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POLR2C Mutations Are Associated With Primary Ovarian Insufficiency in Women

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Context: Primary ovarian insufficiency (POI) results from a premature loss of oocytes, causing infertility and early menopause. The etiology of POI remains unknown in a majority of cases.

Objective: To identify candidate genes in families affected by POI.

Design: This was a family-based genetic study.

Setting: The study was performed at two academic institutions.

Patients and Other Participants: A family with four generations of women affected by POI (n = 5). Four of these women, three with an associated autoimmune diagnosis, were studied. The controls (n = 387) were recruited for health in old age.

Intervention: Whole-genome sequencing was performed.

Main Outcome Measure: Candidate genes were identified by comparing gene mutations in three family members and 387 control subjects analyzed simultaneously using the pedigree Variant Annotation, Analysis and Search Tool. Data were also compared with that in publicly available databases.

Results: We identified a heterozygous nonsense mutation in a subunit of RNA polymerase II (*POLR2C*) that synthesizes messenger RNA. A rare sequence variant in *POLR2C* was also identified in one of 96 women with sporadic POI. *POLR2C* expression was decreased in the proband compared with women with POI from another cause. Knockdown in an embryonic carcinoma cell line resulted in decreased protein production and impaired cell proliferation.

Conclusions: These data support a role for RNA polymerase II mutations as candidates in the etiology of POI. The current data also support results from genome-wide association studies that hypothesize a role for RNA polymerase II subunits in age at menopause in the population.

Freeform/Key Words: premature ovarian failure, whole genome sequencing, RNA polymerase II

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; cDNA, complementary DNA; CLRT, composite likelihood ratio test; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; gDNA, genomic DNA; GWAS, genome-wide association study; LOD, logarithm of odds; MAF, minor allele frequency; mRNA, messenger RNA; POI, primary ovarian insufficiency; pVAAST, pedigree Variant Annotation, Analysis and Search Tool; SD, standard deviation; shRNA, short hairpin RNA; Tris, tris(hydroxymethyl)aminomethane; VAAST, Variant Annotation, Analysis and Search Tool.

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Primary ovarian insufficiency (POI), which is characterized by early ovarian follicle depletion and consequent loss of ovarian function, affects 1% of women before the age of 40 years [1, 2]. With the exception of karyotype abnormalities and *FMR1* premutations, the etiology in an estimated 65% of cases remains unknown [3].

An autoimmune etiology is also purported to account for a substantial proportion of POI, from 4% to 30% [1, 4, 5]. It is often assigned in the presence of any coincident autoimmune disease [1, 4, 5]. However, an autoimmune etiology has been confirmed in only a small number of cases in which autoimmune oophoritis was documented by an infiltration of lymphocytes into the theca layer of the follicle [4, 5], with eventual follicle destruction and ovarian insufficiency. Adrenal insufficiency and/or the presence of adrenal antibodies has been demonstrated in a majority of proven autoimmune oophoritis cases because the autoimmunity targets enzymes common to adrenal and ovarian steroidogenic cells [4]. Autoimmune oophoritis has not been demonstrated in the presence of more common autoimmune diseases such as autoimmune thyroiditis, which is found only slightly more frequently in women with POI than in the general population [4]. Therefore, an autoimmune etiology for POI may be assigned in cases in which it is not the primary causal factor.

In addition to karyotype abnormalities and *FMR1* premutations, there is overwhelming evidence for additional genetic causes for POI [6]. Familial cases are estimated to account for approximately 12.7% of POI [7]. The availability of next-generation sequencing technology using familial cases has resulted in a growing list of genes causing POI in the last 2 years. Whole-exome sequencing in consanguineous and large families demonstrated a diverse set of causal genes important for mitochondrial function (*LARS2, HARS2, CLPP*), homologous recombination and meiosis (*STAG3, SYCE, HFM1*), DNA damage repair (*MCM8, MCM9, CSB-PGBD3, NUP107*), and messenger RNA (mRNA) transcription (*SOHLH1*) and translation (*eIF4ENIF1*) [8, 9]. Using whole-genome sequencing in a family with POI and associated autoimmune disease, the current study demonstrates mutations in a previously unreported gene and family, *POLR2C,* adding RNA polymerase II as an etiologic factor. The presentation of the women with POI in the family described provides additional evidence that associated autoimmune disorders may be coincidental rather than causal.

