

Identification of Multiple Gene Mutations Accounts for a new Genetic Architecture of Primary Ovarian Insufficiency

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Context: Idiopathic primary ovarian insufficiency (POI) is a major cause of amenorrhea and infertility. POI affects 1% of women before age 40 years, and several genetic causes have been reported. To date, POI has been considered a monogenic disorder.

Objective: The aim of this study was to identify novel gene variations and to investigate if individuals with POI harbor mutation in multiple loci.

Patients and Methods: One hundred well-phenotyped POI patients were systematically screened for variants in 19 known POI loci (and potential candidate genes) using next-generation sequencing.

Results: At least one rare protein-altering gene variant was identified in 19 patients, including missense mutations in new candidate genes, namely *SMC1 β* and *REC8* (involved in the cohesin complex) and *LHX8*, a gene encoding a transcription factor. Novel or recurrent deleterious mutations were also detected in the known POI candidate genes *NOBOX*, *FOXL2*, *SOHLH1*, *FIGLA*, *GDF9*, *BMP15*, and *GALT*. Seven patients harbor mutations in two loci, and this digenicity seems to influence the age of symptom onset.

Conclusions: Genetic anomalies in women with POI are more frequent than previously believed. Digenic findings in several cases suggest that POI is not a purely monogenic disorder and points to a role of digenicity. The genotype-phenotype correlations in some kindreds suggest that a synergistic effect of several mutations may underlie the POI phenotype. (*J Clin Endocrinol Metab* 101: 4541–4550, 2016)

Pprimary ovarian insufficiency (POI) is characterized by amenorrhea with elevated gonadotropin levels affecting 1% of women before the age of 40 years. It is a familial disorder in 4–30% of cases (1). POI encompasses a broad spectrum of disorders due to two main mechanisms: follicle dysfunction and follicle depletion (2). Identification of single

germline mutations in some women with POI and murine studies have provided substantial insight into factors regulating ovarian differentiation and development. POI can be triggered by autoimmune disorders, viral infections, iatrogenic causes, or genetic factors (3). It may also be part of syndromic disorders such as Turner, fragile X, or blepharo-

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Abbreviations: AMH, anti-Müllerian hormone; POI, primary ovarian insufficiency; WT, wild-type protein.

phimosis ptosis epicanthus inversus (BPES) syndromes (4). The first genetic anomalies to be linked to POI were X chromosome abnormalities, deletions, *FMR1* premutations, and *BMP15* variants (5). To date, relatively few genes have been implicated in the pathogenesis of POI (6, 7). Recently, rare deleterious mutations in genes involved in meiosis, such as *STAG3* (8), *HFM1* (9), and *SYCE1* (10), in DNA repair, such as *MCM8* and *MCM9* (11, 12) and also in genes encoding transcription factors, including *FIGLA* (13, 14) and *SOHLH1* (15) have been reported in POI. Moreover, eukaryotic translation initiation factor 4E nuclear import factor 1 (*eIF4ENIF1*) has been recently identified in cases of dominantly inherited POI (16). Further, mutations in *NOBOX* (17, 18) and *BMP15* have been found in 6% and <10% of women with POI, respectively (5). However, in most cases, the genetic cause of POI is unknown, suggesting that new causative genes are yet to be discovered.

Primary amenorrhea with pubertal failure represents the earliest-onset of POI, whereas some women develop secondary amenorrhea after puberty (2). Importantly, two clinical features remain unexplained: 1) the overall sporadic nature of POI, and 2) observations of patients harboring the identical mutation yet developing POI either early in life (puberty) or later (20s or 30s). We postulated that defects affecting more than one gene in a given patient might explain this variability. To test this, we used multiplex sequencing technology to analyze 19 genes with either known or suspected roles in POI pathogenesis in a cohort of 100 patients.

Patients and Methods

Patients

One hundred women with idiopathic POI were recruited following Institutional Review Board approval (reference PHRC No. A0R03 052, approved by Bicêtre Ethical committee [CPP No. PP 16–024 Ile-de-France VII]). Patients exhibited primary or secondary amenorrhea before age 40 years with FSH values >20 IU/L measured in two samples at least 1 month apart. Turner syndrome, X-chromosome karyotypic abnormalities, and *FMR1* premutations were excluded and none of the patients had circulating ovarian antibodies. FSH and LH plasma levels were measured via RIAs (Immunotech and Beckman Coulter, respectively) (19). Serum anti-Müllerian hormone (AMH) concentrations were measured in duplicate using a sandwich ELISA method (AMH/MIS Elisa; Immunotech, Beckman-Coulter). The lower detection limit of the assay was 0.7 pmol/L. The mean interassay coefficient of variation was 8.6% and the mean intra-assay was 4.0% at 22 pmol/L.

Informed consent was obtained from all participating individuals prior to blood sample collection and molecular studies.

Ovarian volume and antral follicle count measurements

Transvaginal Ovarian ultrasound examination was performed by an experienced ovarian ultrasonographer, with a Voluson E8 Expert (Voluson E8 EXP BT13, 3D-4D, General Electric Systems, VELIZY, France) with a 6–12 MHz transvaginal transducer. Ultrasound measurements were taken in real time, according to a standardized protocol. The highest possible magnification was used to examine the ovaries. The ovarian volume was estimated using the equation: $\pi/6$ (transverse diameter) \times (anteroposterior diameter) \times (longitudinal diameter). Each antral follicle was identified and then measured by taking the mean of two diameters.

