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Mutations in TUBB8 and Human Oocyte Meiotic Arrest

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BACKGROUND

Human reproduction depends on the fusion of a mature oocyte with a sperm cell to form a fertilized egg. The genetic events that lead to the arrest of human oocyte maturation are unknown.

METHODS

We sequenced the exomes of five members of a four-generation family, three of whom had infertility due to oocyte meiosis I arrest. We performed Sanger sequencing of a candidate gene, TUBB8, in DNA samples from these members, additional family members, and members of 23 other affected families. The expression of TUBB8 and all other β-tubulin isotypes was assessed in human oocytes, early embryos, sperm cells, and several somatic tissues by means of a quantitative reverse-transcriptase–polymerase-chain-reaction assay. We evaluated the effect of the TUBB8 mutations on the assembly of the heterodimer consisting of one α-tubulin polypeptide and one β-tubulin polypeptide (α/β-tubulin heterodimer) in vitro, on microtubule architecture in HeLa cells, on microtubule dynamics in yeast cells, and on spindle assembly in mouse and human oocytes.

RESULTS

We identified seven mutations in the primate-specific gene TUBB8 that were responsible for oocyte meiosis I arrest in 7 of the 24 families. TUBB8 expression is unique to oocytes and the early embryo, in which this gene accounts for almost all the expressed β-tubulin. The mutations affect chaperone-dependent folding and assembly of the αβ-tubulin heterodimer, disrupt microtubule behavior on expression in cultured cells, alter microtubule dynamics in vivo, and cause catastrophic spindle-assembly defects and maturation arrest on expression in mouse and human oocytes.

CONCLUSIONS

TUBB8 mutations have dominant-negative effects that disrupt microtubule behavior and oocyte meiotic spindle assembly and maturation, causing female infertility. (Funded by the National Basic Research Program of China and others.)
SUCCESSFUL HUMAN REPRODUCTION starts when a metaphase II oocyte fuses with a sperm cell to form a fertilized egg. In human oocytes, the meiotic cell cycle begins in the neonatal ovary and pauses at prophase I of meiosis until puberty, when a surge of luteinizing hormone stimulates the resumption of meiosis and ovulation. This leads to progression of the oocyte from metaphase I to metaphase II.1,2 Oocytes arrested in prophase I have an intact nucleus, termed the germinal vesicle, whereas oocytes that have resumed meiosis are characterized by the breakdown of the germinal vesicle. After germinal-vesicle breakdown, metaphase I is completed by extrusion of a polar body and asymmetric division. Mature oocytes are again arrested at metaphase II.3 In most mammals, this is the only stage at which oocytes can be successfully fertilized.1

In vitro fertilization (IVF) now accounts for 1 to 3% of births worldwide.4 It is common for some oocytes to remain immature after ovarian stimulation and administration of human chorionic gonadotropin,5 but complete arrest of oocyte maturation has been reported in only a few women,6,7 and nothing is known about the genetic cause of this phenotype.

RESULTS
TUBB8 AND ARREST OF HUMAN OOCYTE MATURATION
We identified a four-generation family (Family 1) with a rare autosomal dominant pattern of inheritance of primary female infertility (Fig. 1). The spouses of the affected women had normal sperm counts, with normal sperm morphologic features and motility. Attempted IVF in Patient III-5 resulted in oocytes that were in metaphase I (Table 1); these oocytes failed to mature even after extended culture in vitro. In Patient III-4, intracytoplasmic sperm injection yielded three oocytes in metaphase I and one with abnormal morphologic features; none of these oocytes had a visible spindle on polarization microscopy.