1. Patients and Methods

A. Case Report

The proband presented with POI at age 30 years (follicle-stimulating hormone 106 IU/L and amenorrhea) (Fig. 1). Her medical history was notable for immune thrombocytopenia and hypothyroidism. Her maternal grandmother and mother also presented with POI at age 36 and 34 years, respectively. Her daughter underwent menarche at age 13 years and had regular cycles until age 16 years, when she presented with amenorrhea, an elevated follicle-stimulating hormone level (134 IU/L), and a low estradiol level (<20 pg/mL). Within 1 year, she was also diagnosed with a positive intrinsic factor blocking antibody and hypothyroidism, with positive thyroid peroxidase antibodies >1000 IU/mL (normal, <35 IU/mL) and thyroglobulin antibodies 58.8 IU/mL (normal, <40 IU/mL). Two additional daughters, currently aged 21 and 18 years, have regular menses. The proband's sister had irregular menstrual cycles starting at age 43 years. She was then diagnosed with atypical ductal hyperplasia and was treated with tamoxifen, at which time her menses ceased. She was also hypothyroid with positive thyroglobulin antibodies and had immune thrombocytopenia with negative platelet antibodies. Karyotype and *FMR1* repeat length were normal and adrenal cortical antibodies and 21-hydroxylase antibodies were negative in the proband and her daughter. These tests were not performed in the mother, grandmother, and sister. The family is of European ethnicity.

B. Additional Subjects With POI

DNA from 96 subjects with nonsyndromic POI diagnosed at varying ages was studied for replication. These patients had a normal karyotype and *FMR1* premutation length and

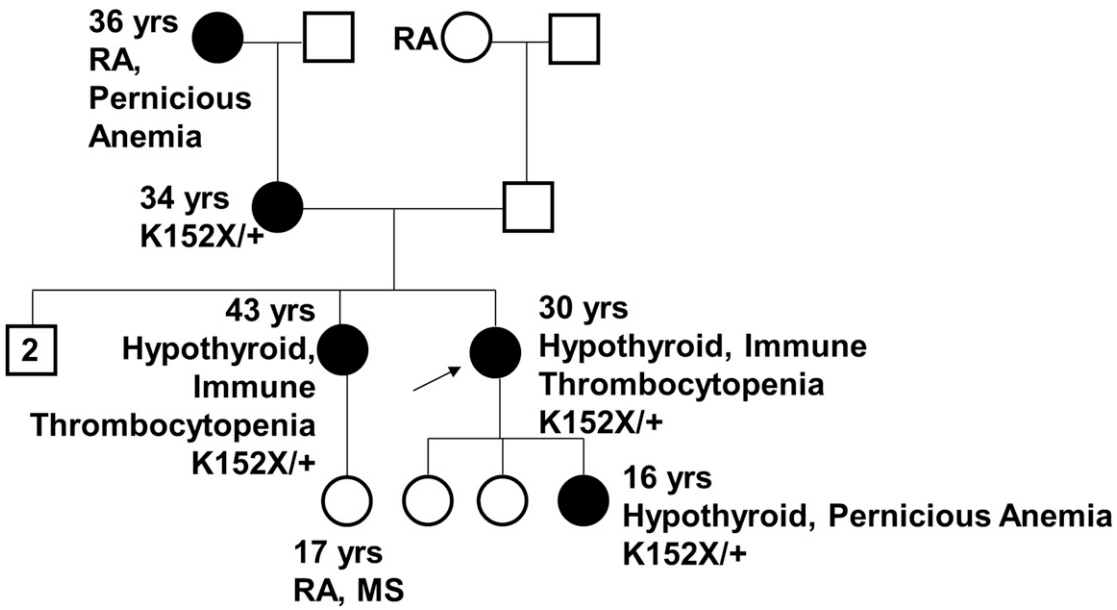

Figure 1. The pedigree shows a family with dominant inheritance of POI (closed circles). The age at menopause and additional autoimmune diagnoses are indicated. The *POLR2C* mutations in all of the affected women changed lysine amino acid number 152 to a stop codon. The second allele was not mutated (indicated with a + symbol). The squares denote men, and the open circles denote women who are unaffected or of unknown status. The square with the number 2 indicates brothers who were not available for phenotyping. RA, rheumatoid arthritis; MS, multiple sclerosis.

negative adrenal cortical antibody test results. Two of the 96 subjects also had a pattern of autosomal dominant inheritance, both with a mother and a sister with POI. Whole-exome sequencing data from the sisters and one mother were available.

C. Controls

Control subjects consisted of 96 unrelated, unaffected whole-genome sequenced controls of European ancestry recruited for health in old age through the Utah Genome Project. In addition, whole-genome sequences from 291 Northern Europeans from Utah, Finnish in Finland and British in England and Scotland samples from the 1000 Genomes Project were used as unaffected controls.

