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ORIGINAL RESEARCH

Recurrent triploidy due to a failure to complete maternal meiosis II: whole-exome sequencing reveals candidate variants

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ABSTRACT: Triploidy is a relatively common cause of miscarriage; however, recurrent triploidy has rarely been reported. A healthy 34-year-old woman was ascertained because of 18 consecutive miscarriages with triploidy found in all 5 karyotyped losses. Molecular results in a sixth loss were also consistent with triploidy. Genotyping of markers near the centromere on multiple chromosomes suggested that all six triploid concep-tuses occurred as a result of failure to complete meiosis II (MII). The proband's mother had also experienced recurrent miscarriage, with a total of 18 miscarriages. Based on the hypothesis that an inherited autosomal-dominant maternal predisposition would explain the phenotype, whole-exome sequencing of the proband and her parents was undertaken to identify potential candidate variants. After filtering for quality and rarity, potentially damaging variants shared between the proband and her mother were identified in 47 genes. Variants in genes coding for proteins impli-cated in oocyte maturation, oocyte activation or polar body extrusion were then prioritized. Eight of the most promising candidate variants were confirmed by Sanger sequencing. These included a novel change in the *PLCD4* gene, and a rare variant in the *OSBPL5* gene, which have been impli-cated in oocyte activation upon fertilization and completion of MII. Several variants in genes coding proteins playing a role in oocyte maturation and early embryonic development were also identified. The genes identified may be candidates for the study in other women experiencing recurrent triploidy or recurrent IVF failure.

Key words: triploidy / oogenesis / meiosis / whole-exome sequencing

Introduction

Triploidy is one of the more common abnormalities to occur in preg-nancy and accounts for ~1% of conceptions and ~10% of early spontan-eous abortions (SAs) (Hassold *et al.*, 1980). It occurs independently of maternal age and may be the result of either digyny (extra haploid set from mother) or diandry (extra haploid set from father) (McFadden and Kalousek, 1991). Digynic triploidy predominates in cases with iden-tifiable fetuses while diandry is slightly more common in early triploid SAs that lack embryos (McFadden and Kalousek, 1991). Some mechanisms for the occurrence of triploidy include failure to extrude the first polar body (PB) at maternal meiosis I (MI); failure to divide or failure to extrude the second PB at meiosis II (MII) or, in the case of diandry, poly-spermy (McFadden and Langlois, 2000; Zaragoza *et al.*, 2000; McFadden *et al.*, 2002). While failure of paternal MI or MII could result in fertilization by a diploid sperm (Zaragoza *et al.*, 2000; Rosenbusch, 2008), there is no conclusive molecular data to support that such sperm contribute to triploid pregnancies (McFadden *et al.*, 2002).

There is little evidence for a propensity to recurrence of triploidy in the general population (Robinson *et al.*, 2001). Hence, the occurrence of two triploid pregnancies from one parent is usually attributed to chance. Recurrent triploidy, defined as three or more triploid concep-tions from one couple, has rarely been reported. Pergament *et al.* (2000) reported on a woman with two triploid pregnancies, with an

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additional 2 triploid embryos identified from 13 embryos produced after IVF using ICSI. It was inferred that these were due to errors of MII division because the oocytes showed the presence of one PB and only two pronuclei were observed at fertilization (Pergament *et al.*, 2000). Brancati *et al.* (2003) reported on a 36-year-old woman with three consecutive digynic triploid pregnancies consistent with either maternal MI or MII errors. Huang *et al.* (2004) similarly reported on a woman who at age 31 had had three triploid losses, all of which were determined to be due to maternal MII errors. An additional case with three triploid losses was reported in a mother with low level 45, X mosaicism, but molecular testing was not performed in this case (Johnson *et al.*, 2000).

We hereby report a case with at least six triploid conceptions, each consistent with a failure of maternal MII. A number of rare genetic variants were identified by whole-exome sequencing, and we discuss the possible candidates responsible for this phenotype.

Materials and Methods

Ethics statement

Ethics approval for this study was obtained from the University of British Columbia Clinical Research Ethics Board (H01-70460, Study of Epigenetics of Reproduction and Fertility; H12-00145, Imprinting in Placenta).

