Trends in Endocrinology & Metabolism

CellPress
REVIEWS

Review

Advances in the Molecular Pathophysiology, Genetics, and Treatment of Primary Ovarian Insufficiency

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Primary ovarian insufficiency (POI) affects ~1% of women before 40 years of age. The recent leap in genetic knowledge obtained by next generation sequencing (NGS) together with animal models has further elucidated its molecular pathogenesis, identifying novel genes/pathways. Mutations of >60 genes emphasize high genetic heterogeneity. Genome-wide association studies have revealed a shared genetic background between POI and reproductive aging. NGS will provide a genetic diagnosis leading to genetic/therapeutic counseling: first, defects in meiosis or DNA repair genes may predispose to tumors; and second, specific gene defects may predict the risk of rapid loss of a persistent ovarian reserve, an important determinant in fertility preservation. Indeed, a recent innovative treatment of POI by *in vitro* activation of dormant follicles proved to be successful.

Highlights

The mechanisms underlying the formation of the ovarian reserve are generally well conserved, from *Drosophila* to mammals. Owing to this high degree of conservation, factors shown to regulate the ovarian reserve in mouse models are all potential candidates for identifying mutations associated with POI in humans.

With the generation of genetically modified mice, much insight has been gained into the mechanisms that control the formation of the ovarian reserve and trigger the activation of primordial follicles.

Comparison with animal models is complicated by the fact that the phenotype of complete gene deletion in knockout models may not be mimicked by single gene mutations.

Recently innovative treatment for POI based on *in vitro* activation of the dormant primordial follicular pool has been developed.

Introduction

Primary ovarian insufficiency (POI), affects ~1% of women before 40 years of age, thus being a relatively frequent syndrome [1]. POI is often diagnosed too late, causing irreversible impairment to the fertility and well-being of the affected women. Recent data indicate that POI is associated with significant morbidity and mortality. Several of these risks are direct consequences of extra-ovarian defects, generated by the gene mutations that underlie some forms of POI [2]. The clinical relevance of POI has exponentially increased only very recently, particularly in economically advanced countries, due to the frequent choice of women to conceive after 30 years of age and the increased life expectancy.

POI can manifest as pubertal delay and primary amenorrhea (PA), secondary amenorrhea (SA), or oligomenorrhea of ≥4 months. Recurrence of menses and pregnancies can occur in up to 22% of cases with SA for up to 4 months [3], but spontaneous resumption of follicle activity is exceptional in cases with long-lasting SA. The POI-associated hypergonadotropic hypogonadisms is defined as elevation of **follicle-stimulating hormone (FSH)** (see Glossary) ≥ 25 IU/L confirmed twice, 30 days apart, in women with SA [1]. The **ovarian reserve (OR)** can be evaluated by transvaginal ovarian ultrasound (US) with antral follicular count and/or by **anti-Müllerian hormone (AMH)** determination. In both PA and SA cases, it is possible to uncover a certain OR by US and AMH measurement [4].

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Low/undetectable AMH indicates a dramatic diminution of the OR, predicting poor success of fertility preservation. However, follicular activity and pregnancy were rescued in POI patients with undetectable serum AMH after ***in vitro* activation (IVA)** and autotransplantation of fresh tissue [5].

Menstrual irregularities, such as oligoamenorrhea or polymenorrhea, can anticipate the onset of SA, but not as a rule.

Many clinicians are unaware of the advantages of early POI diagnosis and fail to provide integrated personal care to address all the clinical needs. Here, we review the pathophysiology, genetics and treatment of POI in order to shed light on: (i) the manifestations that should alert clinicians, and (ii) the novel multidisciplinary approaches for improved clinical management.

Iatrogenic POI frequently occurs in cancer survivors of young age. A variety of environmental factors, such as infections or pollutants like phthalates, bisphenol A, and polycyclic aromatic hydrocarbons from cigarette smoke, have a harmful impact on reproduction and are implicated in about 10% of POI [6]. Pollutants can affect ovarian follicles mainly by binding to estrogen or aryl hydrocarbon receptors, severely affecting follicle growth and viability [7]. Moreover, pollutants can cause germline epigenetic modifications, thereby accounting for transgenerational inheritance of reduced OR [8]. About 5%–30% of POI may have autoimmune origin [9], which is of potential interest because an early diagnosis may allow prompt treatment and eventually prevent damage to the OR.

The incidence of familial cases of premature ovarian failure was reported to vary from 4% to 31% [10,11]. Thorough evaluation of alleged affected relatives showed a lower incidence (12.7%) than the original family history suggested [12]. Pedigree studies on affected families showed a mode of inheritance suggestive of autosomal dominant or recessive transmission with highly variable expressivity or X-linked inheritance with incomplete penetrance [13]. Approximately 2–6% of women with sporadic POI have a premutation of the *FMR1* gene [14]. Other known genetic causes are responsible for a small proportion of POIs.

Most causes of POI are unknown. Understanding the underlying molecular mechanisms is essential to develop strategies for prevention, early diagnosis, and improved management of POI.

A great leap in the genetics of POI was achieved by the major methodological progress of **next generation sequencing (NGS)** and in particular **whole exome sequencing (WES)**. The knowledge of more than 60 genes has enabled genetic diagnosis by NGS and provided a flow chart for the diagnosis and treatment of POI (see below). A novel innovative treatment of the infertility of these patients has recently emerged.