The study was approved by the Partners Human Research Committee for all POI subjects. The study was approved by the institutional review board of the University of Utah for the 96 unrelated controls. All subjects gave written informed consent.

D. Genetic Studies

DNA was extracted from whole blood from the proband, mother, and daughter using the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA). Whole-genome libraries were prepared using the KAPA Hyper Prep kit and were sequenced on the Illumina X Ten sequencing platform to a depth of >55× by NantOmics (Culver City, CA). The 96 unrelated women with sporadic POI underwent whole-exome sequencing using the Illumina HiSequation 2000 by the High-Throughput Genomics (Illumina, Inc., San Diego, CA) and Bioinformatics Analysis Core (Huntsman Cancer Institute, University of Utah, Salt Lake City, UT). DNA libraries were prepared using the Agilent SureSelectXT Human All Exon + UTR (v5) (Agilent Technologies, Inc., Santa Clara, CA).

Variants were called according to the UGP 1.3.0 variant calling pipeline (http://weatherby.genetics.utah.edu/UGP/wiki/index.php/UGP_Variant_Pipeline_1.3.0). In brief, reads were aligned to GRCh37, decoy sequence, and phiX using bwa (v0.7.10). Duplicates were marked

using samblaster (v0.1.22). The resulting BAM files were polished using GATK (v3.3-0) IndelRealigner and BaseRecalibrator. Variants were called using GATK HaplotypeCaller, limiting to regions targeted in the Agilent SureSelectXT Clinical Research Exome kit. Genotyping was done using GATK GenotypeGVCFs on the variant calls plus the controls. Variant calls were recalibrated with GATK VariantRecalibrator.

E. Genomic Analysis

Data were analyzed using the pedigree Variant Annotation, Analysis and Search Tool (pVAAST) in the proband, mother, and daughter, and data were compared with jointly genotyped controls (total n = 387) [10]. pVAAST was parameterized with unknown_representatives=no, inheritance_model=dominant, informative_site_selection=3, genotyping_error_rate=1e-4, penetrance_lower_bound=0.995, penetrance_upper_bound=1, max_prevalence_filter=0.1, lod_score_filter=yes, clrt_score_filter=yes, nocall_filter=yes, nocall_filter_cutoff=2, inheritance_error_filter=yes. Data were also analyzed using Omicia Opal 4.15 in a three-person cohort analysis including the proband, mother, and daughter (https://app.omicia.com; Omicia, Inc., Oakland, CA). The software prioritizes variants using the Variant Annotation, Analysis and Search Tool (VAAST) and therefore does not take into account the family relationships between individuals [11]. However, because the analysis was focused on variants shared by all three women, the results are similar to pVAAST. Opal variants were filtered to exclude noncoding and synonymous variants and variants reported at a frequency of greater than 1% in three publicly available databases: the 1000 Genomes Project [12]; the Exome Aggregation Consortium (ExAC) database, which includes whole-exome sequencing from more than 60,000 subjects without severe pediatric disease [13, 14]; and the Exome Variant Server, which includes whole-exome sequencing from subjects with cardiovascular or cerebrovascular disease, hypertension, or hyperlipidemia and unaffected controls [15]. In addition, nonpathogenic variants found in other families (n = 2) with a known mutation in a different gene and variants in genes known to demonstrate an excess of false positives were filtered [16]. HLA genes, which also demonstrate an excess of false positives, were analyzed on the basis of associated autoimmune features in the family. Mutations were prioritized by the Omicia score, which ranks variants according to expected protein effects using SIFT (http://sift.jcvi.org/), Mutation Taster (http://www.mutationtaster.org/), PolyPhen (http://genetics.bwh.harvard.edu/pph2), and phyloP [17–19]. In both analyses, the Phenotype Driven Variant Ontological Re-ranking tool (Phevor) feature was used to further prioritize genes for analysis using premature ovarian failure and POI as filters [8, 20].