Patient and family history details

The proband at age 34 had experienced 18 consecutive SAs all between 5.5 and 12 weeks of gestation and no live births (G18SA18). Her first loss was at age 26. Five SAs were karyotyped with three being diagnosed as 69, XXX and two as 69, XXY. A sixth loss was evaluated by diagnostic array comparative genomic hybridization (oligo-based array) after culture failure and did not show aneuploidy, but triploidy cannot be detected with this approach. Parental karyotypes were normal. Normal test results were obtained for anticardiolipin antibodies, lupus anticoagulant, thrombophilia screen and thyroid-stimulating hormone. The proband is otherwise in good health and had no difficulty conceiving. There is no reported consanguinity. The proband's family history is significant in that the proband's mother, who is also otherwise healthy, was reported to have had 18 SAs, as well as 2 live births (the proband and her brother). The reproductive history of the brother is unknown. The maternal grandmother was not thought to have experienced recurrent miscarriage.

Previous investigations for a mutation in *NLRP7* and *C6orf221 (KHDC3L)* in the proband were negative (Manokhina *et al.*, 2013).

Molecular genotyping

DNA was extracted using standard procedures from all six available chorionic villus samples from miscarriage specimens, as well as from blood samples from the proband and both of her parents. Parental and meiotic origin of the extra set of chromosomes was determined by comparing microsatellite marker inheritance patterns in the triploid placenta to that of the maternal DNA as we have done previously (McFadden *et al.*, 2002; McFadden and Robinson, 2006). Markers used to distinguish MI from MII errors mapped near (<5 centiMorgans (cM) from the centromere (http://cedar.genetics. soton.ac.uk/pub) and were from multiple chromosomes (Table I). Digyny is expected to be associated with inheritance of either both maternal alleles or a double dose of one maternal allele at all loci, whereas diandry would be expected to show two copies of a non-maternal allele. Digyny as the result of an MI error should show non-reduction at all centromeric markers, while a failure of MII should show reduction to homozygosity at all markers near the centromeres.

Methylation analysis

Recurrent triploidy has been suggested to be a consequence of *NLRP7* mutation in some cases (Slim *et al.*, 2011), and *NLRP7* mutations have been associated with the loss of maternal methylation imprints; therefore, we wanted to confirm that such methylation imprints were set appropriately in these cases. We estimated methylation at the following imprinting control regions: *H19* (ICR1), *KvDMR1* (ICR2) and *SGCE* by pyrosequencing. The assays used and results in normal placenta were described previously (Bourque *et al.*, 2011).

Whole-exome sequencing

Whole-exome sequencing on the proband and both her parents was performed by Perkin Elmer using the Illumina Hiseq 2000 machine for 100 bp Paired end sequencing (Instrument control software 1.4.8; RTA version 1.12.4.2). Following targeted enrichment using the SureSelect (Agilent Technologies Canada, Inc.) 50 Mb v3 Capture kit, we obtained a yield of 20–22 Gb of sequence data (~84 000 000 reads) per individual. The mean exome coverage was 89-fold, with at least 80% of the exome covered more than 30 times. Mapping to the reference genome sequence (hg19) and alignment was done using both Bowtie and BWA algorithms in order to effectively align reads with substitutions as well as short indels. The −m1 option specifies bowtie to discard any reads that can be mapped to more than one location. Using samtools, sam.files were then converted to bam.files removing unaligned reads as well as alignments with a quality lower than a Phred quality score at 20 for the entire read. For local realignment, we used the GATK aligner, which searches for missing deletions in Bowtie + BWA procedure to reduce false positives. Variant calling was performed by using samtools mpileup. Single-nucleotide variants were filtered for minimum mapping quality of a Phred quality score at 30 allowing 99.9% base call accuracy.