Establishment of the OR

Primordial follicles (PFs) constitute the entire OR. Mechanisms that regulate the formation of the PF pool and the rate by which it is used will determine the duration of the fertile lifespan (Figures 1 and 2). OR formation is a similar process in humans and mice, and in recent years, much insight has been gained into the molecular mechanisms involved.

At 20 weeks of gestation, a pair of human ovaries contains close to 7 million germ cells [15]. A rapid loss of follicles in fetal life results in about 1–2 million oocytes at birth. Prior to puberty, the number declines further, to 300 000–400 000. During reproductive life, the number declines

Gonads, Unit of Genetic Stability, Stem Cells and Radiation: UMR 967, INSERM; CEA/DRF/iRCM/SCSR; Univ. Paris Diderot, Sorbonne Paris Cité; Univ. Paris-Sud, Université Paris-Saclay, Fontenay aux Roses, F-92265, France
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steadily until a critical threshold of 1000 PFs is reached [15]. Below this threshold, ovulations cannot be supported, yielding menopause.

Oocytes derive from **primordial germ cells (PGCs)**, which emerge from the extraembryonic mesoderm and migrate to the genital ridge (Figure 1). Upon arrival in the gonad, the PGCs will yield interconnected oogonia which, together with aggregation of germ cells, create so-called germ cell cysts [16]. Somatic cell-derived factors, in particular bone morphogenetic proteins (BMPs) and Wnt3/β-catenin, control the commitment, migration, and proliferation of PGCs. These signaling pathways induce the reacquisition of pluripotency, which is driven by Prdm1 and Prdm14. Subsequently, pluripotency genes (*Pou5F1* encoding Oct4, *Nanog, Klf4*, *Lin 28a*) and germline specific genes (early genes: *Nanos3*, *Kitlg*, *Tfapc2*, *Dppa3*; late genes: *Ddx4* or *Vasa*, *Mael, Dazl*) play important roles in the migration, proliferation, and survival of PGCs [16]. After cessation of mitosis, the oogonia enter **meiosis** (13.5 days postconception in mouse and asynchronously ~10–12 weeks of gestation in humans) and become oocytes (Figures 1 and 2). They will progress through the initial stages of prophase I until arrest in the diplotene stage of prophase I. The initiation of meiosis is dependent on expression of *Dazl*, which induces responsiveness to retinoic acid, which in turn induces Stra8 (stimulated by retinoic acid)-dependent and -independent pathways, and subsequently the activation of the synaptonemal complex (SC) proteins Sycp1, 2, and 3. Recently, Taf4b was identified as an upstream regulator of several meiotic genes, including *Stra8* and *Dazl,* in both mice and women [17]. Additionally it recently appeared that post-transcriptional mechanisms involving the Ythdc2/Meioc complex are mandatory for the proper mitotic/meiotic transition [18,19].

The next developmental step in follicle formation is the breakdown of germ cell cysts between E17.5 and 5 days postpartum in mice and at midgestation (10–13 weeks of gestation) in humans (Figure 1). Upon breakdown, pregranulosa cells are recruited to encapsulate a single oocyte to form PF, and oocytes undergo meiotic arrest. Gdf9, Bmp15, FoxL2, Nobox, Figla, Notch2, and Adam10 include some of the factors that affect the timing of cyst breakdown and differentiation of pregranulosa cells [20,21]. Furthermore, estrogen signaling plays an inhibitory role in cyst breakdown [22]. At the cessation of mitosis, the number of oogonia has increased exponentially. However, it is estimated that during cyst breakdown, two-thirds of the oocytes are lost through programmed cell death, including apoptosis and autophagy [23].

Genetic modification in mice has been beneficial for identification of the crucial genes in establishment of the OR. Mutations in several of these genes have been identified in women with POI.

Recent Advances in the Genetics of POI in Humans

The genetic causes of POI are highly heterogeneous, with isolated or syndromic forms. Reproductive and extra-reproductive features of syndromic POI are described in Table 1. The genes involved are listed in Figures 1–3 and Table 1.

Meiosis, DNA Repair, and POI

Mutations of meiotic and DNA repair genes are responsible for syndromic and nonsyndromic POI (Figures 2 and 3). There have been recent major advances in the identification of these genes as a cause of POI through NGS studies.

Oocytes enter into and progress through meiosis prophase I during fetal life. Mutations in meiotic genes usually impair meiotic progression and trigger oocyte death, as evidenced by several mouse models [24].

Glossary

Anti-Müllerian hormone (AMH): a growth factor produced by the granulosa cells of growing follicles. Serum AMH level is an indirect marker of the ovarian reserve and declines with increasing age.

Follicle-stimulating hormone (FSH): a pituitary-derived hormone that stimulates estrogen production, follicle growth, and selection of the preovulatory follicle. Serum FSH levels are elevated upon ovarian aging due to the loss of negative feedback signals. Normal FSH concentrations (IU/l) are: during follicular phase, 3.5–9.0; ovulatory phase, 7.0–21.5; luteal phase, 1.7–7.0; postmenopause, 26–140.

Homologous recombination (HR): a process that assures faithful repair of double strand breaks, one of the most dangerous DNA damages. HR relies on the invasion of a similar DNA matrix (the homologous chromosome during meiosis) as a template to repair the broken DNA. The products of this repair can either be a local replacement of DNA sequence or exchange of large chromosome fragments, respectively termed non-crossover and crossover. The meiotic crossovers are mandatory for proper segregation of chromosomes, thus precisely halving the genome in gametes.