F. RNA Expression

RNA was isolated from whole blood from the proband and two women with POI from another cause using the QIAamp RNA Blood Mini Kit and were subsequently treated with DNAse digestion and RNA cleanup according to the manufacturer's instructions (Qiagen, Valencia, CA) [21]. Reverse transcription was performed with SuperScript VILO Master Mix (Life Technologies, Carlsbad, CA) using SuperScript III RT and random primers. Quantitative real-time polymerase chain reaction was performed for the expression of *POLR2C* and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) as an endogenous control using PowerUp SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Primers were designed to span *POLR2C* exons 6 and 7 to avoid amplifying genomic DNA. The GAPDH primer pair was validated to have 90% to 100% efficiency in standard curve reactions [22]. Primer sequences were as follows: *POLR2C* forward 5′-CCGAGATAATGACCCCAATG-3′, *POLR2C* reverse 5′-TTTTGGCATAGGCTCGAAGT-3′, *GAPDH* forward 5′-ACCCACTCCTCCACCTTTG-3′, *GAPDH* reverse 5′-CTCTTGTGCTCTTGCTGGG-3′. Samples were examined in triplicate and at two dilutions. mRNA levels of *POLR2C* were determined using the $2^{-\Delta\Delta C}{}_{T}$ method to calculate relative quantification and to correct for expression of endogenous controls and were compared between the subject and two controls using unpaired *t* tests.

G. PA-1 Cell Model

PA-1 cells (American Type Culture Collection, Manassas, VA), derived from a metastatic teratocarcinoma of the ovary consisting of germ cells with a diploid female karyotype prior to the first meiosis, were selected as a model [23]. The expression pattern in these cells is similar to that of embryonic stem cells [24] and was confirmed in the current study (Supplemental Table 1). Embryonic stem cells, germ cells, and carcinoma cells have a number of similarities [25]. Therefore, PA-1 cells were used as a model of early germ cells in the current study [26, 27].

PA-1 cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cells were seeded at a density of 2×10^4 in a 96-well plate with nine wells for each experimental condition, repeated three times. When cells reached a density of 80%, they were transfected using PolyJet (SignaGen Laboratories, Rockville, MD) with 0.125 μg short hairpin RNA (shRNA) *POLR2C* plasmid or 0.125 μg control shRNA containing 25 nucleotides corresponding to the small interfering RNA gene silencer sequence plus hairpin (Santa Cruz, Dallas, TX). Time to confluence was recorded for each condition after puromycin selection. Twenty-one hours after transfection, cells were treated with 1 μg/mL puromycin to select for stably transfected cells. Selected cells were observed daily to document cell death and subsequent doubling time when grown to 80% confluence.

Two days after control cell death was complete, cells were incubated with 5-bromo-2′-deoxyuridine (BrdU) for 2 1/2 hours, and incorporation was assessed using a colorimetric immunoassay after fixing and permeabilizing cells and denaturing DNA according to the manufacturer's instructions (Calbiochem; EMD Millipore Corporation, Temecula, CA). Absorbance was measured at 450 nm using a spectrophotometric plate reader (ThermoMax Microplate Reader; Molecular Devices, Sunnyvale, CA). The experiment was repeated three times.

In a second set of experiments, PA-1 cells were transfected as above and protein was extracted. Cells were grown to 80% confluence and were washed with phosphate-buffered saline, digested with 0.25% trypsin-EDTA, and collected at 300× g for 5 minutes. Cells were homogenized in a radioimmunoprecipitation assay buffer supplemented with 1× protease and phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL) on a shaker for 30 minutes at 4°C. Samples were centrifuged at 12,000 rpm for 20 minutes at 4°C, and supernatants were collected. Homogenates were diluted with 4× Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) and heated at 95°C for 10 minutes. Next, 83 μg of protein from each sample was separated on a 10% polyacrylamide gel by SDS-PAGE for 75 minutes at constant 100 V in tris(hydroxymethyl)aminomethane (Tris)-glycine buffer (25 mM Tris-HCl; 192 mM glycine; 3.5 mM SDS; pH 8.3), then transferred to nitrocellulose membranes overnight at constant 30 V in transfer buffer (25 mM Tris base; 190 mM glycine; 1.7 mM SDS; 20% ethanol). Nitrocellulose membranes were blocked in 5% nonfat dry milk in TBST (20 mM Tris base; 137 mM NaCl; 0.05% Tween 20; pH 7.6) for 1 hour and incubated with primary antibody against POLR2C (Origene, Rockville, MD; RRID:AB_2636846) at a 1:1000 dilution in TBST with 5% nonfat dry milk at 4°C overnight. After three washes in TBST, membranes were incubated with secondary donkey antirabbit antibody at 1:2000 dilution (Life Technologies, Frederick, MD) in TBST with 2% nonfat dry milk and washed three times with TBST for 15 minutes. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate and X-ray film. Membranes were subsequently stripped (63 mM Tris-HCl; 69 mM SDS; 0.7% 2-mercaptoethanol; pH 6.7) and probed with β-actin antibody at a 1:15,000 dilution (Novus Biologicals, Littleton, CO; RRID:AB_10001612). Band density was analyzed using VisionWorks LS software (UVP, Upland, CA). The experiment was repeated three times.

