fertilization failure and healthy controls were recruited from the Ninth Hospital affiliated with Shanghai Jiao Tong University. Peripheral blood samples were taken for DNA extraction. The control first polar body (PB1) oocytes used in this study were matured *in vitro* from oocytes at the GV or metaphase I (MI) stage. Some PB1 oocytes from the affected individuals were donated for investigation after they provided written and informed consent. The study was approved by the ethics committee of the Medical College of Fudan University and the reproductive center of the Ninth Hospital affiliated with Shanghai Jiao Tong University.

Genetic Studies

Genomic DNA was extracted from peripheral blood based on a previously published protocol.⁸ Whole-exome capture (Agilent) and Illumina sequencing were performed following the standard protocols. Basic bioinformatics analysis included mapping the raw FASTQ files to the human reference sequence (NCBI Genome build GRCh37); variant calling; annotation with the GRCh37, dbSNP (version 138), 1000 Genomes, Exome Aggregation Consortium (ExAC, version 0.3.1) databases, and our in-house exome database; and functional prediction with the SIFT and PolyPhen-2 programs. Homozygosity mapping was performed with HomozygosityMapper.⁹ Because three of the four families were consanguineous, homozygous and compound heterozygous variants were prioritized in the analysis. All candidate mutations had to meet the following inclusion criteria: (1) be absent in other family members, (2) have a frequency less than 0.1% (for homozygous variants) or 1% (for compound-heterozygous variants) in public databases, and (3) be located in or near a homozygous region greater than 2.0 Mb.

Expression Vector Construction and Mutagenesis

The full-length coding sequence of *WEE2* was amplified from control human oocyte cDNA. The *WEE2* product was then cloned into the pCMV6-Entry vector to introduce a FLAG-tag (Origene) using AsiSI and MluI (New England Biolabs) dual-enzyme digestion. All FLAG tags were fused in the N termini and were in frame with the frameshifted C termini. Site-directed mutagenesis was performed to introduce four variants—p.Asp234His, p.Glu75Valfs*6, p.Thr493Asnfs*39, and p.His337Tyrfs*24—into the pCMV6-Entry vector according to the instructions of the KOD-Plus-Mutagenesis Kit (Toyobo Life Science).

Immunofluorescence

In order to determine the effects of these mutations on *WEE2*, immunofluorescence was performed in mouse oocytes, HeLa cells, and affected individual oocytes as previously described.⁸ Briefly, in mouse oocytes, wild-type or mutant *WEE2* cRNAs were microinjected into GV oocytes, which were then cultured for 12 hr and fixed for immunofluorescence. HeLa cells were transfected with wild-type or mutant *WEE2* constructs, and cells were fixed for immunofluorescence after culturing for 30 hr. The oocytes of the affected individuals and healthy control subjects were fixed in 2% paraformaldehyde for immunofluorescence. An anti-*WEE2* antibody was used at 1:100 dilution (Abcam). DAPI was used at 1:600 dilution (Beyotime) for DNA visualization. An anti-FLAG antibody was used at 1:500 dilution (Sigma).

All images were captured on a confocal laser-scanning microscope (Leica).

Western Blotting

To evaluate the effects of the mutations on *WEE2* phosphorylation, HeLa cells were transfected with wild-type or mutant *WEE2* constructs and incubated for 36 hr. Total protein was extracted by RIPA lysis buffer (Shanghai Wei AO Biological Technology), and immunoprecipitation was performed using anti-FLAG beads. Western blotting was conducted using a phospho-serine antibody (Immunechem Canada) to detect *WEE2* phosphorylation. To determine the effect of *WEE2* mutations on MPF activity, transfected HeLa cells were used for western blotting. Antibodies against Cdc2 (Abcam) and pY15-Cdc2 (Abcam) were used at 1:1,000 dilution, and mouse anti-vinculin (MIM: 193065) (1:5,000 dilution, Sigma) was used as the internal control. The secondary antibodies were goat anti-rabbit IgG (1:5,000 dilution, Abmart) or goat anti-mouse IgG (1:5,000 dilution, Abmart) conjugated to horseradish peroxidase.

Oocyte Collection and Microinjection

GV oocytes were collected from the ovaries of 7- to 8-week-old ICR mice. GV oocytes were cultured at 37°C in M2 medium (Sigma) supplemented with 200 μM of 3-isobutyl-1-methylxanthine (Sigma) to prevent GV breakdown. For collecting MII oocytes, 7.5 IU of human chorionic gonadotropin was administered 48 hr after injection of pregnant mare's serum gonadotropin. Approximately 10 pl of the mRNA solution (500 ng/μL) was injected per oocyte. GV oocytes were fixed for immunofluorescence 12 hr after injection. MII oocytes were counted for pronuclear formation 10 hr after injection.