Figure 1 (facing page). Pedigrees and TUBB8 Status of Families Affected by Oocyte Arrest.
Pedigrees of seven families with inherited or de novo TUBB8 mutations are shown. Squares denote male family members, circles female family members, solid symbols affected family members, and open symbols unaffected family members. Short double horizontal lines indicate that there were no offspring. The arrows indicate the probands in Families 1 and 2. Family 1 includes five affected women with long-term primary infertility. The V229A mutation in TUBB8 was inherited from the father of each affected woman. Family member IV-2 is a 7-year-old girl carrying the mutation who could not be evaluated for fertility. The D417N, M363T, R2K, and M300I mutations were identified in Families 2, 5, 6, and 7, respectively; paternal transmission is evident. The S176L and R262Q mutations identified in Families 3 and 4 are de novo. Sanger sequencing chromatograms are shown near the pedigrees. W denotes wild type.

STUDY PARTICIPANTS
Patients with oocyte maturation arrest from 24 families were referred from the Reproductive Medicine Center at Ninth Hospital, which is affiliated with Shanghai Jiao Tong University, the Shanghai Ji Ai Genetics and IVF Institute, and the Shaanxi Maternal and Child Care Service Center. The institutional review board of Shanghai Medical College of Fudan University approved the study, and the participants provided written informed consent. Additional information is provided in the Methods section in the Supplementary Appendix, available with the full text of this article at NEJM.org.

METHODS
STUDY PROcedures
We sequenced the exomes of three affected women and two unaffected women from Family 1, using Agilent SureSelect Whole Exome capture and Illumina sequencing technology. A variant was considered to be a candidate mutation if it was present in all three affected women, was absent in the two controls, and had not previously been reported or if it had a prevalence below 0.1% in the 1000 Genomes variant database, the NHLBI (National Heart, Lung, and Blood Institute) Exome Sequencing Project database, and the Exome Aggregation Consortium. We performed Sanger sequencing of the candidate gene in members of Families 1 through 24 (see the Methods section in the Supplementary Appendix for further details). We assayed gene expression in oocytes, predicted the effects of the candidate mutations on protein structure, and tested the functional effects of the mutations in vitro, in HEIa cells, in yeast, and in mouse and human oocytes (see the Methods section in the Supplementary Appendix).
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(Fig. 2A and 2B), and the morphologically abnormal oocyte later failed to become fertilized. Whole-exome sequence analysis in five members of Family 1 (three affected and two unaffected) implicated a single mutation—a heterozygous missense c.T686C (p.V229A) mutation in the coding region of TUBB8, a gene that encodes a primate-specific β-tubulin isotype, the func-

<table>
<thead>
<tr>
<th>Family 1</th>
<th>Family 2</th>
<th>Family 3</th>
<th>Family 4</th>
<th>Family 5</th>
<th>Family 6</th>
<th>Family 7</th>
</tr>
</thead>
</table>

- **Primary infertility**
- **Unevaluated fertility**
tion of which had not been determined. This mutation cosegregated with female infertility in Family 1 and was characterized by paternal transmission (Fig. 1). We subsequently identified six other \textit{TUBB8} mutations in seven infertile patients with a similar phenotype of oocyte maturation arrest: two patients in Family 2 (c.G1249A, p.D417N), a patient in Family 5 (c.T1088C, p.M363T), a patient in Family 6 (c.G5A, p.R2K), and a patient in Family 7 (c.G900A, p.M300I) (all these mutations were paternally transmitted), as well as de novo mutations in a patient in Family 3 (c.C527T, p.S176L) and a patient in Family 4 (c.G785A, p.R262Q) (Fig. 1). No mutations in \textit{TUBB8} were detected in 17 additional patients who were from the 17 other families with primary infertility. Each patient in Families 2 through 7 underwent two to five failed IVF or intracytoplasmic sperm injection attempts. Almost all oocytes harvested during these attempts were arrested at metaphase I, and none had a visible spindle (Table 1, and Fig. S1 in the Supplementary Appendix). Immunostaining of one metaphase I oocyte each from the patients in Families 1, 2, 3, 4, and 6 revealed either an abnormal spindle or no detectable spindle (Fig. 2C). Thus, all the patients were devoid of mature metaphase II oocytes.