2. Results

A. Genomic Results

There were 739 gene variants shared by the proband, daughter, and mother (Supplemental Table 2). After pVAAST analysis in the proband, daughter, and mother, variants in 16 genes

were identified as candidates (Table 1). There were no previous reports of mutations in these genes causing POI. The pVAAST score, VAAST score, and Phevor analysis prioritized the nonsense mutation in *POLR2C* as the top candidate and most likely to be important for the POI phenotype ($P = 0.0005$) (Table 1 and Supplemental Tables 3 and 4). Other candidates were present at >1% frequency in the population databases (n = 9) [12–15], tolerant of mutations (n = 3), present in other families with different known gene mutations (n = 1), or functionally less likely (n = 3). Sanger sequencing confirmed the c.454A>T base pair change (NM_032940) in exon 7 in the three affected family members and the proband's sister. The mutation changes a lysine to a premature stop codon, p.Lys152Ter. One African subject in the ExAC database was found to have a different nonsense mutation at p.Gln157Ter, for a frequency of 0.000008270 [12]. The sex and phenotype of the patient were not known.

An additional rare sequence variant was identified in an African American member of the 96-person POI cohort with sporadic POI and no syndromic features. The variant was found at base pair c.206-3C>T, changing a splice receptor site at exon 4, confirmed by Sanger sequencing (Supplemental Fig. 1). The variant is found at a frequency of 0.0001 and 0.0003 in the 1000 Genomes and Exome Variant Server databases, respectively [12, 15]. She had no autoimmune disease or other medical problems.

Additional heterozygous variants were identified in the other RNA polymerase I and II subunits in women with POI (Supplemental Table 5). Two were variants in POLR1A and POLR1C that are predicted to be deleterious.

There was only one variant in any gene previously identified to cause POI (*i.e.*, *NOBOX*) (Supplemental Table 2). However, the variant in *NOBOX*, p.Lys664Arg, was found in the controls, at a minor allele frequency (MAF) 0.017 in 1000 Genomes, and at a MAF 0.027 in the ExAC database with 10 homozygotes identified [12, 14]. Of note, the *RIPK3* nonsense mutation c.1264C>T p.Arg422Ter (rs146886719), which had a similar *P* value to *POLR2C* (Table 1), was also sequenced in the four affected family members and was not identified in the proband's sister. The same *RIPK3* nonsense mutation was identified in an additional woman with familial POI, but it was isolated to that subject and was not found in her affected mother or sister.

B. Autoimmune Findings

Gene variants predisposing to autoimmune disease were also examined in the proband, mother, and daughter [28, 29]. No rare variants associated with autoimmune disease were shared among the three affected family members (Supplemental Table 6). There were several common variants shared in the subjects: *TNFAIP3* rs2230926 (MAF=0.22), *C1QTNF6* rs229527 (MAF=0.45), and *IL23R* rs11209026 (MAF=0.022). There was also a rare *HLA-DRB1* stop-gained variant that was not confirmed using Sanger sequencing.

C. RNA Expression

Sequencing complementary DNA (cDNA) from the proband demonstrated a small T peak in the electropherogram, decreased in amplitude compared with that in genomic DNA, and not found in a control subject (Fig. 2). *POLR2C* expression was decreased approximately 60% in the lymphocytes of the proband compared with that in two women with POI from another cause (relative quantification, 0.60 ± 0.055 vs 1.39 ± 0.23; $P < 0.02$).

D. PA-1 Cell Model

Cells transfected with *POLR2C* shRNA and control shRNA maintained 10% to 20% confluence during selection. Over the next 3 days, cells transfected with *POLR2C* shRNA remained at 15% ± 6% confluence (range, 10%–30%), whereas those transfected with control shRNA demonstrated 60% ± 14% confluence (range, 40%–80%). BrdU analysis at 3 days after selection, corrected for cell confluence, demonstrated decreased incorporation in shRNA knockdown compared with shRNA control (0.056 ± 0.008 vs 0.087 ± 0.017; $P < 0.05$) (Fig. 3).