Next-generation sequencing

Genomic DNA was extracted from peripheral blood. Mutations were sought in the coding exons and flanking splice sites of the 19 POI genes (Supplemental Table 1). Primers were designed using Ampliseq Designer™ software. Library preparation used the Ion Plus Fragment Library Kit, with 50 ng of genomic DNA. Adapter ligation, nick repair, and amplification were performed according to the Ion Torrent protocol (Life Technologies). Emulsion PCR and enrichment steps were carried out with the Ion One Touch™ Template kit. Amplicon libraries were sequenced on the Ion Torrent PGM 316-chip system, and barcoding was performed with the Ion Xpress™ Barcode Adapters' kit. The Ion Sequencing kit v2 was used for all sequencing reactions, according to the recommended protocol. After sequencing, reads were mapped to the human genome 19 (*hg19*) assemblies with the Torrent Mapping Alignment Program. Single-nucleotide variants and small insertions/deletions (indels) were identified using Torrent Variant Caller (Life Technologies) and Nextgene software. Identified mutations and rare variants were confirmed using Sanger sequencing.

In silico prediction of variants

The prevalence of variants was determined using the Exome Aggregation Consortium (ExAC), a coalition of investigators who aggregate and harmonize exome sequencing data from a variety of large-scale sequencing projects making summary data available for the wider scientific community. The data set provided on this website includes 60 706 unrelated individuals sequenced as part of various disease-specific and population genetic studies. To assess the possible functional effect of amino acid variants, PolyPhen-2 v.2.2.5 (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT (<http://sift.jcvi.org/>) algorithms were used.

Plasmids

pCMV6 (Myc-Flag tagged)-hSOHLH1, -hFIGLA, -hSMC1- β , and -hREC8 were purchased from Origene. pGEMT-hLHX8 was purchased from Sino Biological Inc. and the cDNA was subcloned into EcoRI and NotI sites of pCMV-HA vector. The *NOBOX* and *FOXL2* expression vectors and DK3 promoter have been described previously (20). Lin28A promoter containing the Lin Binding Element (region –644 to –494) was obtained by PCR and subcloned into SacI and XhoI sites of pGL4.26 Luc/miniP vector (Promega). pVP16-Er α was purchased from Addgene.

Antibodies

Several primary antibodies were used for Western-blot analysis: Anti-HA High Affinity (Roche Diagnostic No. 11867423001), anti-cMyc (Clontech, No. 631206), anti-Flag M2 (Sigma, F104), anti-V5 (Sigma, V8012), antiactin (Millipore, clone C4), anti- α tubulin (Sigma, clone DM1A), and anti-ER α (Santa Cruz Biotechnology, sc-543).

Mutagenesis

All variants were generated using QuickChange XLII Kit (Stratagene) (18) and confirmed by Sanger sequencing. Primers flanking the mutations (upon request) were used for PCR amplifications (5' \rightarrow 3') forward and reverse sequences. The PCR products were sequenced using BigDye Terminator v1.1 and analyzed on a 3130xl Genetic Analyzer (Applied Biosystems).

Cell culture and transfection

Cell lines were tested for mycoplasma contamination before experiments. CHO and HEK293T cells were grown in DMEM: F12 medium with L-glutamine (Invitrogen) and supplemented with 5% fetal bovine serum at 37°C in an atmosphere containing 95% air/5% CO₂. Transient transfections of cells were carried out using FuGENE6 reagent (Roche), according to the Manufacturer's protocol.

Expression

Expression of tagged wild-type and mutated proteins in CHO cells was detected by Western blotting. Briefly, cells were lysed in 65mM Tris, pH 6.8, 4% SDS, 1.5% β -mercaptoethanol and held at 100°C for 5 minutes. Proteins were separated on 7–8% SDS-PAGE and electro-transferred onto a nitrocellulose membrane. For immunoblotting, the membranes were probed overnight at 4°C with relevant antibodies and then incubated with appropriated fluorescent secondary antibodies (Pierce). Bound immunoglobulins were revealed by fluorescent detection (Odyssey, Li-Cor).

Transcriptional assays

CHO and HEK 293T cells seeded on 96-well plates were transfected with plasmids encoding the different wild-type and mutated transcription factors, appropriate firefly luciferase reporters, and Renilla luciferase (internal control) as previously reported (20). Firefly and Renilla luciferase activities were measured consecutively with dual-luciferase assays (Promega) and a TriStar reader (Berthold), and are expressed as relative light units.

The capacity to activate downstream signaling was compared between mutated transcription factors and their wild-type counterparts. Each assay was performed independently three times and included six replicates.

Statistical analysis

PRISM 5 software (GraphPad) was used for statistical analysis. Data were analyzed using one-way ANOVA with Kruskal-Wallis post-tests. Data are shown as mean \pm SEM.

$P < .05$ were considered statistically significant. The allele frequency of each variant was compared with the one reported in ExAC database using Fisher's exact test.