These results and an analysis of allele-specific expression in a single S176L oocyte (Fig. S2A and S2B in the Supplementary Appendix) implied that the \textit{TUBB8} mutations were likely to have been responsible for oocyte maturation arrest in Families 1 through 7. The location and strict evolutionary conservation of the affected residues are shown in Figure S3 in the Supplementary Appendix.

**\textit{TUBB8} and \(\beta\)-\textit{Tubulin Supply in Human Oocytes**

Microtubules are dynamic polymers assembled from heterodimers consisting of one \(\alpha\)-\textit{tubulin} polypeptide and one \(\beta\)-\textit{tubulin} polypeptide (\(\alpha/\beta\)-\textit{tubulin}).\(^{13}\) Nine \(\beta\)-\textit{tubulin} isotypes are expressed in mammals; these are primarily distinguished by variations in the acidic C-terminal tail that affect specific cellular functions.\(^{14}\) Mutations in \textit{TUBB1}, \textit{TUBB2A}, \textit{TUBB2B}, \textit{TUBB3}, \textit{TUBB4A}, and \textit{TUBB4B} have been described; these cause a broad range of diseases, usually involving microtubule-based defects in neuronal migration.\(^{15-17}\) Although \textit{TUBB8} has the sequence of a \textit{tubulin} gene, its function is unknown. It is unusual in that it exists only in primates. We hypothesized that in primates, \textit{TUBB8} influences microtubule behavior in oocytes, in which the self-organization of microtubules and motor proteins direct meiotic spindle assembly and chromosome orientation.\(^{18,19}\) Strikingly, we found that \textit{TUBB8} is the only \(\beta\)-\textit{tubulin} isotype expressed at a high level at different stages of human oocyte development and is essentially absent in mature sperm and in somatic tissues such as the brain and liver (Fig. S4 in the Supplementary Appendix). Immunostaining of human oocytes at different developmental stages with the use of an ostensibly specific anti-TUBB8 antibody — or an anti-FLAG

<table>
<thead>
<tr>
<th>Family and Patient No.</th>
<th>Mutation</th>
<th>Age (yr)</th>
<th>Duration of Infertility</th>
<th>No. of Previous IVF or ICSI Cycles</th>
<th>Total No. of Oocytes Retrieved</th>
<th>Stage or Stages of Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family 1, Patient III-4</td>
<td>V229A</td>
<td>37</td>
<td>8</td>
<td>1</td>
<td>4</td>
<td>3 in MI, 1 with abnormal morphologic features</td>
</tr>
<tr>
<td>Family 1, Patient III-5</td>
<td>V229A</td>
<td>32</td>
<td>10</td>
<td>2</td>
<td>21</td>
<td>21 in MI</td>
</tr>
<tr>
<td>Family 2, Patient II-2</td>
<td>D417N</td>
<td>37</td>
<td>9</td>
<td>9</td>
<td>37</td>
<td>7 in GV, 30 in MI</td>
</tr>
<tr>
<td>Family 3, Patient II-1</td>
<td>S176L</td>
<td>34</td>
<td>10</td>
<td>4</td>
<td>43</td>
<td>3 in GV, 40 in MI</td>
</tr>
<tr>
<td>Family 4, Patient II-1</td>
<td>R262Q</td>
<td>37</td>
<td>10</td>
<td>2</td>
<td>12</td>
<td>12 in MI</td>
</tr>
<tr>
<td>Family 5, Patient II-1</td>
<td>M363T</td>
<td>25</td>
<td>4</td>
<td>3</td>
<td>18</td>
<td>1 in GV, 17 in MI</td>
</tr>
<tr>
<td>Family 6, Patient II-1</td>
<td>R2K</td>
<td>33</td>
<td>7</td>
<td>2</td>
<td>54</td>
<td>2 in GV, S2 in MI</td>
</tr>
<tr>
<td>Family 7, Patient II-1</td>
<td>M300I</td>
<td>26</td>
<td>6</td>
<td>2</td>
<td>26</td>
<td>26 in MI</td>
</tr>
</tbody>
</table>

* GV denotes germinal vesicle, ICSI intracytoplasmic sperm injection, IVF in vitro fertilization, and MI metaphase I.
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antibody, in the case of a human germinal-vesicle oocyte microinjected with TUBB8–FLAG complementary RNA — confirmed that TUBB8 is indeed localized to the spindle (Fig. S5 in the Supplementary Appendix).