Table 1. Candidate Coding Gene Variants Found in the Proband, Mother, and Daughter With Primary Ovarian Insufficiency After pVAAST Analysis

Gene Symbol	Chr	Starting Base Pair	rsID	Base Pair Change	Protein Change	Consequence	Alleles in Ctls (n = 387)	MAF ExAC[a]	pVAAST Rank[b]	CLRT Score[c]	LOD Score[d]	pVAAST *P* Value[e]
POLR2C	chr16	57503887	—	c.454A>T	p.Lys152Ter	Stop	0	—	1	19.66	0.54	0.0005
ICAM5	chr19	10402996	—	c.959A>T	p.Tyr320Phe	Missense	0	—	2	11.28	0.54	0.0005
RIPK3	chr14	24806303	rs146886719	c.1264C>T	p.Arg422Ter	Stop	0	0.0016	3	19.66	0.54	0.0008
RIPK3	chr14	24806902	rs34106261	c.899C>T	p.Thr300Met	Missense	37	0.051	—	—	—	—
HAUS1	chr18	43703331	rs202180722	c.666+1G>A	—	Splice donor	0	0.00031	4	13.02	0.54	0.0008
DENND6B	chr22	50750953	rs749252524	c.1559C>T	p.Ala520Val	Missense	0	0.00024	5	12.09	0.54	0.001
MYO18A	chr17	27421108	rs143502477	c.4711G>A	p.Ala1571Thr	Missense	105	0.0080	6	8.732	0.54	0.0011
LOC152586	chr4	141372656	rs62346874	c.1024G>A	p.Arg342Ter	Stop	485	0.012	7	12.36	0.54	0.0027
GUCA1C	chr3	108672558	rs143174402	c.52G>T	p.Glu18Ter	Stop	7	0.0038	8	13.653	0.54	0.0059
LOC100996571	chr21	27923508	—	c.98C>G	p.Ser33Ter	Stop	592	—	9	10.289	0.54	0.0078
ZAN	chr7	100389677	rs149104440	c.7616C>T	p.Arg956Ter	Stop	105	0.015	10	12.929	0.54	0.0090
NBPF10	chr1	145368664	rs201638494	c.10642G>T	p.Gly3548Ter	Stop	17	0.0096	11	12.458	0.54	0.0094
FAM149A	chr4	187088248	rs9991339	c.1291C>T	p.Arg431Trp	Missense	12	0.031	12	8.165	0.54	0.014
CYTL1	chr4	5021021	rs35755546	c.152C>T	p.Ser51Leu	Missense	20	0.029	13	6.194	0.54	0.014
COL6A5	chr3	130150310	rs61744488	c.5250A>C	p.Glu1750Asp	Missense	341	0.053	14	2.490	0.54	0.015
ACOT9	chrX	23723711	rs56378612	c.934G>C	p.Glu312Gln	Missense	18	0.022	15	2.963	0.54	0.016
TNK1	chr17	7290695	rs7220814	c.1413-2A>G	—	Splice acceptor	41	0.093	16	3.417	0.54	0.032

Abbreviations: chr, chromosome; CLRT, composite likelihood ratio test; ctl, control LOD, logarithm of odds; rsID, reference SNP cluster ID.
[a]MAF ExAC refers to the minor allele frequency in the ExAC Database [14].
[b]The pVAAST rank is assigned according to the pVAAST gene and variant scores along with the *P* value.
[c]The gene CLRT score assesses the allele frequency differences in the case and the control populations and whether a variant is likely to be deleterious according to SIFT, PolyPhen, MutationTaster, and phyloP variant scoring algorithms. It also incorporates the LOD score.
[d]The LOD score integrates linkage information as a log-likelihood ratio that a variant is a disease-susceptibility variant.
[e]The probability of observing the gene score by random chance.

POLR2C protein levels were decreased with shRNA knockdown compared with control shRNA as assessed by relative density controlled for β-actin (0.066 ± 0.019 vs 0.15 ± 0.071 density units; $P < 0.05$) (Fig. 3).

3. Discussion

We identified a *POLR2C* nonsense mutation in a family with a dominant inheritance pattern of POI. *POLR2C* mRNA expression was decreased in lymphocytes from the proband. shRNA knockdown of *POLR2C* decreased the growth rate and DNA replication in the PA-1 embryonic cell line as a model of early germ cells. These data are consistent with the hypothesis that *POLR2C* haploinsufficiency affects germ cell proliferation, resulting in a decreased final oocyte complement and POI.

POLR2C is the third largest subunit of the highly conserved 12-subunit complex that makes up RNA polymerase II, the polymerase responsible for transcription of mRNA [30]. The nonsense mutation results in loss of three of seven conserved regions in the protein, four β-strands, and one α-helix, and the portions of the protein that interact with the largest RNA polymerase II subunit and required transcription initiation factors [30, 31]. Depleting any one of the 12 subunits, including *POLR2C*, results in failure of RNA polymerase II assembly, accumulation of the remaining subunits in the cytoplasm, and failure to move to the nucleus [32].