Statistical Analysis

Data are representative of at least three independent experiments. Significance was analyzed by one-way ANOVA, and $p < 0.05$ was considered statistically significant. Quantitation of immunofluorescence and western blotting results was performed with the ImageJ software.

Results

Clinical Characteristics of the Affected Individuals

All four female affected individuals had normal menstrual cycles and had been diagnosed with primary infertility of unknown cause for several years. The male partners of these affected individuals had normal sperm counts and their sperm had normal morphologic features and motility. To further exclude male factors, we acquired *in vitro*-matured MII oocytes from other affected individuals and found that these oocytes could be fertilized after injecting sperm of the male partners of the four affected individuals, indicating that the sperm were functionally normal. The affected individuals in family 1, family 2, and family 3 were from consanguineous families. The proband in family 1 was 37 years old and her parents were cousins. She underwent two failed ICSI attempts. Three PB1 oocytes were retrieved, but none of them could be fertilized (Table 1). The proband in family 2 was 34 years old, and based on the

Table 1. Clinical Characteristics of Affected Individuals and Their Retrieved Oocytes

Case	Age (years)	Duration of Infertility (years)	IVF/ICSI Cycles	Total No. of Oocytes Retrieved	GV Oocytes	MI Oocytes	PB1 Oocytes	Fertilized Oocytes
Family 1	37	9	2	3	0	0	3	0
Family 2	34	7	1	11	2	1	8	0
Family 3	29	6	3	19	0	1	18	0
Family 4	27	4	1	20	0	0	20	0

Abbreviations: GV, germinal vesicle; MI, metaphase I; PB1, the first polar body.

result of her homozygosity mapping her parents also had some biological relationship (Figure S1). She had one failed ICSI attempt, in which eight PB1 oocytes were retrieved but could not be fertilized (Table 1). The proband in family 3 was 29 years old. Her grandfather on her father's side and her grandmother on her mother's side were cousins. She had one failed IVF attempt and two failed ICSI attempts. Altogether, 18 PB1 oocytes were retrieved but none could be fertilized (Table 1). The proband in family 4 was 27 years old, and she had one failed ICSI attempts. During her first ICSI cycle, 20 PB1 oocytes were retrieved but none could be fertilized (Table 1). The morphology of the retrieved oocytes before ICSI is shown in Figure S2.

Identification of Homozygous Mutations in *WEE2*

After whole-exome sequencing, bioinformatics filtering analysis, and homozygosity mapping (Figure S1), *WEE2* was the only gene that segregated with the phenotype in each of families 1, 2, and 3. Sanger sequencing confirmed mutations in *WEE2* in these families (Figure 1A). The proband in family 1 had a homozygous missense mutation c.700G>C (p.Asp234His). The affected individuals in families 2 and 3 both had homozygous frameshift protein-truncating mutations. The proband in family 2 had a c.1473dupA (p.Thr493Asnfs*39) mutation, while the proband in family 3 had a c.220_223delAAAG (p.Glu75Valfs*6) mutation. Another homozygous frameshift protein-truncating mutation c.1006_1007insTA (p.His337Tyrfs*24) was identified in the proband in family 4 by direct sequencing of all exons of *WEE2*. The allele frequency of p.Glu75Valfs*6 is 0.00008296 (10/120,538) in the ExAC database, while other three mutations were not found in the ExAC database. The specific information for the mutations is shown in Table 2. The positions of these mutations are shown in Figure 1B along with their conservation in different species.

Localization and Expression of Mutant Protein in Cells and Oocytes

Light microscopy showed that oocytes from affected individuals could not form pronuclei after fertilization (Figure 2A). Immunofluorescence staining in two affected individual oocytes suggested that the mutations caused loss of *WEE2* protein or protein degradation (Figures 2B and S3A). To evaluate the effects of the mutations on pro-