***In vitro* activation (IVA):** although menstrual cycles cease in POI patients, some of them retain residual dormant ovarian follicles. A new infertility treatment has been developed, which enables POI patients to conceive using their own eggs, by activation of the residual dormant follicles through *in vitro* manipulation of signaling pathways responsible for follicular quiescence.

Luteinizing hormone (LH): a pituitary-derived hormone that triggers ovulation. Serum LH levels increase upon ovarian aging due to the loss of negative feedback signals.

Meiosis: meiosis is the universal cellular process in eukaryotes that allows formation of the haploid reproductive cells.

DNA double strand breaks (DSBs): DSBs are programmed DNA breaks generated early during prophase I and catalyzed by the sporulation 11 homolog (SPO11) enzyme. DSBs are concentrated in

During prophase I, meiosis requires the establishment of the SC and the generation and repair of **DNA double strand breaks (DSBs)** [25]. Cohesin rings surrounding the chromosomes contribute to proper formation of the SC. Stromal antigen 3 (*STAG3*), Recombination 8 (*REC8*), Structural Maintenance of Chromosomes 1B (*SMC1B*), and Radiation Sensitive 21-Like (*RAD21L*) encode proteins belonging to the cohesin family and are specific to meiosis. Exome sequencing revealed that the two copies of *STAG3* are inactivated by a truncating mutation in patients with POI from a consanguineous family [26]. Of note, one patient had bilateral ovarian tumors. Inactivation of *Stag3* in mice impairs meiotic progression and leads to oocyte death [27]. *SMC1B* and *REC8* have also been proposed to be associated with POI [28]. The SC is formed by several proteins organized in lateral and central elements [25]. A homozygous mutation of the Synaptonemal Complex Central Element Protein 1 (*SYCE1*) was described in two sisters with POI in a consanguineous family [29], consistent with infertility observed in animal models [30].

Mini Chromosome Maintenance 8 and 9 are helicase members of the MCM family. MCM8-9 complex is required for **homologous recombination (HR)**-mediated repair of DSB, facilitating DNA resection by the MRN complex [31]. Lack of *Mcm8* or *Mcm9* in mice induces meiotic defects, oocyte degeneration, and ovarian tumors. Regarding *MCM8*, the analysis of three consanguineous sisters with hypothyroidism and POI revealed the presence of a pathogenic variant [32]. The study of several other consanguineous families allowed the identification of homozygous variants for MCM8 and MCM9 in the affected patients [33–36].

For MCM8 and MCM9, the repair of chromosomal breaks in fibroblasts or lymphocytes of the patients was found to be altered [32,33].

Meiotic DSB repair requires the loading of two recombinases, RAD51 and its meiotic paralog DMC1, on DNA. The activities of DMC1 and RAD51 are regulated by many factors, including homologous-pairing protein 2 homolog (HOP2/PSMC3IP). Only one homozygous mutation in *DMC1* has been reported in women with POI [37]. The study of a Palestinian family using homozygosity mapping and NGS allowed the detection of a homozygous microdeletion in the *PSMC3IP* gene [38]. The possibility of a meiotic defect in the patients studied was not examined directly.

The recombination intermediates need to be stabilized to promote the formation of crossovers. This step requires helicases such as HFM1 and the dimer MSH4-MSH5. Exome sequencing has uncovered composite heterozygous mutations in *HFM1* in a cohort of patients with sporadic POI and SA [39,40], in agreement with the phenotype of the *hfm1*$^{-/-}$ mice. Exome sequencing recently identified a deleterious homozygous donor splice-site mutation in *MSH4* in a case of familial POI. This mutation was associated with the generation of internally deleted MSH4 protein [41]. Similarly, a homozygous mutation in *MSH5* in two sisters with POI has recently been reported [42]. The adverse effect of this mutation was confirmed in a mouse model and proven to impair DNA repair.

The final step of recombination is the resolution of recombination intermediates. The resolution of the double Holliday junctions is believed to rely on the heterodimer MLH1-MLH3, and the exonuclease EXO1. Mice lacking either Mlh1 or Mlh3 are sterile. Human mutations reported in *MLH1* are largely associated with colorectal cancer and Lynch syndrome, with no systematic impact on fertility.

Three RecQ helicases, namely BLM (Bloom syndrome), RECQL4 (RecQ protein-like 4), and WRN (Werner syndrome) are proposed to be involved in meiotic recombination, albeit their

'hotspots' designated by PR domain containing 9 (PRDM9), through the deposition of trimethylation on lysine 4 of histone 3.

Next generation sequencing (NGS): also known as high-throughput sequencing, describes modern sequencing technologies that allow the sequencing of thousands to millions of DNA molecules simultaneously. It allows sequencing multiple genes and multiple individuals at the same time.

Nonhomologous end-joining (NHEJ): a DSB repair pathway often opposed to HR. NHEJ directly ligates broken DNA ends together. It is believed to result in low repair fidelity in the absence of a homologous sequence to guide DNA repair, as in HR.

Ovarian reserve (OR): a term describing the quality and number of resting oocytes within primordial follicles, and considered as a female's reproductive potential.

PI3K/Akt signaling pathway: the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway, regulating various stages of folliculogenesis. Studies in most genetic mouse models have revealed an essential role of this pathway in primordial follicle activation.