Slow growth of PA-1 cells after *POLR2C* knockdown is consistent with *in vitro* and *in vivo* studies demonstrating that *POLR2C* haploinsufficiency results in a decreased rate of growth under conditions that favor rapid growth, such as germ cell proliferation. In *Saccharomyces cerevisiae,* haploinsufficiency of the *POLR2C* homolog RBP3 confers a slow growth phenotype in enriched medium [33]. The same phenotype can be observed with RBP3 mutation A159G, which abolishes a required transcription activation site and would be deleted in the human nonsense mutation described. The slow growth phenotype is not apparent in minimal medium [33], suggesting that haploinsufficiency of RBP3 affects only rapidly dividing cells.

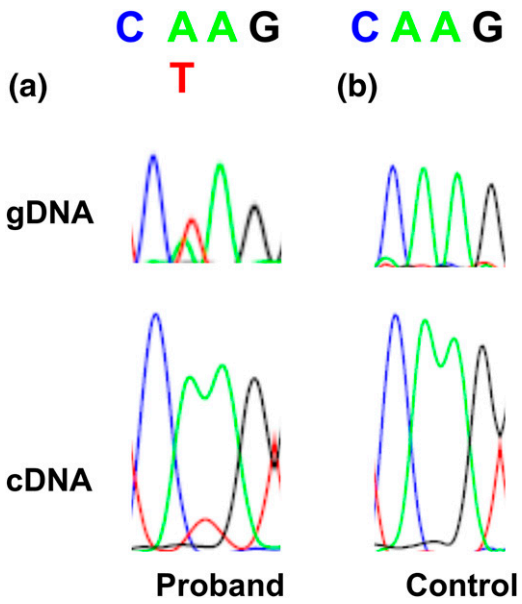

Figure 2. Genomic DNA (gDNA) and cDNA sequences are shown. Electropherograms show the gDNA and cDNA sequences of the proband and an unrelated control with normal age at menopause. A heterozygous T allele changing the amino acid codon from AAG (lysine) to TAG (stop) is present in the gDNA of the proband, and a small T peak remains in the cDNA of the proband.

In the ovary, *POLR2C* haploinsufficiency likely disrupts the rapid mRNA synthesis that occurs during germ cell proliferation and oocyte maturation [34, 35]. *POLR2C* is expressed in oocytes [36]. The most prominent increase in DNA transcription involving RNA polymerase II occurs in germ cell mitosis and growth of oocytes from the primordial stage to the early antral stage [26], which pauses when the oocyte is fully grown [35]. The PA-1 germ cell model [23] demonstrated decreased growth after shRNA knockdown compared with control knockdown, supporting a role for *POLR2C* haploinsufficiency as a cause for a decreased germ cell complement and resulting POI. There are other occasions in which rapid cell growth may be required in humans, such as in wound healing, but the family had no additional clinical manifestations with the exception of autoimmune diseases. We are limited by the absence of a mammalian model to test the relative effect of the stop-gained mutation on rapidly dividing germ cells and cell division in the rest of the organism.

The nonsense mutation in *POLR2C* informs genome-wide association studies (GWASs) examining age at menopause in the population. Recent GWASs implicated two additional subunits of RNA polymerase II in age at menopause: *POLR2E* and *POLR2H* [37]. However, the GWAS variants did not reside in the coding regions of the genes. Rather, these genes were identified as the best biological candidates in the locus based on their involvement in DNA damage and repair pathways [37]. The current study provides support for a role for RNA polymerase II subunits in age at menopause. However, the precise relationship between common variation at these genome-wide important loci and age at menopause will need to be defined.

Other heterozygous variants predicted to be damaging were found in RNA polymerase I and II subunits in women with sporadic POI (Supplemental Table 5). The identified *POLR2C* splice site variant has been identified in other African American subjects, albeit rarely, and will need to be assessed in larger, ethnically matched cohorts to determine pathogenicity. Taken together, the data suggest other RNA polymerases may play a role in the etiology of POI.