Results

Genetic analysis

In this series of 100 patients with idiopathic POI, we identified 40 variants in 14 different genes. All variants were validated by Sanger sequencing (Table 1). After excluding the most frequent variants and those affecting residues with low cross-species conservation, the predicted in silico consequence of each variant was analyzed and functionality tested when possible.

Three new candidate genes are associated with POI

We identified mutations in *SMC1 β* , *REC8*, and *LHX8* three genes not previously been implicated in POI. Both *SMC1 β* and *REC8* are involved in meiosis while *LHX8* encodes a transcription factor. Independent studies have already implicated meiosis-related gene mutations in POI (8–10), and several murine models of meiotic-genes exhibit phenotypes resembling human POI. We found two mutations in *SMC1 β* (I221T and Q1177L) and two mutations in *REC8* (Q154R and R300L). *REC8* Q154R variant has never been described and the R300L variant is rare (Figure 1A), both are highly conserved across species and predicted to be deleterious by Polyphen2 (Table 1). They are both correctly expressed (Figure 1A). The *SMC1 β* I221T variant has not been previously described and is weakly expressed in vitro (Figure 1B). The *SMC1 β* Q1177L variant is highly conserved.

We also detected the first reported variant of the *LHX8* — encoding a transcription factor. Protein alignment revealed that the A325V variant affects an amino acid located near the critical homeodomain of the protein that is highly conserved across species and is predicted to be damaging (Table 1). This variant was normally expressed in vitro (Figure 1C). Functionality was tested on the promoter of *Lin28A* (a protein regulating primordial germ cell development) with significant reduced transcriptional activity of the *Lin28A* promoter (Figure 1D).

Novel or recurrent deleterious mutations were identified in known POI loci

We identified five *NOBOX* variants (G91W, R117W, K371T, D452N, and P619L) that we have previously demonstrated to be loss-of-function mutations (17, 18). We also found a novel *FOXL2* Y5N variant. The tyrosine residue is highly conserved across species and this variant is predicted to be damaging (Figure 2A). The altered protein was correctly expressed in vitro (Figure 2B). We then tested its functional activity using a *DK3* promoter containing forkhead response element and *NOBOX* binding elements sequences as previously described (20). Overexpression of either

Table 1. Summary of 14 Candidate Gene Variations

Category	Gene	Sequence Variation	AA Change	dSNP	ExAC Allele Frequency	Polyphen	SIFT	Conservation (GERP)	
Meiosis	SMC1-β	c.662T>C	I221T	—	1.657e-05	Benign (0.060)	Deleterious (−3.09)	ND	
		c.1189C>G	Q397E	rs149617002	0.0009939	Benign (0)	Neutral (−0.51)	4.85	
		c.2235C>G	E745D	—	ND	Benign (0.009)	Neutral (−0.68)	ND	
		c.2243G>A	C748Y	rs56174639	0.1279	Benign (0.001)	Neutral (−1.11)	3.35	
		c.2273 G>A	R758Q	rs9614653	0.1489	Damaging (0.57)	Deleterious (−3.20)	3.51	
		c.2733A>G	E911D	rs147786046	0.001338	Benign (0.1)	Neutral (−2.06)	1.54	
		c.3165C>G	F1055L	rs61735519	0.05524	Damaging (1)	Deleterious (−5.35)	2.25	
		c.3530A>T	Q1177L	rs61737925	0.003193	Damaging (0.763)	Neutral (−1.91)	5.61	
		REC8	c.461A>G	Q154R	—	ND	Damaging (0.985)	Neutral (−2.22)	ND
			c.899G>T	R300L	rs145684232	0.002268	Damaging (0.798)	Neutral (−2.29)	ND
Transcription factor	STAG3	c.48G>T	L16F	rs11531577	0.1792	Benign (0.002)	Neutral (−0.30)	−0.53	
	DMC1	c.773 A>G	M200V	rs2227914	0.01048	Benign (0.009)	Neutral (−2.21)	5.6	
	LHX8	c.974C>T	A325V	rs34889650	0.001969	Damaging (0.998)	Neutral (−1.26)	5.36	
	NOBOX	c.131C>A	R44L	rs115206969	0.001756	Benign (0)	Neutral (−0.28)	−2.11	
		c.271G>T	G91W	rs77587352	0.002413	Damaging (0.999)	Neutral (−0.30)	−1.98	
		c.349G>A	R117W	rs7800847	0.0068	Damaging (0.978)	Damaging (0.96)	3.47	
		c.1112A>C	K371T	rs189306575	0.0002189	Damaging (0.937)	Neutral (−2.30)	3.11	
		c.1354C>T	D452N	rs112190116	0.01596	Benign (0)	Neutral (1.73)	2.07	
		c.1856C>T	P619L	rs146227301	0.002124	Benign (0.008)	Deleterious (−2.74)	—	
	FOXL2	c.13T>A	Y5N	—	ND	Damaging (0.998)	Neutral (0)	—	
c.536C>G		A179G	rs7432551	0.07913	Damaging (0.829)	Neutral (−2.44)	—		
ESR1	c.16C>T	H6Y	rs139960913	0.003604	Damaging (0.997)	Neutral (−0.54)	5.19		
	c.236C>G	P79R	—	ND	Benign (0.074)	Neutral (−0.48)	—		
TGF-β ligand		c.352T>C	S118P	rs200075329	0.009761	Damaging (1)	Deleterious (−2.56)	4.89	
		c.1726T>C	S576P	—	1.649e-05	Damaging (0.974)	Neutral (−0.44)	5.18	
		c.521C>T	S174L	rs114620737	0.00119	Benign (0.092)	Neutral (−1.76)	−2.01	
		c.652C>A	P218T	—	ND	Benign (0.002)	Neutral (−0.13)	ND	
		c.916C>A	L306M	rs144035874	0.004032	Damaging (1)	Neutral (−0.73)	3.17	
	FIGLA	c.122C>T	A41V	—	ND	Damaging (0.957)	Neutral (−1.91)	ND	
	BMP15	c.13A>C	S5R	rs113099187	0.01587	Damaging (0.838)	Neutral (−0.76)	1.24	
		c.308A>G	N103S	rs41308602	0.08748	Benign (0.002)	Neutral (−0.28)	0.75	
		c.443 T>C	L148P	rs114823607	0.003386	Damaging (1)	Deleterious (−7.05)	5.42	
		c.538G>A	A180T	rs104894767	0.01051	Benign (0.123)	Neutral (−0.01)	−2.37	
GDF9		c.307C>T	P103S	rs61754583	0.003072	Damaging (1)	Deleterious (−6.03)	−2.37	
Enzyme		c.442 A>G	T148A	—	ND	Benign (0.129)	Neutral (−1.26)	—	
		c.1360C>T	R454C	rs61754582	0.00294	Damaging (1)	Deleterious (−2.74)	5.24	
		LATS1	c.286 C>T	R96W	rs55945045	0.003544	Damaging (0.978)	Neutral (−1.40)	1.05
		c.610A>G	S204G	rs34793526	0.007851	Benign (0.451)	Neutral (−0.50)	4.9	
		GALT	c.776G>A	R259Q	—	ND	Damaging (0.999)	Neutral (−2.25)	—
	c.940A>G		N314D	rs2070074	0.09201	Benign (0)	Neutral (−0.59)	4.4	