The variants were annotated based on their frequency in the overall population and as being 'known' or 'novel' according to whether they had been reported in the public databases dbSNP or 1000 Genomes, variants were further assessed for their potential to disrupt protein function using the software 'Sorting Intolerant from Tolerant' (SIFT) (http://sift.bii.a-star. edu.sg/www/SIFT_AWS_help.html) (Ng and Henikoff, 2003), PolyPhen-2 (Adzhubei *et al.*, 2010) and Mutation Taster (Schwarz *et al.*, 2014). We deleted non-exonic variants and variants indicated as synonymous to obtain 10 000–15 000 rare (<0.01 minor allele frequency (MAF)) or novel variants for each individual. Given the hypothesized autosomal-dominant nature of the predisposition, heterozygous variants shared between the proband and her mother and not present in the father were filtered.

Confirmational Sanger sequencing

Eight selected rare non-synonymous candidates were confirmed using Sanger sequencing. An additional set of 17 blood DNA samples derived from women with a documented single MII digynic triploid pregnancy, ascertained as part of a previous study of origin of triploidy (McFadden and Robinson, 2006), was sequenced with primers for these candidate variants in order to test for the recurrence of the detected variants for the stratified population.

Primers were created using the Primer3 v0.4.0 software (http://bioinfo. ut.ee/primer3-0.4.0) and the UCSC reference genome (GRCh37/hg19). Primer sequences were as follows: *MBD4*, F: TACTGTCCCCCAGCAAAC AT, R: CACTAGGCTTCTAGTTGGGCTAA; *OSBPL5*, F: CTTCCGGTGT GCAGGATGT, R: AGGTGTTGTTCCCAGATGCT; *PLCD4*, F: CAAGAA AAGCAACCCAGTCA, R: CATTAGGAAGGCAGCCAGAG; *YES1*, F: GA GGATATGGATTTTGTCCTCA, R: TCCCAGTCCATTCTTACTGACA;

Table I Molecular genotyping results for the proband, her parents and six SAs.