Primordial germ cells (PGCs): the primary cells that form the progenitors of gametes. PGCs will populate the embryonic gonads and differentiate into either oocytes or spermatocytes.

Whole exome sequencing (WES): sequencing by NGS, the protein-coding region of the human genome (exome) that represents <2% of the genome, but contains most known disease-related variants.

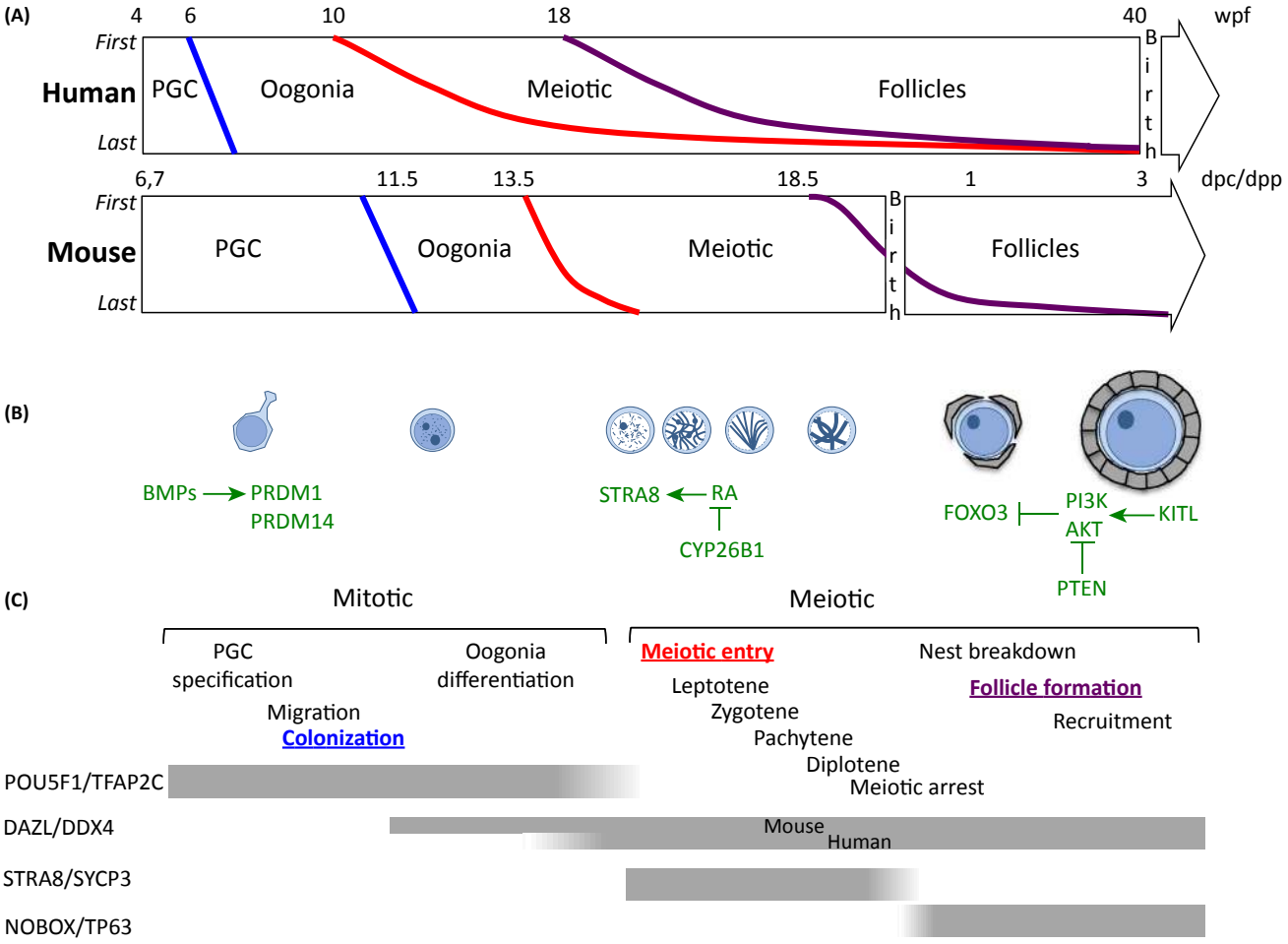

Figure 1. Formation of the Ovarian Reserve in Humans and Mice. (A) Chronology of female germ cell development from primordial germ cell (PGC) specification until follicle formation (purple). Appearance of oogonia and meiotic cells are defined respectively by the blue and red lines [weeks post fertilization (wpf), days post conception (dpc), days postpartum (dpp)]. (B) Schematic presentation of the various germ cell stages. From left to right, migratory PGCs, oogonia, and pre-leptotene-, leptotene-, zygotene-, pachytene-, and diplotene-arrested oocytes in primordial and growing (primary) follicles are presented in blue. Key signaling pathways regulating PGC specification, meiotic entry, and follicle activation are provided in green. (C) Frequently used markers of the various germ cell stages. POU5F1 (OCT4), POU class 5 homeobox 1, and TFAP2C (AP2 γ) are retrieved in PGC and oogonia with stem cell potential. Deleted in azoospermia-like (DAZL) and DDX4 (VASA), DEAD box polypeptide 4, mark the gametogenic competency acquired when mouse germ cells colonize the gonad and later during oogonial differentiation in the human ovary. STRA8 is expressed at the mitotic/meiotic switch, in the pre-leptotene stage, and SYCP3 is a synaptonemal complex protein that labels the axes of the chromosomes during meiotic prophase I. NOBOX, NOBOX oogenesis homeobox, and tumor protein 63 (TP63), are retrieved in the nuclei of diplotene-arrested oocytes enclosed into follicles. BMPs, Bone morphogenic proteins; CYP26B1, cytochrome P450, family 26, subfamily B, polypeptide 1; FOXO3, forkhead box O3; KITL, kit ligand; PI3K, phosphoinositide-3-kinase; PRDM, PR domain containing 1, with ZNF domain (PRDM1/BLIMP1); PTEN, phosphatase and tensin homolog; RA, retinoic acid; STRA8, stimulated by retinoic acid 8.