STRUCTURAL IMPLICATIONS

We mapped the mutant TUBB8 residues onto the atomic structure of tubulin (Protein Data Bank [PDB] code 3JAS). S176 is located at a longitudinal interface between assembled dimers, where it interacts with α-tubulin (Fig. S6A in the Supplementary Appendix), and lies in a key region (V177-S178-D179) in the β-T5 loop that changes on guanosine triphosphate (GTP) hydrolysis. It is likely that the S176L mutation disrupts longitudinal interactions, thus inhibiting microtubule assembly. M363 may interact with V288 within the M-loop region of the protein (Fig. S6B in the Supplementary Appendix); the M363T mutation could potentially weaken the interaction with V288 and thus cause microtubule instability. D417 and R262 interact, forming a salt bridge on the outside surface of the microtubule (Fig. S6C in the Supplementary Appendix); this bridge would be broken by the D417N and R262Q mutations. D417 is involved in kinesin binding, and its mutation would probably negatively affect the interaction between microtubules and kinesin and possibly other microtubule-associated proteins. M300 and V229 are buried within the β-tubulin subunit, and their mutation could destabilize its folding (Fig. S6B in the Supplementary Appendix). Finally, R2 is located at the α–β interface within the hetero-
The dimer (Fig. S6A in the Supplementary Appendix); its substitution could affect dimer assembly and stability.

**α/β-Tubulin Heterodimer Assembly In Vitro**

The de novo generation of tubulin heterodimers requires the concerted action of a spectrum of chaperones: prefoldin (PFD), the ATP-dependent cytosolic chaperonin (CCT), and five tubulin-specific chaperones (TBCA, TBCB, TBCC, TBCD, and TBCE) that function in concert downstream of CCT as a GTP-dependent heterodimer-assembly nanomachine. To investigate potential folding defects incurred as a result of mutation, we monitored the assembly of 35S-labeled wild-type and mutant-bearing TUBB8 polypeptides kinetically. None of the mutations had any influence on translational efficiency (Fig. S7A in the Supplementary Appendix). However, the TUBB8 mutant proteins showed a spectrum of quantita-

![Figure 3. Effects of Wild-Type and Mutant Forms of TUBB8 on Microtubules in HeLa Cells.](image-url)

HeLa cells were transfected with constructs engineered to express C-terminally FLAG-tagged TUBB8 (wild type and mutant). Panel A shows the results of immunostaining with antibody to the FLAG epitope to detect expression of the transgene (green) and immunostaining with antibody to α-tubulin to detect the endogenous microtubule network (red). Shown are examples of cells expressing the various transgenes, with microtubules assembled into a normal or abnormal interphase network or with complete obliteration of the microtubule network; in the latter cells, the FLAG label appears as a diffusely mottled pattern throughout the cytoplasm. The bar indicates 10 μm. Panel B shows a quantitative analysis of the microtubule phenotypes shown in Panel A. Low expression of mutant TUBB8 was typically associated with the normal phenotype, whereas intermediate and high expression of mutant TUBB8 was typically associated with the abnormal and obliterated phenotypes. Approximately 200 transfected cells expressing either wild-type or mutant TUBB8 were examined in each of three separate experiments. The mean percentages of cells assigned to each phenotypic category (normal, abnormal, or obliterated microtubule network) are shown; error bars indicate standard deviations.
tive differences in the characteristic flow of label from the PFD–β-tubulin and CCT–β-tubulin binary complexes to TBCA–β-tubulin and TBCD–β-tubulin, as compared with the wild-type control (Fig. S7B and Table S2 in the Supplementary Appendix). These data reveal a range of heterodimer-assembly defects caused by the TUBB8 mutations. Some of these defects are attributable either to changes in the equilibria that govern the de novo assembly of heterodimers or to misfolding; most lead to a diminished yield of assembled heterodimers.