Marker	Chromosomal band	Map start position (hg19)	Distance to centromere (cMa)	Proband mother	Proband father	Proband	SA5 69, XXY		SA10		SA11 69, XXX		SA16 69, XXX		SA17 69, XXY		SA18 69, XXY		Inferred partner genotype
DXS1003	Xp11.3	46419301		ab	a	aa	aa	ui	aaa	ui	aaa	ui	aaa	ui	aa	ui	aa	ui	*a*
DXS6941	Xp11.3	48235256		aa	a	aa	aa	ui	aaa	ui	aaa	ui	aaa	ui	aa	ui	aa	ui	*a*
AR	**Xq11.2**	**66800000**	**1**	**bc**	**a**	**ab**	**bb**	**R**	**bbc**	**R**	**bbc**	**R**	**aac**	**R**	**aa**	**R**	**NA**		*c*
D3S1271	**3q12.3**	**100734730**	**1**	**bb**	**ab**	**ab**	**aab**	**ui**	**aab**	**ui**	**aaa**	**R**	**aab**	**R**	**aab**	**ui**	**bbb**	**R**	*ab*
D5S822	**5q11.1**	**50511131**	**<1**	**ab**	**ac**	**bc**	**bbb**	**R**	**bbb**	**R**	**bbc**	**ui**	**bbb**	**R**	**bcc**	**ui**	**ccc**	**R**	*bc*
D12S87	**12p11.2**	**30387939**	**<5**	**de**	**ab**	**be**	**dee**	**R**	**bbc**	**R**	**bbd**	**R**	**bbc**	**R**	**dee**	**R**	**cee**	**R**	*cd*
D13S141	**13q12.11**	**20724459**	**<5**	**ab**	**bb**	**ab**	**aab**	**ui**	**aaa**	**R**	**aab**	**ui**	**aaa**	**R**	**aaa**	**R**	**aaa**	**R**	*aa or ab*
D13S175	13q12.11	20848380	<5	bc	cc	bc	bbc	R	bbb	R	abb	ui	bbb	R	bbb	R	bbb	R	*ab or bb*
D13S1236	13q12.11	22695996		be	cd	de	ade	N	aee	R	cee	R	aee	R	aee	R	aee	R	*ac*
D13S115	13q12.11	22842462		dd	ac	cd	bcd	N	bdd	R	ddd	R	bcd	N	bdd	R	bdd	R	*bd*
D13S232		23799762		ab	cd	bc	bcd	N	bbd	R	bbd	R	bcd	N	bbd	R	bbd	R	*dd?*
D16S403	16p12.2	23037651		dd	bc	bd	abd	N	abd	N	abb	R	abd	N	bbd	ui	bdd	ui	*ab or ad*
D16S401	16p12.1	24685957		ac	bc	cc	acc	ui	acc	ui	acc	ui	acc	ui	acc	ui	acc	ui	*aa?*
D16S3093	16p12.1	26745405		ab	bb	bb	bbd	ui	bbd	ui	bbd	ui	bbd	ui	bbc	ui	bbc	ui	*cd*
D16S753	**16p11.2**	**31273504**	**<2**	**bd**	**cc**	**bc**	**NA**		**NA**		**acc**	**R**	**acc**	**R**	**ccc**	**R**	**bbc**	**R**	*ac*
D16S3105	**16q12.1**	**46792100**	**<2**	**aa**	**aa**	**aa**	**aaa**	**ui**	**aaa**	**ui**	**aaa**	**ui**	**aaa**	**ui**	**aaa**	**ui**	**aaa**	**ui**	*aa?*
D16S3044	**16q12.1**	**47437536**	**<2**	**bd**	**ab**	**bd**	**dde**	**R**	**dde**	**R**	**bbe**	**R**	**bbe**	**R**	**bbc**	**R**	**bcd**	**N**	*ce*
D16S409	16q12.1	48775854		bb	ab	ab	bbb	R	bbb	R	aab	ui	aab	ui	aab	ui	abb	ui	*ab or bb*
D16S515	16q23.1	76517028		ab	cd	ad	acd	N	acd	N	acd	N	acd	N	aad	ui	aad	ui	*cd or ac*
D16S402	16q13.3	83293414		ad	cc	ac	abc	N	aac	ui	acc	ui	acc	ui	abc	N	abc	N	*ab or bc*
D18S1093	18q12.2	34019263		ad	cd	ad	acd	N	acd	N	abd	N	abd	N	acd	N	aac	R	*bc*
D18S1127	18q21.2	53371163		bd	ad	bd	dde	R	bde	N	bcd	N	cdd	R	bde	N	bbe	R	*ce*
D18S488	18q22.3	69404462		bd	ee	de	cde	N	cde	N	ade	N	cee	R	cde	N	ade	N	*ac*
D22S686	22q12.2	21393073		ab	ac	ac	aaa	R	aac	ui	aaa	R	aac	ui	aac	ui	aaa	R	*aa or ac*
D22S277	22q13.2	34543413		ad	bc	cd	cdd	ui	acd	N	cdd	ui	cdd	ui	acd	N	acd	N	*ac or ad*

For all cases and all informative centromeric markers (bolded), there was a reduction of maternal heterozygosity to homozygosity. Microsatellite genotyping results are designated by a letter with 'a' being the largest amplicon. For SAs, the transmission of maternal heterozygosity is indicated as N (non-reduced), R (reduced) or ui (uninformative).

aCentimorgans.

NLRP10, F: AGCAGACCACCCAGCAAA, R: ACGTGCTCCCTTCTCAT CAC; *BNC2*, F: TTCGGAGGGAGCTAAAGACC, R: CGAAGTTTCTCCC ACACCTT; *CSF-1R*, F: AGGGAAGAGCCAGAAAGGAG, R: AAGTGCT GGGAGGGCTTG; *CEP250*, F: CTGGGGCAAGAGAGAGAAGA, R: CC AGGACAGAGGCTTGTAGG. Sequencing reactions were performed with the Big Dye Terminator v3.1, run on an Applied Biosystems 3730xl DNA Analyzer (Life Technologies, USA), and evaluated with Chromas 2.33 (Technelysium Pty Ltd, Australia) and SeqDoC (Crowe, 2005) software.