Abbreviation: ND, not determined.

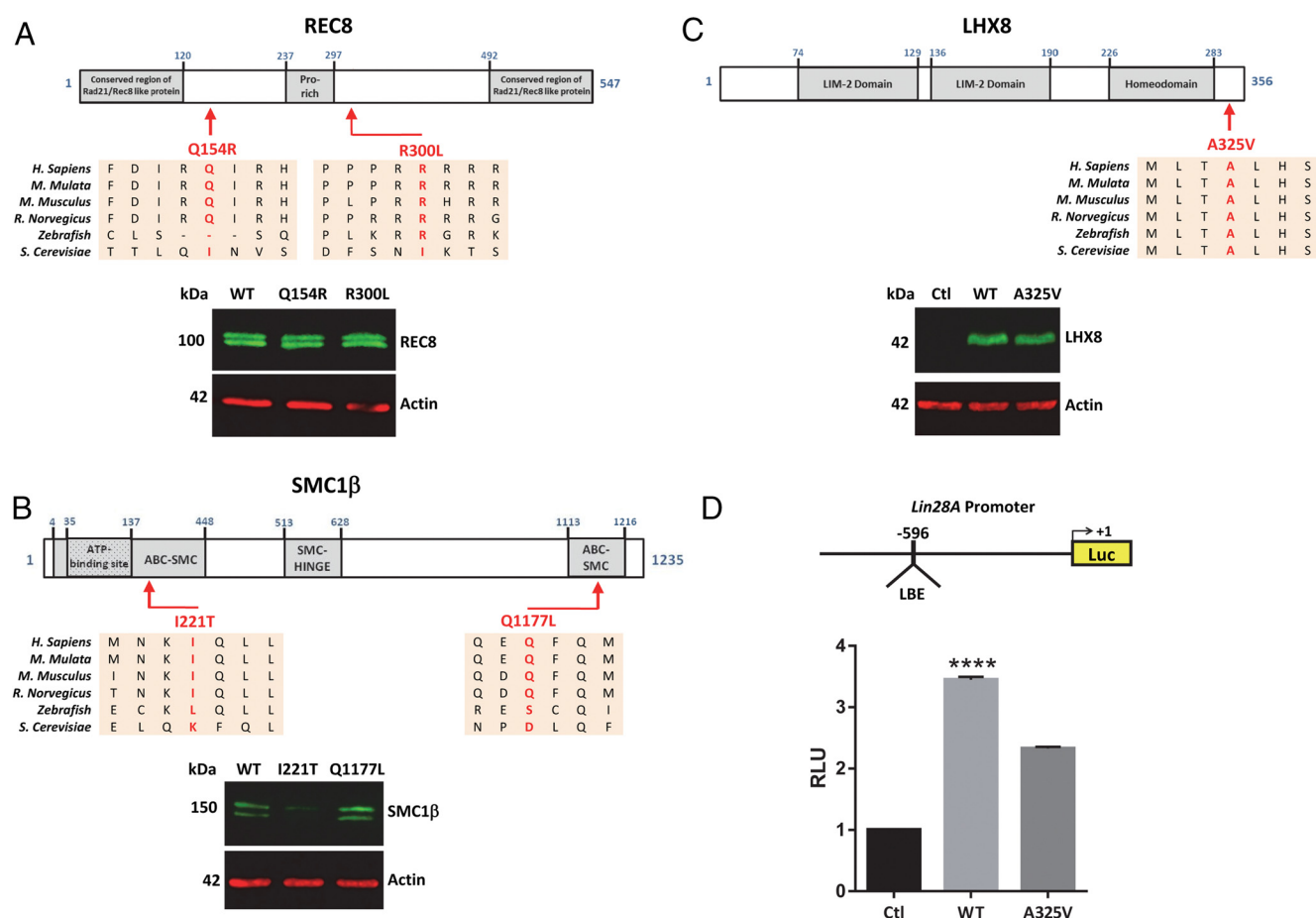
NOBOX or FOXL2 alone led to a significant transactivation of the promoter ($P < .01$). However, the co-overexpression of both wild-type transcription factors led to a significantly decreased activity compared with overexpression of each factor in isolation. We assessed the effect of the FOXL2 Y5N, and found the NOBOX/FOXL2 Y5N complex was unable to decrease the activity of *DK3* promoter consistent with loss-of-function (Figure 2C).