function in mammals is not fully elucidated. These RecQ helicases are also mutated in human syndromes manifesting in premature aging, cancer, and often POI or reduced fertility [43–45].

Cockayne Syndrome B (*CSB/ERCC6*) encodes a protein involved in DNA repair. A heterozygous mutation in the *CSB*-piggyBac transposable element derived 3 (*PGBD3)* fusion gene-induced POI with the mutated protein exhibiting an altered response to DNA damage [46].

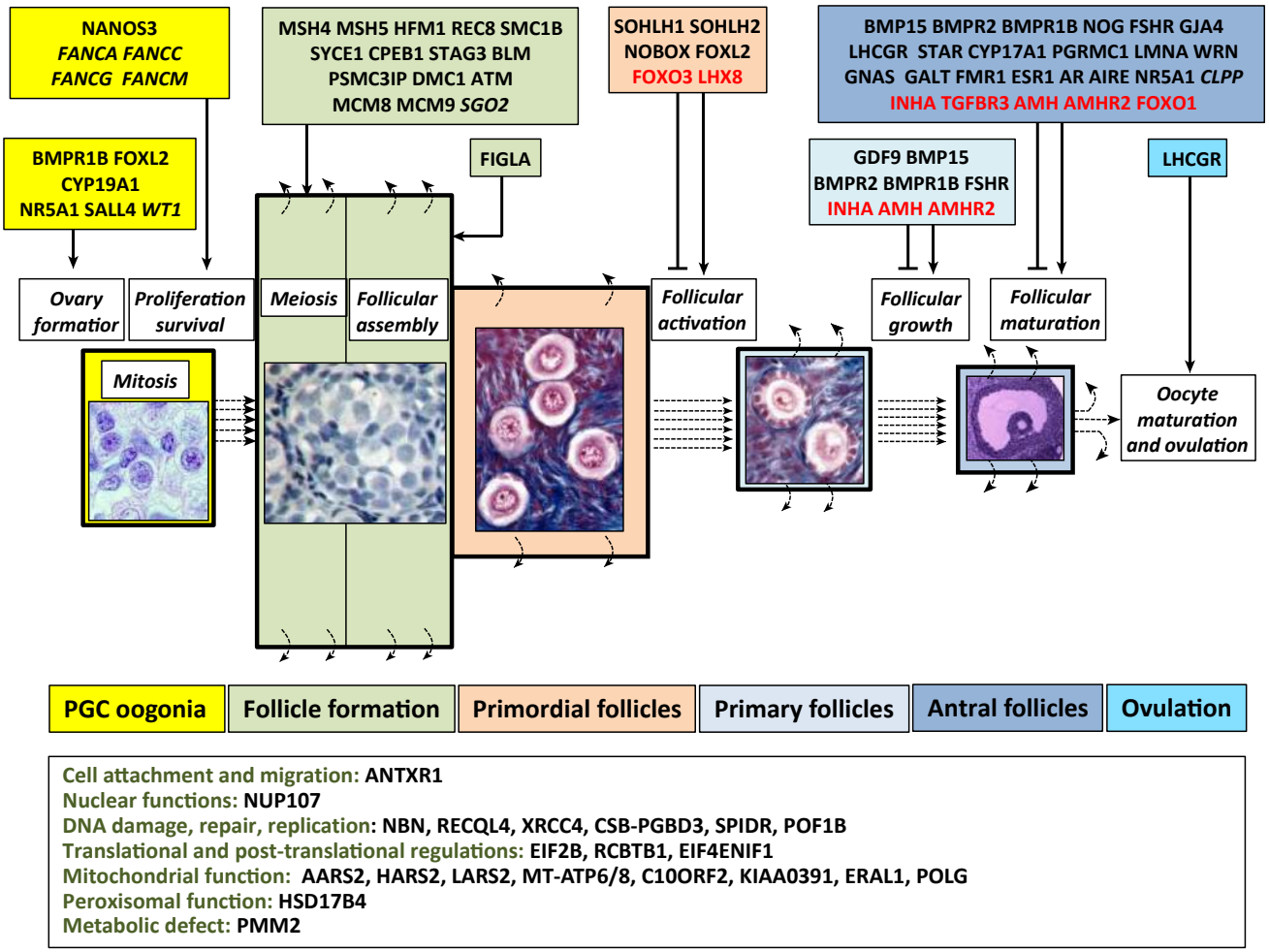

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Figure 2. Human Genes Associated with Primary Ovarian Insufficiency and Their Physiological Importance in Oogenesis, Folliculogenesis, and Other Functions. Genes with *in vivo* mutations associated with primary ovarian insufficiency (POI) in humans and the physiological importance of these genes in ovarian function are indicated. Oogenesis and folliculogenic processes are represented by a transit between different compartmental stages, depicted as boxes containing the cell populations. At each stage of oogenesis and folliculogenesis, an important part of the germ cell will die by apoptosis, depicted by a concomitant decrease in box sizes. The first compartment (yellow box) corresponds to the primordial germ cells (PGCs) when they differentiate into oogonia, the second compartment (green box) corresponds to follicle formation, which involves meiosis and follicular assembly processes and ends with establishment of the ovarian reserve of primordial follicles (pink box). From this reserve, follicular activation leads to the formation of primary follicles (light blue box). Then the growing follicles can reach the antral stage (dark blue box) and ovulate, or degenerate by atresia. Genes whose mutations are associated with POI are indicated at each stage of these developmental processes. The involvement of each gene at a specific stage of ovarian or follicular development is based on *in vivo* (presence/absence of follicles in biopsy samples, detection of antral follicles using ovarian ultrasound scanning or AMH measurements in women carrying mutations) or/and *in vitro* observations (culture experiments using human ovarian cortex or granulosa cells). For genes depicted in italic, information on their stage specific role is only available from mouse models. Genes associated with POI for which the stage-specific role is unknown are listed in the box, with their biological function (green font). The stimulating and inhibiting factors are depicted in black and red font, respectively. See text for references and [13].

Lastly, although meiotic DSB repair appears to rely on HR, a second process for DSB repair, the **nonhomologous end-joining (NHEJ)**, allows the direct ligation of broken DNA ends to each other. X-ray repair cross-complementing protein 4 (XRCC4) and Ligase 4 (LIG4) are two proteins absolutely required for NHEJ. Syndromic POI was reported in a female patient with

Table 1. Clinical Presentations of Syndromic POI.[a]