Results

A maternal MII origin of triploidy

Molecular genotyping results are shown in Table I. Although paternal DNA was not available, the genotype could be inferred at many loci. All markers were consistent with maternal origin of the extra haploid set of chromosomes. As three alleles were observed at many loci, endo-reduplication of one pronucleus could be excluded and a maternal meiotic error could be inferred. A subset of microsatellite markers was chosen based on being very near (<5 cM) to the centromere. For all cases and all informative centromeric markers, there was a reduction of maternal heterozygosity to homozygosity, indicating that the triploids all resulted from a failure of the maternal MII division. In one case (SA18), the closest marker to the centromere on 16q (D16S3044) showed non-reduction, while the closest marker to the centromere on 16p (D16S753) showed reduction to homozygosity, indicating a recombin-ation event had occurred close to the centromere. Hence, this case was uninformative for the chromosome 16 centromere, but all other centromeric markers in this case were reduced to homozygosity, con-sistent with maternal MII failure. As the additional marker analysis demonstrates apparently normal levels of recombination, we assume that MI was normal.

Normal methylation at imprinted loci

The proband, her mother and five triploid samples were evaluated for methylation level at three imprinting control regions: *SGCE*, *H19* (ICR1) and KvDMR1 (ICR2). The triploid samples exhibited values in the expected range for dignic triploid placental villi (for miscarriages) (Bourque *et al.*, 2011), while the adult blood samples showed

methylation consistent with that observed for control blood (Penaher-rera *et al.*, 2010) (Supplementary data, Table SI).

Whole-exome sequencing

All filtered variants were manually inspected for gene function in relation to the phenotype. Specifically, genes known or predicted to be involved in cell cycle regulation, oocyte maturation, MII resumption or extrusion of the second PB and those involved in other basic cellular functions were prioritized. Within the prioritized variants, those involving genes conserved across species and harboring significant mutations predicted to be damaging were considered to be the most promising candidates. SIFT, PolyPhen-2, MutationTaster and the Human Splice Finder scores were reviewed for prediction of functional effects of the variants selected. Public databases, including 1000 Genomes and the exome variant server from the NHLBI GO Exome Sequencing Project, were reviewed for MAFs of those variant alleles. PubMed, OMIM, NCBI Gene, EST and Uni-ProtKB were reviewed for previous publications regarding candidate genes and related genes, as well as phenotypes in humans and other species, and functional and expression data. Reads were inspected for coverage using the Integrated Genome Browser to assess the likelihood of a variant being a true call before using Sanger sequencing for validation of selected variants in the proband and her mother.

We identified rare non-synonymous candidate variants predicted to be protein damaging and shared between the proband and her mother in 47 genes (Supplementary data, Table SII). The literature on gene expression in ovary or oocyte is heterogeneous and expression depends on the tissue used, developmental stages as well as the men-strual cycle, making it difficult to eliminate candidates based on expres-sion patterns. Over 7000 genes are expressed in the MII oocyte alone (Kocabas *et al.*, 2006). We selected eight genes for follow-up validation because they code for proteins known to be involved in cell cycle regu-lation or oocyte maturation (*CSF1R*, MIM 164770; *MBD4*, MIM 603574; *BNC2*, MIM 608669; *CEP250*, MIM 609689) or to be involved in MII re-sumption and extrusion of the second PB (*PLCD4*, MIM 605939; *OSBPL5*, MIM 606733; *YES1*, MIM 164880) (Table II). We also studied *NALP10* (MIM 609662) because it is closely related to the *NALP* gene family that was reported to be required for early embryonic development in the mouse and potentially involved in reproductive disorders in humans

Table II Eight candidate genes involved in cell cycle regulation or oocyte maturation with rare non-synonymous candidate variants that are shared between the proband and her mother.