tein localization, wild-type and mutant constructs were transfected into HeLa cells. As Figure 2C indicates, the nuclear localization of p.Asp234His and p.Thr493Asnfs*39 was not affected but the protein level was slightly reduced, while p.His337Tyrfs*24 and p.Glu75Valfs*6 were significantly degraded as indicated by the very faint immunofluorescence signal (Figures 2C and S3B). This is consistent with the results of immunofluorescence in mouse GV oocytes injected with wild-type or mutant cRNAs (Figures 2D and S3C). To evaluate the functional effects of the mutations *in vitro*, western blot analysis was performed with cells transfected with wild-type and mutant *WEE2* constructs. The amounts of p.Asp234His and p.Thr493Asnfs*39 altered proteins were significantly lower than wild-type, while the p.His337Tyrfs*24 and p.Glu75Valfs*6 altered proteins were nearly undetectable (Figures 3A and S4A). Thus, although the *WEE2* antibody (epitope: amino acids 305–396) would not recognize the truncated proteins predicted to be produced by the p.His337Tyrfs*24 and p.Glu75Valfs*6, considering the corresponding results of Flag-tagged *WEE2* in mouse oocytes and HeLa cells, we believe that these two mutations lead to protein degradation.

Phosphorylation of Cdc2 and *WEE2* Was Impaired by *WEE2* Mutations *In Vitro*

It has been shown previously that serine phosphorylation of mouse *WEE2* is required for egg activation, which in turn is required for successful fertilization.⁷ We first determined the effects of the mutations on human *WEE2* serine phosphorylation. As shown in Figures 3B and S4B, the serine phosphorylation of *WEE2* was significantly reduced for the p.Asp234His, p.His337Tyrfs*24, and p.Glu75Valfs*6 mutations, while it was significantly increased for the p.Thr493Asnfs*39 mutation. Compared with the other mutations, p.Thr493Asnfs*39 resulted in a truncated protein with a lower detection level (Figure 3A), which might further cause abnormally increased phosphorylation. *Wee2*-mediated phosphorylation of Cdc2 is also required for pronuclei formation,⁷ and we therefore determined the effects of the *WEE2* mutations on tyrosine 15 phosphorylation (pY15) of Cdc2 *in vitro*. As shown in Figures 3C and S4C, all case-derived mutations in *WEE2* decreased the level of pY15-Cdc2. To further confirm the impairment of mutations on pronuclei formation, we injected