Diagnosis	Menstrual history	Ovarian phenotype	Particular features	OMIM #	Gene(s) involved
WT1-related XX-DSD	PA or SA	Streak gonads, partial ovarian dysgenesis	Nephropathy, diaphragmatic hernia	194070	*WT1*
SF1-related XX-DSD	PA or SA	Streak gonads, partial ovarian dysgenesis	Adrenal insufficiency	612964	*NR5A1/SF1*
BPES	PA or SA	Rare or absent follicles	Blepharophimosis, ptosis, epicanthus inversus	110100	*FOXL2*
FMR1 premutation	SA	Follicle depletion	X-linked mental retardation in family. Fragile X tremor/ataxia syndrome	300624	*FMR1*
Autoimmune polyendocrinopathy syndrome. APS-PGA type 1	PA or SA	Autoimmune oophoritis	Addison disease, candidiasis, vitiligo, hypoparathyroidism, diabetes mellitus, hepatitis, malabsorption, keratopathy, alopecia	240300	*AIRE*
Autoimmune APS-PGA type 3		Autoimmune oophoritis	Autoimmune thyroid disease, atrophic gastritis, vitiligo		
Pseudohypopara thyroidism	SA	Follicular cysts but no corpora lutea in one case	Brachydactyly, short stature, hypocalcemia and hyperphosphatemia, hypothyroidism, obesity	#103580	*GNAS*
Galactosemia	PA	Streak ovaries or few nonmaturated follicles	Neonatal jaundice, failure to thrive, cirrhosis, cataract, intellectual disability, food intolerance, hypoglycemia, renal dysfunction.	230400	*GALT*
Disorders of glycosylation (CDG1A)	PA	Absent ovaries in some patients by US or laparoscopy	Growth retardation, microcephaly, encephalopathy, peripheral neuropathy, retinitis pigmentosa, cardiac myopathy, hepatomegaly, nephrotic syndrome, psychomotor retardation	212065	*PMM2*
Ataxia telangiectasia	PA		Cerebellar ataxia, telangiectasia, recurrent infections, malignancies, and increased levels of alpha fetoprotein	208900	*ATM*
Nijmegen breakage syndrome	PA or SA	Streak gonads, small ovaries	Prenatal growth retardation, progressive mental deterioration, microcephaly, recurrent infections, increased risk for neoplasias such as lymphoma	251260	*NBN*
Fanconi anemia	PA or SA	Decreased number of primordial follicles	Pancytopenia, small stature, microcephaly, ear anomalies, heart defects, kidney malformations, radial aplasia and thumb deformities, intellectual disability, café-au lait-spots	#227650 #227645 #614082	*FANCA* *FANCC* *FANCG*
XRCC4-related disorder	SA		Short stature, microcephaly, developmental delay, diabetes mellitus	#616541	*XRCC4*
Bloom syndrome	SA	Possibly accelerated follicular atresia	Premature aging with chromosomal instability, short stature, skin rashes and telangiectatic skin on sun-exposed areas, increased risk for neoplasias, immunodeficiency	#210900	*BLM*
Werner syndrome	SA	Possibly accelerated follicular atresia	Premature aging with chromosomal instability, pre- and postnatal growth deficiency, sclerodermic skin changes, cataract, arteriosclerosis, increased cancer risk, diabetes mellitus	#277700	*WRN*
Rothmund-Thomson syndrome	SA	Gonadotropin resistance	Short stature, cataract, saddle nose, teeth anomalies, premature graying of hair	#268400	*RECQL4*

Table 1. (continued)