Gene		Single nucleotide polymorphism	Minor allele frequency	Protein change*	Protein damage
PLCD4	Phospholipase C, delta 4	2,219501020,1,T/C	Novel	L696P	Damaging
OSBPL5	Oxysterol binding protein-like 5	11,3123481,1,C/G	Novel	G385R	Damaging
YES1	Proto-oncogene tyrosine-protein kinase Yes	18,745840,1,T/C	C: 0.009	I198V	Tolerant
MBD4	Methyl-CpG-binding domain protein 4	3,129150385,1,C/G	G: 0.004	D250H	Damaging
CSF1R	Colony stimulating factor 1 receptor	5,149435645,1,G/A	Novel	T833M	Damaging
NLRP10	NOD-like receptor family pyrin-domain containing 10	11,7982144,1,C/A	A: <0.001	D339Y	Damaging
CEP250	Centrosomal protein 250 kDa	20,34092555,1,A/G	G: 0.006	N2120D	Not scored
BNC2	Zinc finger protein basonuclin-2	9,16436952,1,G/C	C: 0.009	L414V	Tolerant

***Refers to:** NP_116115.1 (*PLCD4*); NP_001137535.1, NP_663613.1 (*OSBPL5*); NP_005424.1 (*YES1*); NP_001263202.1 (*MBD4*); NP_001275634.1, NP_005202.2 (*CSF1R*); NP_789791.1 (*NLRP10*); NP_009117.2 (*CEP250*); NP_060107.3 (*BNC2*).

(Meyer et al., 2009; Peng et al., 2012). The GEO database (http://www.ncbi.nlm.nih.gov/geoprofiles/) confirms expression of all candidate genes in the oocyte at the germinal vesicle and MII stage in the mouse, while microarray BIOGPS database (http://biogps.org) show positive expression in the ovary in normal human tissues.

Follow-up Sanger sequencing

The eight candidate variants were validated in the proband and her mother. None of the studied candidate variants were detected in the additional group of 17 samples from women with digynic MII triploid fetus in their pregnancy history (Table II).

Discussion

As triploidy is relatively common at conception, experiencing two cases of triploid miscarriage is most often due to chance (Robinson et al., 2001). There are few reports of recurrent (3+) triploidy in the literature (Pergament et al., 2000; Brancati et al., 2003; Huang et al., 2004). The present case is distinctive by having more losses in total and more confirmed triploid losses than any previously reported case. As the proband's mother had 2 live births from 23 pregnancies, a small number of eggs may mature and fertilize normally. The patient reported by Pergament et al., had a live birth using IVF and preimplantation genetic screening (PGS), having had two triploid pregnancies and two additional triploid conceptuses identified after IVF. Although the clinical utility of PGS is still debated, the use of IVF may enable the selection of fertilized eggs that have extruded a second PB and/or are found to be euploid by PGS.

Origin of triploidy

We performed detailed molecular genotyping that demonstrated that the recurrent triploidy was a consequence of an inherent failure to complete MII, as has also been observed in ∼75% of sporadic digynic triploidy (McFadden and Langlois, 2000; Zaragoza et al., 2000). Other described origins for triploidy, including fertilization of a binucleate/diploid [giant] oocyte (derived from a tetraploid progenitor) and endoreduplication (Rosenbusch, 2008) were excluded by molecular genotyping.

Usually, MII is initiated immediately after completion of MI during the oocyte maturation phase. At the time of ovulation, MII is arrested at metaphase. MII resumes after the sperm enters the egg. Sperm entry initiates a signaling cascade that likely involves activation of phospholipase C (PLC) (Suh et al., 2008; Kashir et al., 2010). PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce diacyl-glycerol and inositol trisphosphate (IP3). IP3 acts to increase calcium levels, which then triggers the extrusion of the second PB (Ullah et al., 2014). Digynic triploidy after assisted reproduction is commonly characterized by non-extrusion of the second PB and formation of three pronuclei (Grossmann et al., 1997; Rosenbusch, 2008). Also, in vitro creation of digynic triploid blastocysts is usually performed using cytochalasin-b, an inhibitor of actin microfilaments, which prevents PB extrusion (Niemierko, 1975; Speirs and Kaufman, 1989). Hence, defects in genes involved in oocyte activation or PB extrusion can be considered candidates explaining recurrent triploidy.