The *SOHLH1* mutants (S174L, P218T and L306M) were found at either low or unknown frequency in the ExAC database (Table 1). These variants were correctly expressed (Figure 2E). We tested the functionality of these variants using a promoter containing E-box motifs as previously described (21). All three were unable to exhibit a transcriptional effect compared with wild-type control (Figure 2F). The *FIGLA* A41V variant located at the N-terminal part of the protein (Figure 2G) is extremely rare. This residue is highly conserved, and the variant showed expression levels similar to wild-type (Figure 2H). However, the tran-

scriptional assay using the luciferase reporter construct revealed this mutation to be loss-of-function (Figure 2I).

Four variations of *ESR1* (H6Y, P79R, S118P, and S576P) were identified each with a very low or unknown frequency in ExAC. It has been previously reported that changing Ser118 to alanine (S118A) alters the unliganded and ligand-induced association of ERα and p160 coregulators with ERα target promoters compared with wild-type (22). This effect is due to an abolished serine phosphorylation site. The other variants (H6Y, P79R, and S576P) have not been previously described. All four were tested and are correctly expressed in vitro. A 17β-estradiol dose-response of ERE-TATA-luciferase gene induction following transient transfection of HEK293T cells with wild-type or the four mutated *ESR1* constructs was performed. All variants had similar transcriptional activity compared with wild-type protein (Supplemental Figure 1).

Genes involved in the TGF-β signaling pathway (*BMP15* and *GDF9*) are critical for normal human fertil-