Diagnosis	Menstrual history	Ovarian phenotype	Particular features	OMIM #	Gene(s) involved
Hutchinson-Gilford progeria	SA	Diminished follicular reserve	Progeria, short stature, low body weight, early loss of hair, lipodystrophy, scleroderma, decreased joint mobility, osteolysis, cardiomyopathy	#176670	*LMNA*
GAPO	SA	Follicle depletion	Growth retardation, alopecia, pseudoanodontia, optic atrophy, high forehead, midface hypoplasia	#230740	*ANTXR1*
Perrault syndrome	PA, SA	Streak ovaries, lack of ovaries, small ovaries	Deafness Neurologic symptoms in PRLTS1, PRLTS3, and PRLTS5	#233400 #614926 #614129 #615300 #616138	*HSD17B4, HARS2, LARS2 CLPP* *C10orf2, CLDN14+ SGO2* *KIAA0391* *ERAL1*
Woodhouse-Sakati syndrome	PA	Streak ovaries	Alopecia, deafness, hypogonadism, diabetes, intellectual disability	#241080	*C2orf37*
Vanishing white matter disease Ovarioleukodystrophy	SA	Ovarioleukodystrophy streak ovaries	Progressive cerebellar ataxia, spasticity, cognitive impairment with white matter lesions on brain imaging. Onset from early infancy to adulthood	#603896 #615889	*EIF2B* *AARS2*
Retinal dystrophy with or without extraocular anomalies	SA		Retinal dystrophy, goiter, intellectual disability, hypogonadism	#617175	*RCBTB1*
Progressive external ophthalmoplegia	SA	Diminished follicle reserve	Ptosis, progressive external ophthalmoplegia, sensorineural hearing loss, axonal neuropathy, muscle weakness, ataxia, dysarthria, dysphagia and late onset Parkinsonism	#157640	*POLG*
Acromesomelic chondrodysplasia with genital anomalies	PA		Severe brachydactyly with radial deviation of the fingers, ulnar deviation of the hands, fusion of the carpal/tarsal bones, aplasia of the fibula, bilateral clubfeet with small broad feet and short toes	#609441	*BMPR1B*
Interphalangeal joint synostosis	SA		Symphalangism, hearing loss	#185800	*NOG*

[a]DSD, Disorder of sexual differentiation; PA, primary amenorrhea; SA, secondary amenorrhea; US, ultrasound. See text for references and [13].

homozygous single nucleotide variant in the *XRCC4* gene [47]. POI was reported in two patients with biallelic truncating mutations in the *LIG4* gene [48]. These patients display short stature, microcephaly, and genomic instability or hypersensitivity to radiation. Similarly, another important DNA repair pathway, the Fanconi anemia (FANC) pathway, exists in numerous progenitor cells, including the germline. This pathway employs at least 20 proteins, including those encoded by the *FANCA*, *FANC*, and *FANCG* genes, and has been associated with POI [49]. Mouse models for several *Fanc* genes (a, c, d, e, f, g, i, m, n, o, p) evidenced gonadal hypoplasia with ovaries showing follicle depletion [50]. This appears to be due to reduced PGC numbers, though meiotic roles are also possible. Very recently a homozygous *FANCM* mutation was shown to underlie a familial case of nonsyndromic POI [51]. FANCM biallelic mutations predispose to cancer, in particular early-onset breast cancer in females and chemosensitivity