The cause of POI in the proband and her daughter was previously attributed to autoimmune POI on the basis of coincident autoimmune diseases including autoimmune thyroiditis, pernicious anemia, and immune thrombocytopenia. However, the proband's affected mother did not have any autoimmune disease. Further, although thyroid autoimmunity and

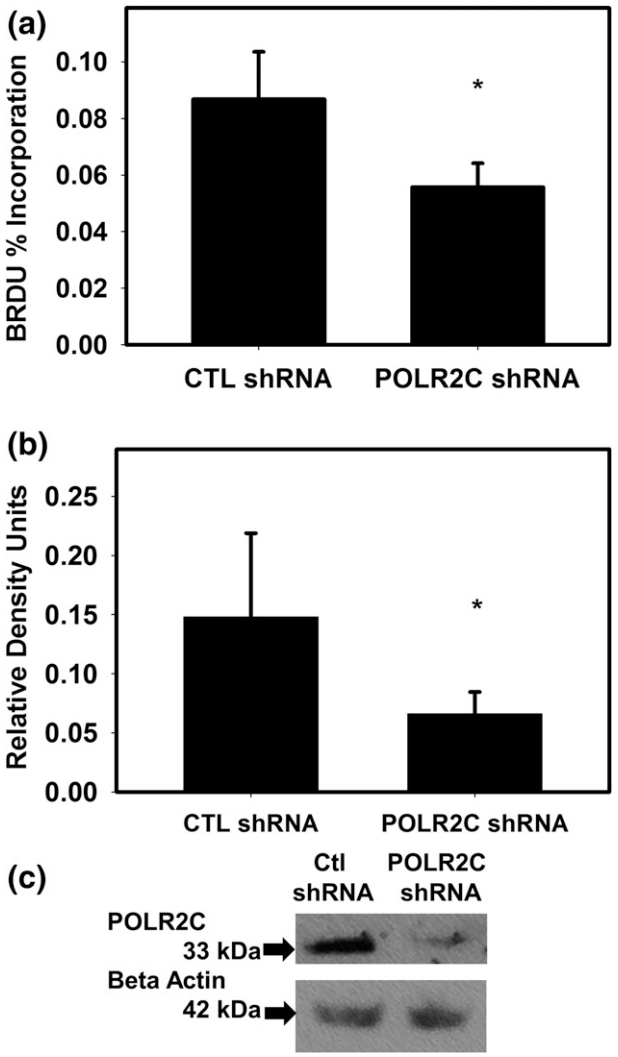

Figure 3. DNA and protein levels are shown. (a) Percent incorporation of BrdU and (b) *POLR2C* protein levels controlled for β-actin in PA-1 cells transfected with control scramble shRNA (Ctl) and *POLR2C* shRNA. Both graphs depict the mean and standard deviation (SD) from three separate experiments. (c) The figure shows representative Western blots of the RNA polymerase 2C and β-actin protein bands from control scramble shRNA and *POLR2C* shRNA transfected cells. There was less BrdU incorporation in the *POLR2C* shRNA transfected cells, consistent with their slower growth and lower RNA polymerase 2C protein levels. * indicates $P<0.05$.

pernicious anemia are the most common disorders reported in association with POI, the prevalence is only slightly higher than in the population, and these disorders have not been linked to anatomically proven oophoritis [5]. The genetic architecture of these common autoimmune diseases is complex, with common variants found in >5% of the population that confer a small risk [38]. Indeed, the family carried several common variants associated with autoimmune disease [28, 29]. However, adrenal insufficiency is the only autoimmune disease found in the presence of anatomically documented autoimmune ovarian insufficiency and oophoritis [4, 5]. Adrenal insufficiency is rare, and the genetic architecture remains unclear [38]. Neither adrenal insufficiency nor adrenal cortex antibodies were found in the proband or her daughter. The family did not have rare mutations in *NLRP1*, *PTPN22,* or *BACH2*, genes associated with autoimmune adrenal insufficiency [38, 39]. Interestingly, the daughter had a common *PTPN22* variant possibly associated with adrenal insufficiency and a common variant in *AIRE* found in the PHD2 domain that interacts with RNA polymerase II. Thus, the

earlier onset of POI in the daughter may be related to an autoimmune predisposition or interaction of *AIRE* and *POLR2C* [40]. Taken together, the data suggest that the cause of POI may not be entirely autoimmune mediated, even in subjects with a strong history of autoimmune disease, and that additional testing to uncover the etiology of POI must be performed in patients with autoimmune disease other than adrenal insufficiency to avoid need for follow-up adrenal function assessments.

The data support a role for mutations in RNA polymerase II subunits, notably *POLR2C,* in the etiology of POI. These findings expand the list of gene mutations causing POI and suggest that autoimmune POI should be diagnosed with caution except in women with associated adrenal autoimmunity. The findings also support the hypothesis that RNA polymerase II subunits are the candidate genes in loci identified in GWASs of age at menopause.

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