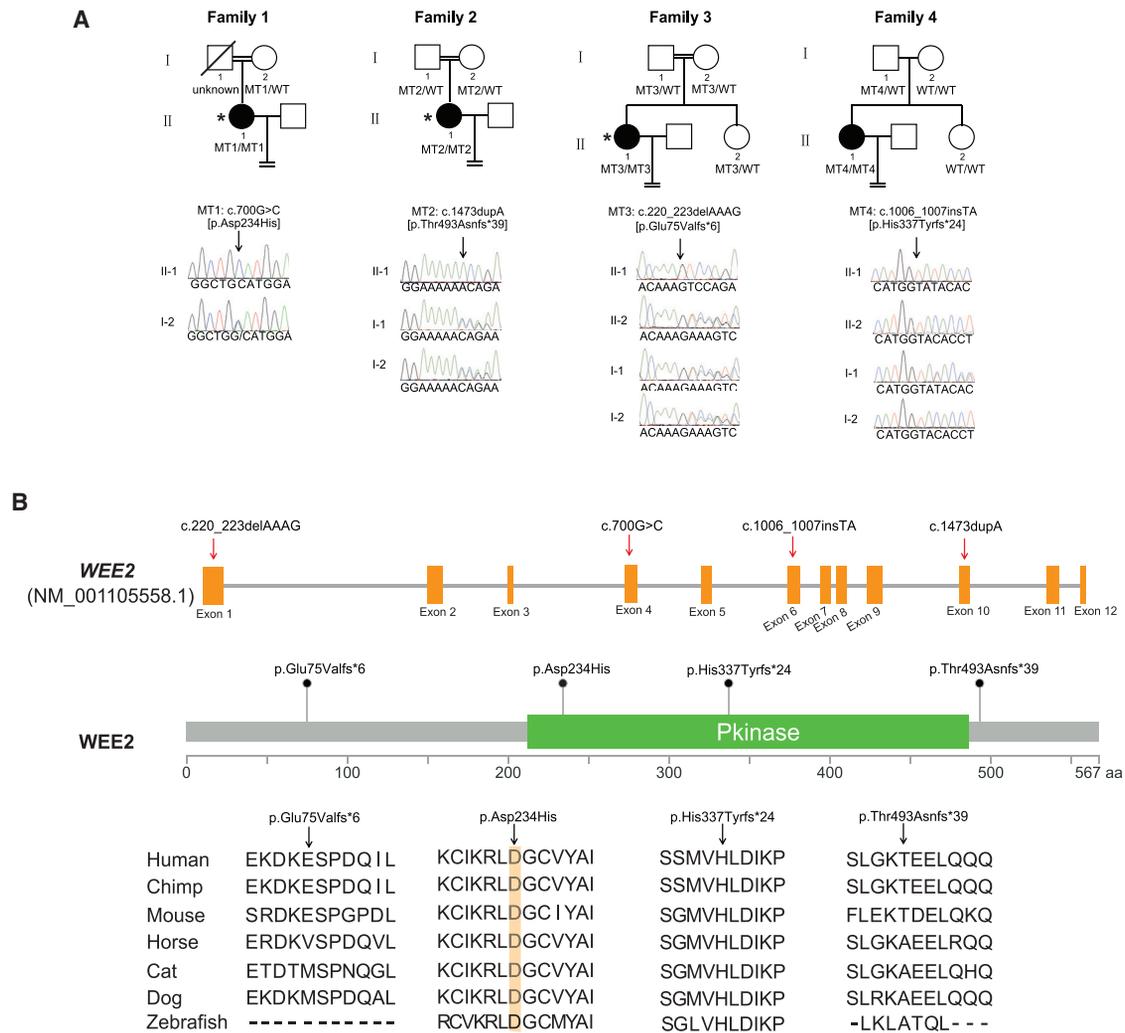


Figure 1. Identification of Mutations in *WEE2*

(A) Four pedigrees presented with the fertilization failure phenotype. Families 1, 2, and 3 were consanguineous families with a recessive inheritance pattern. Sanger sequencing confirmation is shown below the pedigrees. Affected individuals undergoing whole-exome sequencing and homozygosity mapping are labeled with an asterisk. The equal sign indicates infertility. Black circles represent the affected individuals.

(B) Locations and conservation of mutations in the *WEE2* gene. The positions of all mutations are indicated in the genomic structure of *WEE2*, and the conservation of the mutated amino acids is indicated by the alignment of seven mammalian species.

wild-type (WT) or mutant *WEE2* cRNA into mouse MII oocytes. As expected, all mutants significantly reduced the rate of pronuclei formation (Figure 3D). We further demonstrated that oocytes injected with mutant *WEE2* cRNAs had low pY15-Cdc2 levels, indicating that

they might have high MPF activity (Figure S4D). Thus, we concluded that mutations in *WEE2* decreased the amount of *WEE2* protein and impaired the phosphorylation of both *WEE2* and *Cdc2* and that this led to the failure of fertilization.

Table 2. Overview of the *WEE2* Mutations Observed in Four Families

Observed in Families	Genomic Position on chr. 7 (bp)	cDNA Change	Protein Change	Mutation Type	SIFT ^a	PPH2 ^a	1KG_eas ^b	ExAC
Family 1	141,418,986	c.G700C	p.Asp234His	missense	D	D	NA	NA
Family 2	141,427,183	c.1473dupA	p.Thr493Asnfs*39	frameshift insertion	NA	NA	NA	NA
Family 3	141,408,777	c.220_223delAAAG	p.Glu75Valfs*6	frameshift deletion	NA	NA	NA	8.3×10^{-5}
Family 4	141,423,059	c.1006_1007insTA	p.His337Tyrfs*24	frameshift insertion	NA	NA	NA	NA

^aMutation assessment by SIFT and PolyPhen2 (PPH2). D, damaging.

^bFrequency of corresponding mutations in the East Asian population of the 1000 Genome (1KG). NA, not available.