Several mouse strains produce oocytes that arrest in MI and fail to progress to MII; however, some of these eggs can be activated upon fertilization resulting in digynic triploid conceptuses (Kaufman and Speirs, 1987). The defect in the LT/Sv mouse strain has been linked to a prolonged spindle assembly checkpoint (Hupalowska et al., 2008). Interestingly, the ability of the LT/Sv oocytes to become activated upon fertilization was dependent on the levels of protein kinase C (alpha, delta and zeta) (Archacka et al., 2008). There have been a number of reports of women experiencing infertility due to arrest of oocytes at MI (Mrazek and Fulka, 2003; Heindryckx et al., 2011). Thus, mutations in genes coding for proteins involved in the oocyte maturation/meiotic cell cycle regulation may also be considered candidates contributing to recurrent triploidy.

Candidate genes involved in MII resumption/extrusion of the second PB

The PLCD4 gene encodes a member of the delta class of PLC enzymes. This gene is predominantly expressed in testes, where it functions in the acrosome reaction upon fertilization (Fukami et al., 2003). Male mice with a disruption of PLCD4 produced no or small sized litters and in vitro fertilization using PLCdelta4−/− sperm resulted in fewer eggs becoming activated (Fukami et al., 2001). While female mice lacking this gene were generally fertile, there was a slight reduction in fertility rates and litter size. Of interest, PLCD4 is specifically up-regulated in the mouse oocyte during MII (Oliveri et al., 2007). Furthermore, mouse eggs injected with IP3, a product of PLC activity, do not emit the second PB (Kurasawa et al., 1989). The mutation in our patient could have resulted in increased or decreased activity and/or interfered with functional PLCD4 in the oocyte, causing a failure of extrusion of the second PB. Such a mutation could also cause increased ability to activate an immature oocyte arrested at MI, thereby resulting in recurrent triploidy, rather than infertility in the presence of an independent oocyte maturation defect.

OSBPL5 encodes a member of the oxysterol-binding protein (OSBP) family, a group of intracellular lipid receptors that play a key role in the maintenance of cholesterol balance in the body. Experiments with cholesterol depletion in mouse disturbed the subcellular localization of the signal molecule c-Src, and subsequent inhibition of Src kinase proteins prevented second PB extrusion (Buschiazzo et al., 2013). Cholesterol repletion recovered the fertilization index and ability to extrude the second PB in cholesterol-depleted oocytes, indicating reversibility of these effects.

The protein tyrosine kinase YES1, the effector molecule of PLCD4, also carried a non-synonymous variant in our case in the highly conserved SH2 domain. Src-kinases Fyn and Yes were found to be specifically up-regulated in mouse oocytes during MII resumption and essential to this process (Luo et al., 2010). Non-specific inhibiting Src family kinase activity blocks MII; in contrast, microinjecting mRNAs of active forms of signal molecules Fyn or c-Yes into ovulated eggs triggered MII without evoking Ca^{2+} increase (Reut et al., 2007). The patient's mutation in YES1 is not predicted to have high damaging potential, but given its involvement in the same pathway as PLCD4, it may enhance the pathway damage in the presence of the PLCD4 mutation.

Candidate genes involved in cell cycle regulation/oocyte maturation

As discussed, the appearance of a defect in MII could result from a defect in the timing of oocyte maturation, such that MI, rather than MII, is completed at the time of sperm activation. Oocyte maturation may be affected by a wide variety of processes from those affecting growth of

follicular support cells to those acting inside the egg nucleus itself. Three protein-coding genes (*CSF1R*, *NALP10*, *MBD4*) potentially involved in oocyte maturation revealed heterozygous variants predicted to be damaging.

CSF1R encodes for the Receptor for Colony Stimulating Factor 1 and almost exclusively mediates the biological effects of this cytokine (Li *et al.*, 2006). Both *CSF1* and *CSF1R* are expressed at high levels in the mature, unfertilized oocyte (Witt and Pollard, 1997; Salmassi *et al.*, 2005). Following ovulation and fertilization, receptor mRNAs are degraded to undetectable levels (Arceci *et al.*, 1992). *CSF1* also regulates cell division in the blastocyst before implantation and is implicated in the regulation of trophoblast differentiation and local immune responses protecting the fetus (Garcia-Lloret *et al.*, 1994; Gorivodsky *et al.*, 1999). Female mice with a homozygous mutation in *CSF1* revealed a reduced fertility rate (Pollard *et al.*, 1991), although this was thought to involve the implantation stage rather than oocyte development.