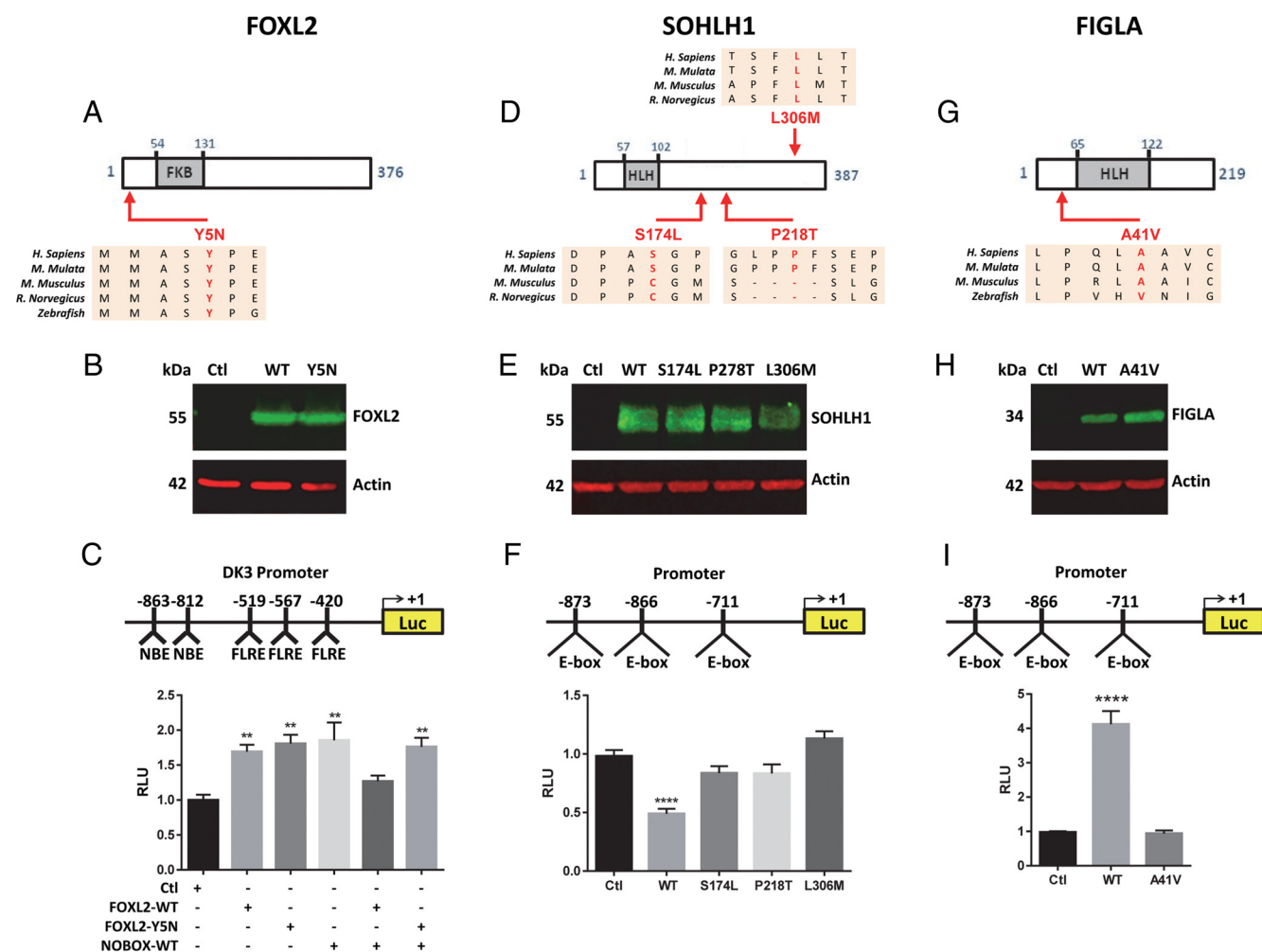


Figure 2. Loss-of-function mutations encoding transcription factors in patients with POI. (A, D, G) represent the structures of the transcription factor genes *FOXL2*, *SOHLH1*, and *FIGLA* respectively, as well as variations of interest and conservation of the relevant amino acid across vertebrates. (B, E, H) The overall expression levels were normal for all variants. Actin was used as a positive control for loading, and the empty vector (Ctl) was used as a negative control for protein expression. C, *FOXL2* Y5N disrupts the transcriptional activity of the NOBOX-*FOXL2* complex, even though it does not alone modify the transcriptional activity of the classical DK3 promoter. F, The *SOHLH1* S174L, P218T and L306M variants do not exert transcriptional activity on the E-box-containing promoter. I, The *FIGLA* A41V variant disrupts the transcriptional activity of the E-box-containing promoter. FKB, forkhead domain; HLH, helix-loop-helix; Ctl, control (empty vector); RLU, relative light units; FLRE, forkhead response element; NBE, NOBOX binding element; E-box, enhanced box. Results are mean of three independent experiments each performed in triplicate. Error bars represent SEM. **, $P < .01$; ****, $P < .0001$.

(2.1 ± 1.9 mL) was below the normal range (between 5 and 8 mL) of a fertile premenopausal woman. Antral follicular count was also very low and correlated with undetectable serum AMH levels (below the normal range, 15.7–48.5 pmol/L).

Interestingly, seven patients (No. 3; 4, A and B [sisters]; 5; 6; 9; 11; and 12) each harbor two variants in two different loci. Notably, one family included two sisters with primary amenorrhea (No. 4, A and B) who were found to harbor the *REC8* Q154R and *GDF9* R454C mutations. The *GDF9* mutation was inherited from the father and the *REC8* mutation from the healthy mother (Figure 3A). Another patient harbors a de novo *FOXL2* mutation and a *GALT* mutation inherited from her father, and she does

not exhibit any signs of an eye phenotype or metabolic, brain, or liver disorders (Figure 3B).

Most patients presenting with primary amenorrhea were found to carry two genetic defects. This was in contrast with the minority of patients with secondary amenorrhea (four of 14 patients). Remarkably, patients harboring two variants had a mean age of onset of 17 years, compared with 27 years for patients carrying only one variant. This strongly suggests that POI occurs significantly earlier in patients harboring multiple variants (Figure 3C).

Discussion

Herein we report that some patients with POI have multiple genetic variants and this finding may partly explain

Table 2. Summary of 20 Mutations of 10 Candidate Genes

	Gene	Sequence Variation	AA Change	dSNP	ExAC Allele Frequency	Frequency in 100 POI Patients	Functional Effect (Test or Reference)	
Meiosis	<i>SMC1-β</i>	c.662T>C	I221T	—	1.657e-05	0.01	ND	
		c.3530A>T	Q1177L	rs61737925	0.003193	0.01	ND	
	<i>REC8</i>	c.461A>G	Q154R	—	ND	0.02	ND	
Transcription		c.899G>T	R300L	rs145684232	0.002268	0.01	ND	
		<i>LHX8</i>	c.974C>T	A325V	rs34889650	0.001969	0.01	+
	<i>NOBOX</i>	c.271G>T	G91W	rs77587352	0.002413	0.05	Bouilly et al. 2011, 2015	
		c.349G>A	R117W	rs7800847	0.0068	0.01	Bouilly et al. 2011, 2015	
		c.1112A>C	K371T	rs189306575	0.0002189	0.01	Bouilly et al. 2015	
		c.1354C>T	D452N	rs112190116	0.01596	0.01	Bouilly et al. 2015	
		c.1856C>T	P619L	rs146227301	0.002124	0.01	Bouilly et al. 2015	
	<i>FOXL2</i>	c.13T>A	Y5N	—	ND	0.01	+	
	<i>SOHLH1</i>	c.521C>T	S174L	rs114620737	0.00119	0.01	+	
	TGF-β ligands		c.652C>A	P218T	—	ND	0.01	+
c.916C>A			L306M	rs144035874	0.004032	0.01	+	
<i>FIGLA</i>		c.122C>T	A41V	—	ND	0.01	+	
<i>BMP15</i>		c.13A>C	S5R	rs113099187	0.01587	0.04	Rossetti et al. 2009	
		c.443 T>C	L148P	rs114823607	0.003386	0.02	Rossetti et al. 2009	
		<i>GDF9</i>	c.307C>T	P103S	rs61754583	0.003072	0.01	Palmer et al. 2007
		c.1360C>T	R454C	rs61754582	0.00294	0.01	Palmer et al. 2007	
		Enzyme	<i>GALT</i>	c.776G>A	R259Q	—	ND	0.01