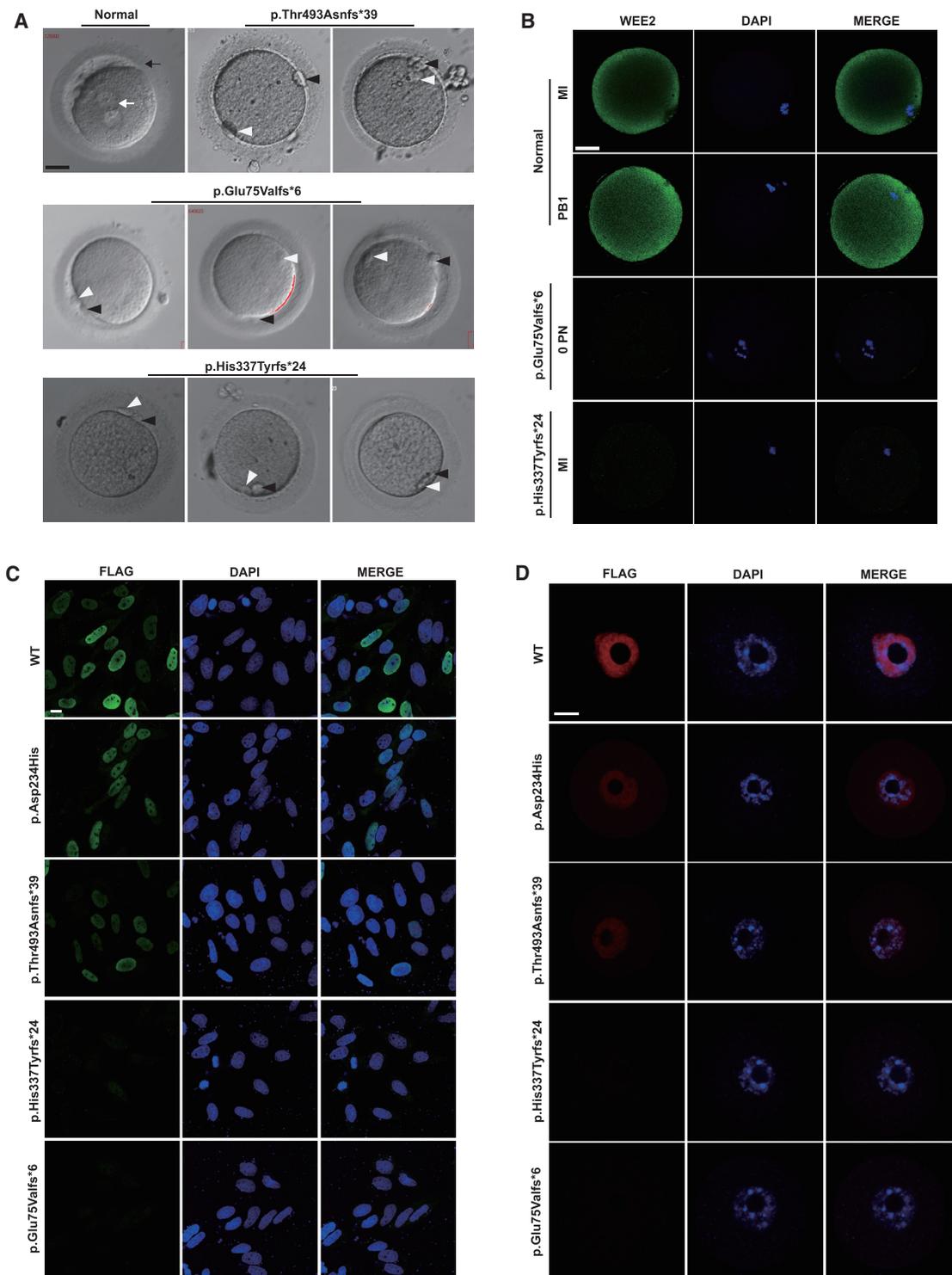


Figure 2. Morphology of Normal Zygotes and Retrieved Oocytes after ICSI and the Effects of the Mutations on WEE2 Protein Levels in Oocytes and HeLa Cells

(A) The morphologies of a normal zygote and affected individual oocytes on day 1 after ICSI. The white arrow indicates the pronuclei, and the black arrow indicates the zona pellucida. The white arrowhead indicates the first polar body, and the black arrowhead indicates the second polar body. Scale bar = 40 μ m.

(B) Immunofluorescence of healthy control and affected individual oocytes with the p.Glu75Valfs*6 or p.His337Tyrf*24 mutation. Abbreviations: MI, metaphase I; PB1, first polar body; PN, pronuclei. Scale bar = 40 μ m.

(C) Protein level and localization of WT and mutant WEE2 in HeLa cells. Scale bar = 10 μ m.

(D) Protein level and localization of WT and mutant WEE2 in mouse GV oocytes. Images were captured by confocal microscopy (Leica). Scale bar = 20 μ m.