**Microtubule Disruption In Vivo**

To determine the influence of the TUBB8 mutations on microtubule behavior in vivo, we transfected FLAG-tagged constructs into cultured (HeLa) cells. In the case of wild-type TUBB8, we observed coassembly into a normal microtubule network (Fig. 3A), except at high levels of transgene expression. In contrast, mutant TUBB8, at intermediate or high levels of expression, was incorporated into microtubules with an abnormal appearance and frequently caused complete loss of the microtubule network (“obliteration”). The obliteration phenotype was most strongly associated with the mutations predicted to interfere with heterodimer stability, β-tubulin folding, or polymerization (V229A, S176L, M363T, R2K, and M300I), whereas an altered microtubule organization was caused by mutations predicted to interfere with kinesin binding (R262Q and D417N) (Fig. 3B).

We repeated these expression experiments using constructs in which we introduced three of the identified TUBB8 mutations into TUBB5 (also termed TUBB), a β-tubulin isotype that, unlike TUBB8, is broadly expressed in mammalian tissues, especially in the developing central nervous system. We found that wild-type TUBB5–FLAG invariably became incorporated into a normal microtubule network, whereas expression of S176L, M363T, and R262Q in the context of TUBB5–FLAG caused a range of abnormal microtubule phenotypes that were similar to those seen in parallel experiments performed in the context of TUBB8–FLAG, but at a greatly reduced frequency (Fig. S8 in the Supplementary Appendix). We conclude that the in vivo expression of mutated TUBB8 results in disruption of microtubule architecture, with the degree of disruption depending on the mutation, and that the disruption is strongly influenced by the β-tubulin isotype context.

To assess the ability of the TUBB8 mutations to interfere with microtubule dynamics, we examined the consequences of embedding them in TUB2 in Saccharomyces cerevisiae (Fig. S9 in the Supplementary Appendix). We obtained strains of diploid yeast bearing each mutation in the heterozygous state, with the exception of S176L. We found that, like the spores of yeast bearing the D417N mutation, those of strains bearing the V229A mutation or the R262Q mutation were not viable, an observation that is consistent with substantive disruption of microtubule function (Fig. S10 in the Supplementary Appendix). Haploid spores harboring R2K, M300I, and M363T were viable but with varying degrees of growth impairment.

As compared with the control strain, strains with the R262Q and D417N mutations had greater resistance to the microtubule-destabilizing drug benomyl, reflecting unusually stable microtubules in vivo. Conversely, the R2K, V229A, M300I, and M363T mutations all decreased benomyl resistance, suggesting that they dominantly destabilize microtubules in vivo (Fig. S11A and S11B in the Supplementary Appendix). Moreover, in cells expressing green fluorescent protein–α-tubulin, astral microtubules in heterozygous V229A and R2K cells depolymerized nearly twice as quickly and the rescue frequency (i.e., the frequency with which catastrophic microtubule depolymerization reverts to microtubule growth) was markedly reduced, as compared with control cells (Fig. S11C in the Supplementary Appendix). Although the depolymerization rates of microtubules in R262Q cells were similar to the rates in control cells, the catastrophe frequency (i.e., the frequency with which growing microtubules suddenly undergo catastrophic depolymerization) and rescue frequency were reduced, and the time spent attenuated (neither growing nor shrinking) was increased (Fig. S11D in the Supplementary Appendix).