Abbreviation: ND, not determined.

the variable phenotypes observed in patients with POI.

The choice of the 19 candidate genes analyzed here was based on the reproductive phenotypes of genetically modified animal models of POI as well as key genes involved in early folliculogenesis. Inherited and de novo point mutations were identified in 10 of these 19 candidate genes. These mutations might underlie POI in 19% of the POI series examined in this study.

Independent studies have previously implicated meiosis-related gene mutations in POI (8–10). We found vari-

ations in *SMC1β* and *REC8*, two genes involved in meiosis, that are part of the cohesin complex (26). The pathogenic role of these mutations in POI is supported by studies of *Smc1β*^{−/−} and *Rec8*^{−/−} female mice that show very early ovarian defects and are sterile as a result of early meiotic arrest in oocytes (27, 28).

Although the mechanisms leading to follicular activation remain poorly understood, mouse models have helped identify several key transcription factors (29). In the present study, the mutation in *LHX8* implicates this gene in POI for the first time. The transcription factor

Table 3. Phenotypes, Genotypes, and Investigation of Gonadal Function in 19 Case Subjects With POI

Amenorrhea	Patient	Age at		Hormone Values		Meiosis		Transcription Factor					TGF-β Ligand		Enzyme	P value ^b
		Diagnosis	Puberty	FSH, ^a IU/L	LH, ^a IU/L	<i>SMC1-B</i>	<i>REC8</i>	<i>NOBOX</i>	<i>FOXL2</i>	<i>SOHLH1</i>	<i>FIGLA</i>	<i>LHX8</i>	<i>GDF9</i>	<i>BMP15</i>	<i>GALT</i>	
Primary	1	16	B1	73	17			G91W								1,5E-03
	2	16	B1	27	30					S174 L, P218T						ND
	3	13	B4P4	43	29	Q1177L								S5R		3,3E-05
	4A	14	B1	59	18		Q154R						R454C			ND
	4B	17	B2P2	40	16		Q154R						R454C			ND
Secondary	5	14	Normal	53	25				Y5N						R259Q	ND
	6	15	Normal	51	21						A41V		P103S			ND
	7	17	Normal	30	35			D452N								9,0E-02
	8	18	Normal	63	21									L148P		9,0E-03
	9	20	Normal	71	28			K371T						S5R		4,8E-05
	10	21	Normal	53	60			G91W								1,5E-03
	11	22	Normal	64	43	I221T		G91W, P619L								7,0E-06
	12	27	Normal	112	50			G91W						S5R		1,7E-06
	13	27	Normal	136	37									L148P		9,0E-03
	14	27	Normal	75	68							A325V				3,2E-01
	15	28	Normal	28	17								S5R			1,1E-03
	16	30	Normal	20	9			R117W								5,0E-02
	17	35	Normal	44	23			G91W								1,5E-03
	18	36	Normal	150	69		R300L									3,5E-02
	19	39	Normal	56	34							L306M				9,0E-02

^a The normal range of basal levels in control subjects is 3–9 IU/L for FSH, 1–5 IU/L for LH.

^b The allele frequency of variant was compared with the one reported in ExAC database using Fisher's exact test. ND, not determined.

Patients with two variants are in bold.