NALP10 is a member of the nucleotide-binding domain leucine-rich repeat containing (NLR) family that serves as major regulators of inflammatory and innate immune response. A number of these family members are expressed in oocyte and involved in reproduction control (Tian *et al.*, 2009), and *NLRP7* is a well-known 'maternal effect gene', in which biallelic mutations are found in women with recurrent biparental hydatidiform mole (Murdoch *et al.*, 2006) and possibly involved in a wider range of pregnancy pathology including triploidy (Murdoch *et al.*, 2006; Meyer *et al.*, 2009; Fallahian *et al.*, 2013). However, *NLRP10* does not appear to be expressed in the oocyte (Tian *et al.*, 2009)

MBD4 (MED1) belongs to a family of proteins with a methyl-binding domain at the N-terminus that functions both in binding to methylated DNA and in protein interactions and a C-terminal mismatch-specific glycosylase domain that is involved in DNA repair. It was deemed of interest as its expression is specifically up-regulated at MII (Oliveri *et al.*, 2007) and it is involved in methylation reprogramming in gametogenesis (Galetzka *et al.*, 2007).

BNC2 encodes basonuclin 2, a transcriptional factor from the group of 'maternal-effect' genes in mice (Ma *et al.*, 2006). It may play a role in the differentiation of spermatozoa and oocytes. Polymorphisms in this gene were associated with serous ovarian cancer. However, the variant in the proband and her mother are assessed as 'tolerated', suggesting minor protein damage.

While we did not identify any damaging mutations in genes known to be involved in meiotic spindle assembly checkpoints (as implicated in the MI arrest in the LT/Sv mouse strain), variants predicted to be tolerated were identified in genes coding for centrosomal proteins (*CEP192*, *CEP250*) and a spindle associated kinesin protein, *KIF9*. Although expressed in MI and MII, knockdown of C-NAP1 (product of *CEP250*) in mouse oocytes did not produce an obvious effect on meiotic progression (Sonn *et al.*, 2011) and *Drosophila* mutants lacking centrosomes can complete MII (Stevens *et al.*, 2007). KIF9 is a regulator of spindle dynamics and affects mitotic progression (Andrieu *et al.*, 2012), although a function in meiosis is unknown.

Summary

In summary, we present a unique patient exhibiting a high number of miscarriages and recurrence of triploid conceptions with a maternal history of unusually frequent miscarriages. Hypothesizing an inherited autosomal-dominant predisposition, we identified candidate genes potentially involved in the etiology of recurrent triploidy using whole-exome sequencing. While we cannot conclude which mutation(s) caused the reproductive failure in this particular case, a limited number of reasonable candidates playing a role in oocyte maturation, oocyte activation or PB extrusion were identified. This shows the great potential of studying families with rare phenotypes to increase our knowledge about mechanisms of reproductive failure. Future patients with recurrent triploidy or recurrent IVF failure due to failed oocyte maturation could be screened for mutations in these same genes in order to confirm our findings. However, considering genetic heterogeneity and complexity, establishing animal models may be the most reasonable approach in the future for studying the functional impact of variants in genes involved in human reproduction.

Supplementary data

Supplementary data are available at http://molehr.oxfordjournals.org/online.

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Authors' roles

I.F., I.M. and W.P.R. were involved in conception and design of the study, data acquisition and analysis and interpretation, drafting of the manuscript and revision of the article. M.S.P. was involved in data acquisition and analysis and interpretation, revision of the manuscript. D.E.M. was involved in conception, data interpretation and revision of the manuscript. K.L. was involved in data acquisition and revision of manuscript. E.N. was involved in data acquisition and analysis and revision of the manuscript. J.M.F. contributed to conception and design of the study and manuscript revision. All the authors were involved in the final approval of the version to be published.

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Conflict of interest

None declared.

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