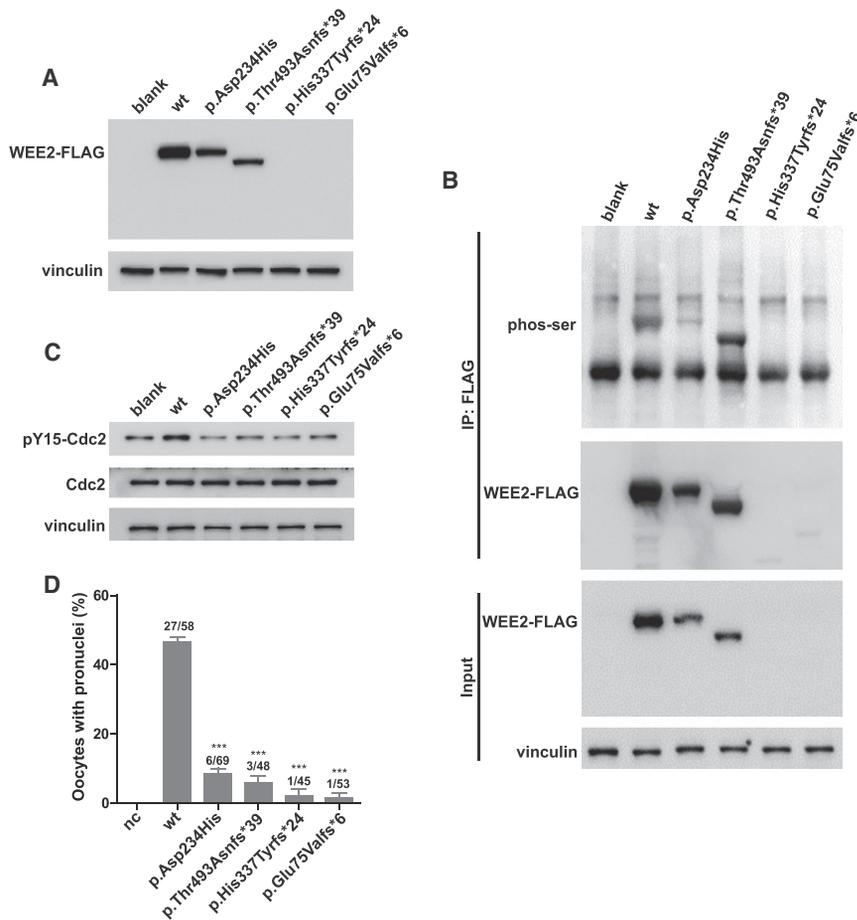


Figure 3. Effects of the Mutations on WEE2 and Cdc2 Phosphorylation and Pronuclei Formation

(A) The effects of the mutations on WEE2 protein level by western blotting in HeLa cells transfected with WT or mutant constructs.

(B) The effects of the mutations on serine phosphorylation of WEE2 in HeLa cells. WEE2 was immunoprecipitated by FLAG beads, and western blotting was performed using a phospho-serine antibody.

(C) The effects of the mutations on tyrosine 15 phosphorylation of Cdc2 in HeLa cells. HeLa cells were transfected with WT or mutant constructs, and 20 μg whole-cell lysate was used for western blotting.

(D) The effects of the mutations on pronuclei formation. A total of 10 pl of the 500 ng/ μL WEE2 crna solution was injected into mouse MII oocytes, and the pronuclei formation rate was calculated 10 hr after WEE2 crna injection. The total number of oocytes used is listed on top of the column. Significance was compared between WT and mutant groups. Three independent experiments were performed, and results are shown as the mean \pm SEM. *** $p < 0.001$.

Phenotypic Rescue by WEE2 crna Injection in Affected Individual Oocytes

To determine whether the phenotype of fertilization failure could be rescued, we directly injected WEE2 crna into oocytes of proband II-1 from family 4 during the recent second ICSI cycle. A total of 13 oocytes were retrieved, of which 11 were PB1 oocytes. Among the 11 PB1 oocytes, 4 were injected with WEE2 crna and then used for ICSI, while the remaining PB1 oocytes acted as controls and were used directly for ICSI without crna injection. In the control group, all oocytes remained unfertilized after ICSI (Figure 4A). However, all four oocytes injected with WEE2 crna were successfully fertilized as indicated by the formation of 2 pronuclei on day 1, and two of the oocytes developed into blastocysts on day 6 (Figure 4B). Preimplantation genetic screening showed that the two blastocysts had normal numbers of chromosomes and had no obvious large repetition/deletion fragments (Figure S5). These results further confirm the effects of WEE2 on fertilization and suggest the possibility for novel treatment options for affected individuals with mutations in WEE2 in the future.

Discussion

In this study, we identified four different homozygous mutations in WEE2 that are responsible for the unique

phenotype of fertilization failure. These mutations reduced the WEE2 protein level in the oocytes and affected the phosphorylation state of WEE2 and Cdc2 *in vitro*, which blocked oocyte MII exit and caused subsequent fertilization failure. Finally, we found that injection of WEE2 crna into affected oocytes led to successful fertilization and blastocyst formation *in vitro*.