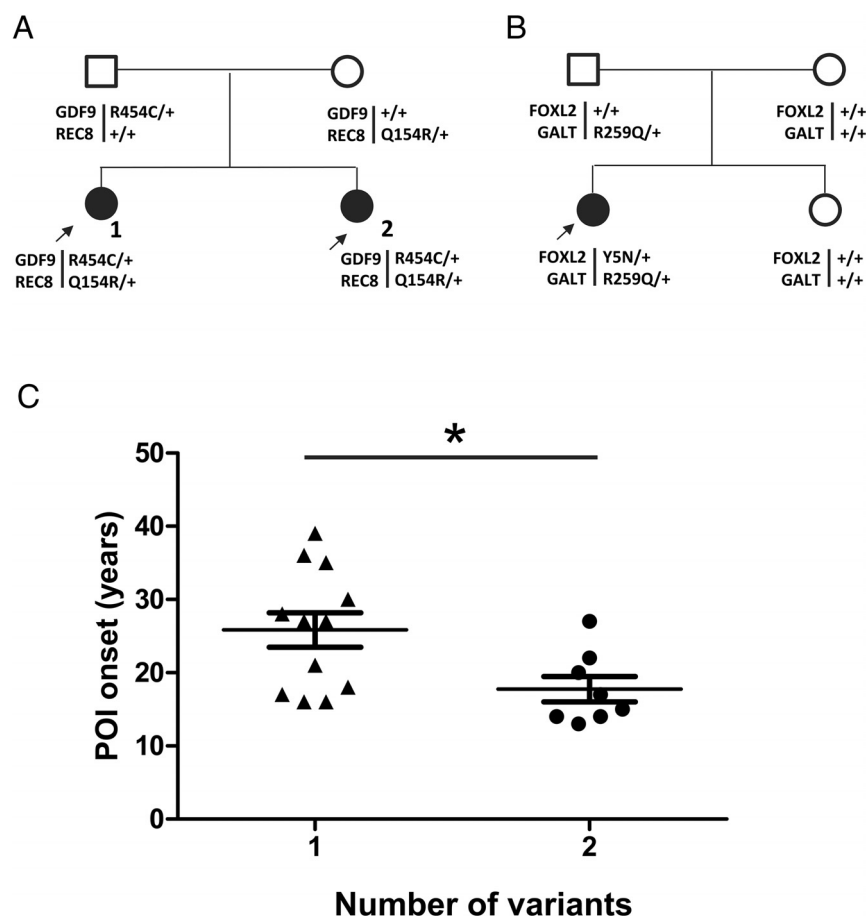


Figure 3. Pedigrees of POI patients harboring mutations, age of POI onset is depicted for women with mono- and digenic forms. A, Two sisters (arrow) with POI harbor *REC8* Q154R and *GDF9* R454C defects. B, The *FOXL2* Y5N and *GALT* R259Q variants were identified in a young girl (arrow). Females are depicted by circled, males by squares. C, Comparison of age of POI onset in patients with only one variant compared with those with two variants ($P < .05$).

encoded by this gene is a germ-cell-specific critical regulator of early oogenesis, acting in part by down-regulating the *Nobox* pathway and targets *Lin28a*, a protein critical for primordial germ cell development (30, 31). Here we found one *LHX8* variant in 100 patients whereas a previous study found no variants in a cohort of 95 Caucasian women with POI (32). This suggests that although *LHX8* mutations may underlie POI, this may be a rare event. Similarly, we found loss-of-function variants in transcription factors already implicated in POI including *NOBOX*, *SOHLH1*, *FIGLA*, and *FOXL2* (7). In particular, the subcellular localization and the aggregation of some *NOBOX* variants formed aggregates (article submitted). This may be of particular relevance for POI pathology as the aggregation of mutant proteins may hamper their translocation to the nucleus.

To date, more than 230 different mutations have been described in the human *GALT* gene (33). Classic galactosaemia is a rare etiology of POI. It is caused by galactose-1-phosphate

uridyltransferase deficiency and leads to a severe disease in the newborn. The germline *GALT* R259Q variant was identified in a patient with a nonsyndromic form of galactosemia, suggesting it may be relevant for genetic counseling. The same patient harbors a new *FOXL2* mutation yet did not exhibit any eyelid defects. This expands our knowledge of the phenotypic consequences of missense mutations in *FOXL2* as it is the second study demonstrating a POI phenotype resulting from a missense mutation outside the forkhead domain (34).

In addition, we found previously described variants in *BMP15* and *GDF9*. These TGF β -ligands play a key role in the molecular dialog between the oocyte and surrounding somatic cells, promoting granulosa cell mitosis and cumulus expansion via paracrine effects.

Altogether, genetic variants were discovered in 19/100 (19%) of the patients with POI. A number of observations, particularly in sporadic cases, suggest that POI is not always a monogenic Mendelian disorder as previously thought. Rather it seems that digenicity or potentially oligogenicity contributes to this disorder. The exhaus-

tion of the follicular reserve is an ongoing phenomenon that can occur before, during or after puberty. One paradigm for studying this is Turner syndrome. It is well-established that POI can emerge as a primary or secondary amenorrhea. A genetic cause of POI (predominantly in cases characterized by secondary amenorrhea) has been well-established in women carrying the fragile X premutation (7). This suggests that multiple genetic events could contribute to an increased decline in the ovarian follicle pool and follicle exhaustion. Using the PGM Ion Torrent technology, we identified seven patients with two heterozygous missense variants, a state associated with early POI onset. These data will be improved by an exhaustive analysis involving genes yet unknown.

The incomplete penetrance of POI has proven difficult to explain, but our findings suggest that digenicity (and perhaps oligogenicity) may play a role. Interestingly, we identified three digenic cases among five pa-

tients presenting with primary amenorrhea, compared with four cases among 14 patients presenting with secondary amenorrhea.

In addition, the digenic patients had earlier POI onset compared with those with a monogenic basis. This is in agreement with a very recent report showing that the addition of two genetic events (even on the same gene) induces early POI (35). Digenic inheritance has previously been suggested in another reproductive disorder namely Congenital hypogonadotrophic hypogonadism (36, 37) as well as cases of congenital heart disease (38).

Given these findings, it seems likely that mutational analysis of multiple genes in apparently monogenic disorders will become increasingly frequent. Further the discovery of several mutated candidates in the same individual may have important implications for genetic counseling.

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