**TUBB8 and Impaired Spindle Assembly**

To establish the causal relationship between mutations in TUBB8 and the infertility phenotype, we microinjected wild-type and mutated TUBB8 RNA in mouse oocytes. For oocytes microinjected with wild-type TUBB8 RNA, maturation was normal approximately 12 hours after germi-
nal-vesicle breakdown, with a clearly visible, barrel-shaped spindle (Fig. S12 in the Supplementary Appendix). In contrast, microinjection of any of the mutated TUBB8 RNAs resulted in maturation arrest and a misshapen spindle, as well as a reduction in the rate of extrusion of the first polar body (rate of 6 to 33% vs. a mean [±SD] rate of 61±2.2% in the wild-type control) (Fig. 4A).

We repeated these experiments using a higher concentration of wild-type, S176L, and D417N TUBB8 RNA (1000 ng per microliter). The higher concentration resulted in severely or completely impaired spindle assembly with both mutated RNAs (Fig. 4B): the phenotype of human oocytes in maturation arrest. We also tested the extent to which the tubulin isotype context contributes to these effects by microinjecting mouse oocytes with RNAs encoding either wild-type or mutant (S176L or D417N) TUBB8 12 hours (mouse) and 16 hours (human) after germinal-vesicle breakdown. The MI oocytes were stained with Hoechst 33342 to visualize chromosomes (blue), β-tubulin to visualize spindles (green), and FLAG to visualize TUBB8 (red).
Although the process of oocyte maturation has been extensively investigated in mice, the genetic cause of human oocyte maturation arrest was previously unknown. We have identified several mutations in TUBB8, which encodes a β-tubulin isotype of hitherto undetermined function. These mutations interfere with human oocyte maturation and either are inherited paternally in an autosomal dominant fashion or arise de novo (Fig. 1 and Table 1). They are in several cases predicted to confer changes in the structure of the tubulin heterodimer (Fig. S6 in the Supplementary Appendix). The mutations were found to affect α/β-tubulin heterodimer folding and assembly in vitro (Fig. S7 in the Supplementary Appendix), to cause a spectrum of striking microtubule phenotypes on expression in cultured cells (Fig. 3), and to alter microtubule dynamics in vivo (Fig. S11 in the Supplementary Appendix). Moreover, the TUBB8 mutations caused oocyte maturation defects on expression in mouse and human oocytes that precisely mimic the infertility phenotype (Fig. 4). We conclude from these observations that mutations in TUBB8 cause oocyte maturation arrest and that TUBB8 has a key role in meiotic spindle assembly and maturation in human oocytes. We note that the data presented here provide the basis for developing diagnostic tools that use the polymerase chain reaction for the identification of women with mutations in TUBB8.

TUBB8 is uniquely expressed in human oocytes and early embryos, where it accounts for most of the β-tubulin that is present (Fig. S4 in the Supplementary Appendix). It follows that human oocyte spindles consist of microtubules polymerized from heterodimers containing a single preponderant β-tubulin isotype. This finding reinforces the hypothesis that different tubulin isotypes can confer unique properties in functionally distinct subsets of microtubules. The ability of mutant TUBB8 to cause microtubule disruption on expression in cultured cells and in oocytes suggests a model in which a dominant and cumulative effect occurs when a critical proportion of mutant heterodimers are incorporated into polymers, leading to greater instability, as compared with microtubules in wild-type cells, and frequently to complete annihilation of microtubules.

The fact that TUBB8 is present only in primates suggests that the gene has had a unique role in the evolution of these species. Males with mutations in TUBB8 are fertile, which is consistent with the absence of TUBB8 expression in mature sperm and highlights differences that distinguish meiosis and spindle formation in primate males and females. We found TUBB8 mutations in a high proportion of infertile women (7 of 24) with oocyte maturation arrest. The absence of an observed mutation in TUBB8 in some of the affected women implies that defects in other, as yet undiscovered genes can also contribute to human oocyte maturation arrest.
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