During fertilization, the specialized meiotic germ cells, the oocyte and sperm, are transformed into a zygote that is capable of developing into all cells and tissues of human body.¹ Fertilization involves several biological changes, including sperm-egg binding,¹⁰ Ca²⁺ oscillations, the release of cortical granules, and the extrusion of the second polar body.^{11,12} Mature oocytes are arrested at the MII stage and must undergo MII exit in order to be fertilized.¹³ Inactivation of the MPF, which is the complex of Cdc2 and Cyclin B1 (MIM: 123836), is imperative for MII exit in mouse oocytes.⁷ Although several receptor proteins have important roles in the process of fertilization in mice,^{14,15} only three genes have been shown to cause failure of fertilization and infertility when deleted *in vivo*.^{16–19}

In this study, we identified different homozygous mutations in WEE2 that cause fertilization failure by reducing the WEE2 protein level and impairing the phosphorylation of WEE2 and Cdc2. It has been shown that in *Wee2* knockdown mouse oocytes MPF has high activity and causes the failure of MII exit and the subsequent formation of pronuclei.⁷ In this study, all mutations we identified were truncated or loss of function and had similar effects

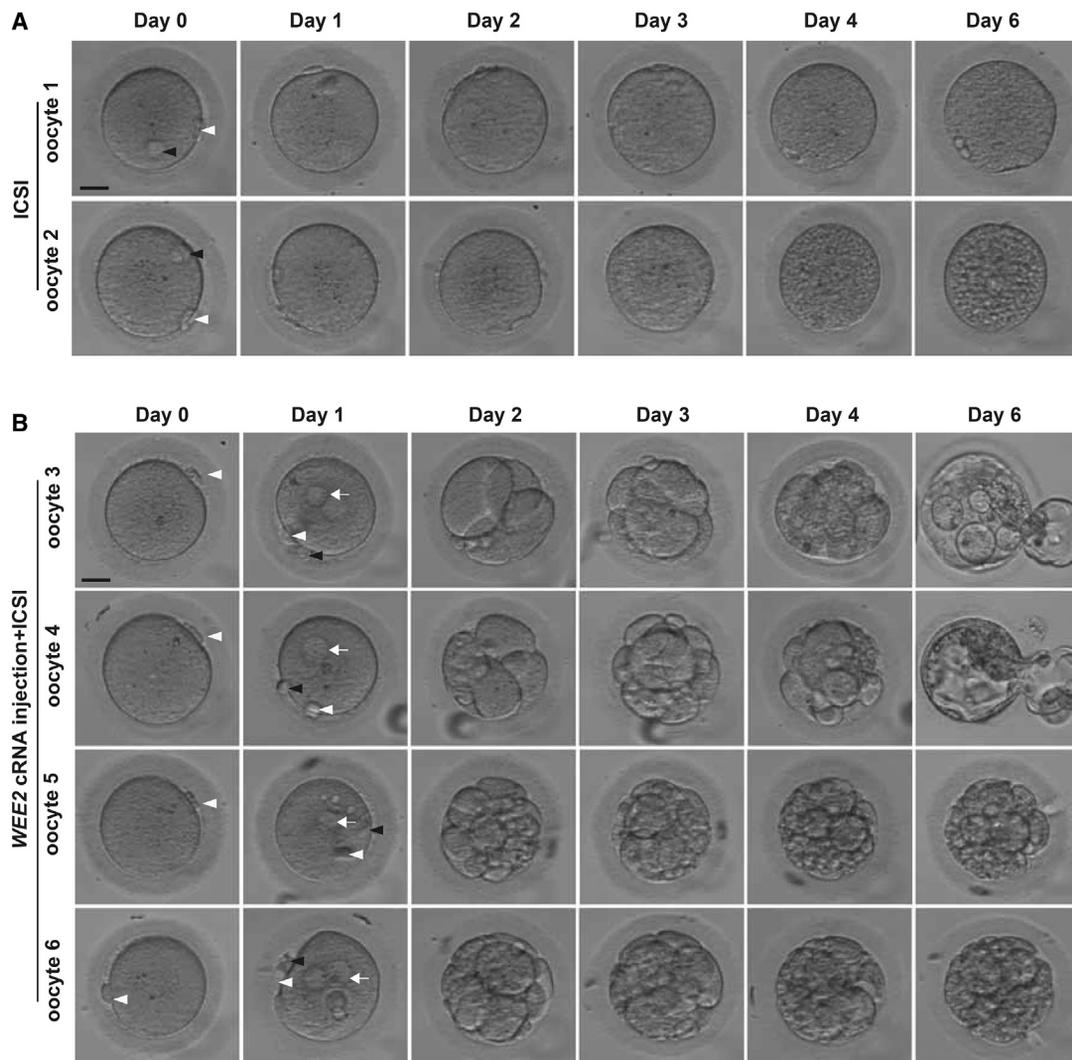


Figure 4. Phenotypic Rescue by *WEE2* cRNA Injection into Oocytes from the Proband from Family 4

(A) In the control group, two retrieved PB1 oocytes underwent ICSI without *WEE2* cRNA injection. Day 0 indicates the time point at 4 hr after ICSI. The white arrowhead indicates the first polar body, and the black arrowhead indicates the second polar body. Scale bar = 40 μm.

(B) In the experimental group, four retrieved PB1 oocytes were injected with *WEE2* cRNA and cultured for 4 hr and then used for ICSI. Day 0 indicates the time point at 4 hr after cRNA injection and just before ICSI. Oocytes were monitored for 6 days after ICSI. White arrows indicate the pronuclei. Scale bar = 40 μm.

as *Wee2* knockdown in mouse oocytes. In addition, all of these mutations decreased the level of pY15-Cdc2 (Figures 3 and S4C). These results indicate that MPF activity is also presumably high in affected individuals' oocytes and that this leads to the phenotype of fertilization failure. By direct injection of *WEE2* cRNA into oocytes from the proband in family 4, we showed that the oocytes could be successfully fertilized and that normal blastocysts could be formed. Further studies in terms of safety and efficacy still need to be performed, but our present results suggest a novel therapeutic approach for these affected individuals with mutations in *WEE2*.

Female infertility can result from several factors, including dysfunctions in ovary development, folliculogenesis, gamete maturation and recognition, early embryonic development, etc.^{20–22} However, the genetic basis for

infertility characterized by abnormalities in human oocyte development, fertilization, and early embryogenesis is not well understood. With the widespread application of IVF for infertile couples, the genetic factors behind the Mendelian phenotypes observed in these procedures are becoming clearer. Recently, we identified mutations in a few novel genes that are responsible for female infertility caused by oocyte maturation arrest (MIM: 616780, 617743) and early embryonic arrest (MIM: 617234).^{8,23–25} For example, mutations in *TUBB8* (MIM: 616768), encoding a primate-specific β-tubulin isotype with previously unknown function, cause oocyte MI arrest by impairing oocyte microtubule stability and spindle assembly.⁸ Different mutations in *TUBB8* also cause multiplicity of phenotype in human oocytes and early embryos.²³ Biallelic mutations in *PATL2* (MIM: 614661) are responsible for

oocytes phenotypic variability, including oocyte arrest at the GV stage and MI stage and oocytes with an abnormally large polar body.²⁴ Mutations in *PADI6* (MIM: 610363) caused early embryonic arrest by destroying the subcortical maternal complex (SCMC) complex.²⁵ In addition, Alazami et al.² identified a single missense mutation in *TLE6* that caused fertilization failure by impairing TLE6 phosphorylation and affecting SCMC formation.² Huang et al. identified a mutation in *ZPI* (MIM: 195000) that is responsible for a unique form of familial infertility characterized by abnormal eggs that lack a zona pellucida.²⁶ These findings indicate that some phenotypes in early human reproductive processes follow Mendelian patterns. Although more than 5,000 Mendelian disorders have been recorded in humans, most of these are related to traits seen after birth. In the genomic era, systematic genetic analysis and deepened as well as broadened phenotype analysis will help uncover novel genes, pathways, and functions involved in human reproduction. Thus, by investigating the Mendelian phenotypes underlying early human reproduction process, the molecular mechanisms behind the processes can be elucidated, and such findings will facilitate genetic diagnoses and help to guide the therapeutic pathway of infertility affected individuals in the future.

Supplemental Data

Supplemental Data include five figures and can be found with this article online at <https://doi.org/10.1016/j.ajhg.2018.02.015>.

Acknowledgments

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Web Resources

1000 Genomes, <http://www.internationalgenome.org/>
dbSNP, <https://www.ncbi.nlm.nih.gov/projects/SNP/>
ExAC Browser, <http://exac.broadinstitute.org/>
GenBank, <https://www.ncbi.nlm.nih.gov/genbank/>
OMIM, <http://www.omim.org/>
PolyPhen-2, <http://genetics.bwh.harvard.edu/pph2/>
RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>
SIFT, <http://sift.bii.a-star.edu.sg/>

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