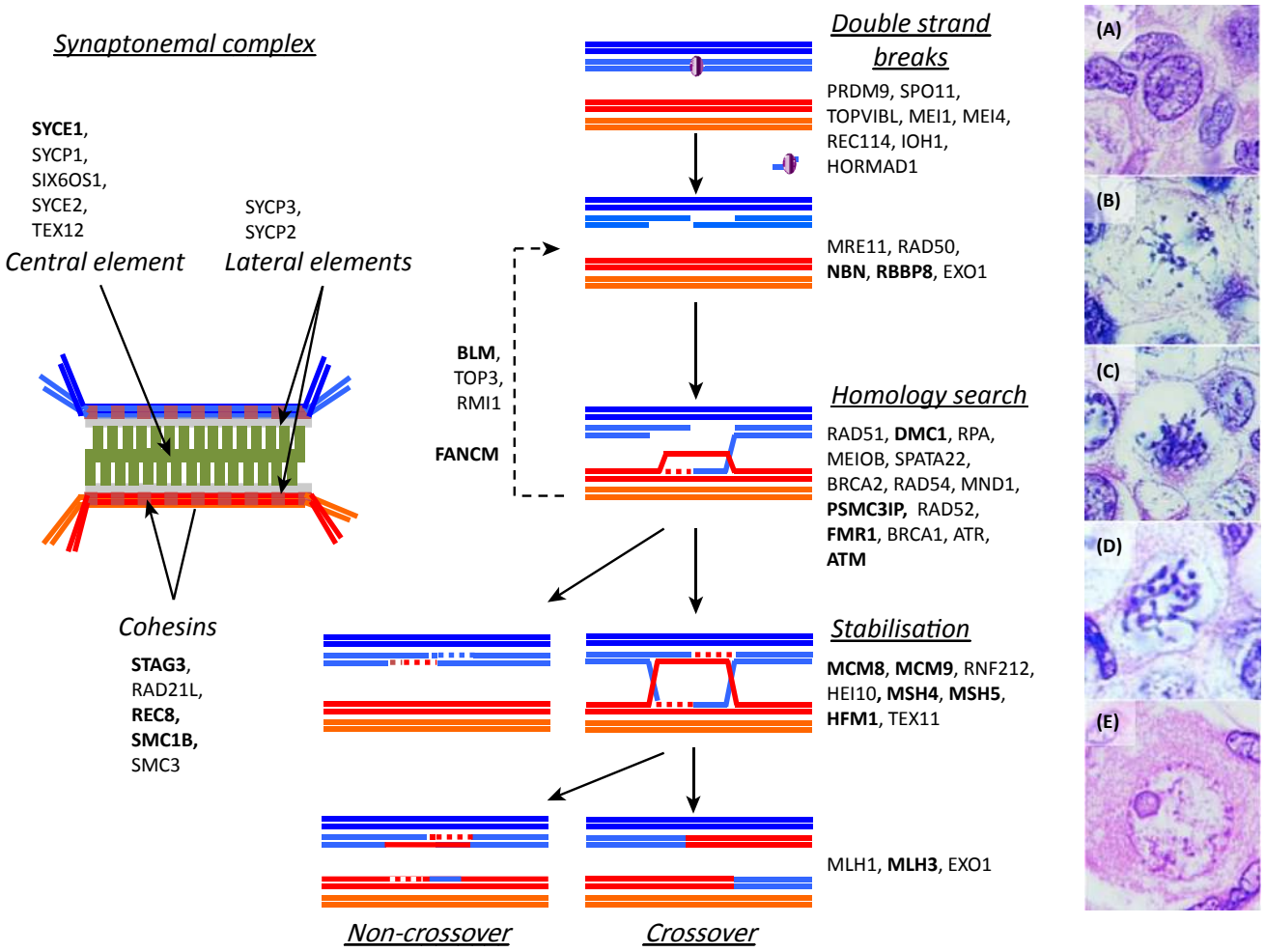

Figure 3. Female Infertility, Meiotic Recombination, and DNA Repair. The various steps of meiotic recombination and the specific genes involved (nonexhaustive, middle panel) are presented in the middle panel. Genes suspected in primary ovarian insufficiency cases are in bold. Left panel shows the structure of the synaptonemal complex required for completing recombination. Right panel presents representative germ cells at oogonial (A), leptotene (B), zygotene (C), pachytene (D), and diplotene (primordial follicle) (E) stages, from human ovaries at 8, 12, 15, 21, and 27 weeks postfertilization, respectively.

[52–54]. These findings clearly support a genetic link between infertility and DNA repair/cancer genes.

The recent identification of a genetic link between POI and tumor/cancer susceptibility genes (*STAG3*, *MCM9*, *FANCM*) makes the genetic diagnosis of all isolated cases of unexplained POI necessary, to perform enhanced genetic counseling and long-term follow-up. Indeed, POI patients can harbor mutations in such 'cancer susceptibility' genes. The large number of genes potentially involved will make these families among the most important involved in POI.

Genes Involved in Syndromic POI (Figures 2 and 3 and Table 1)

The clinical presentations of syndromic POI are highly variable and are presented in Table 1.

Perrault Syndrome (PS)

Perrault syndrome (PS) is a genetically heterogeneous autosomal recessive syndrome, mainly characterized by ovarian dysfunction and sensorineural deafness (Table 1). Recently, a growing number of genes involved in PS were identified by NGS. These genes are implicated in mitochondrial functions or metabolism. In mouse models, genetic changes that cause perturbation in mitochondrial protein translation lead to hearing loss as a result of tissue-specific apoptosis [55]. Given the role of apoptosis in ovarian development, inappropriately timed apoptosis may also lead to POI. *HARS2* [56] and *LARS2* [57,58] encode mitochondrial histidyl or leucyl-tRNA synthetases involved in translation of mitochondrially encoded genes. *CLPP* encodes a highly conserved endopeptidase component of a mitochondrial ATP-dependent proteolytic complex, involved in degradation of unfolded or misfolded polypeptides [59–61]. *C10orf2* encodes Twinkle, a mitochondrial primase-helicase essential for mitochondrial DNA replication [62,63], yielding a mitochondrial DNA depletion syndrome and progressive external ophthalmoplegia. Very recently, mutations of *ERAL1 and KIAA0391* were involved in PS. ERAL1 protein binds to the mitochondrial 12S rRNA and is involved in assembly of the small mitochondrial ribosomal subunit affecting mitochondrial respiration and function [64]. *KIAA0391* encodes RNase P (PRORP) the metallonuclease subunit of the mitochondrial RNase P complex responsible for the 5′-end processing of mitochondrial precursor tRNAs [65].

Apart from mitochondrial functions, mutations in a multifunctional peroxisomal enzyme involved in fatty acid ß-oxidation and steroid metabolism, 17β-hydroxysteroid dehydrogenase type 4 [*HSD17B4*, also known as D-bifunctional protein *(DBP)*] also cause PS [66–68]. Mutations of this gene were already identified in autosomal recessive mode in a severe disorder of peroxisomal fatty acid β-oxidation.

A combination of two homozygous mutations leading to a coincidental PS, one in *CLDN14* involved in deafness and the other in shugoshin-like 2a (*SGO2*) encoding shugoshin2, likely involved in POI, have been described [69]. During meiosis in the mouse, SGO2 maintains the integrity of the cohesion complex that tethers sister chromatids. Unsolved cases of PS persist, indicating that novel genes will still be discovered [70,71].

Premature Aging Syndromes

Laminopathy due to mutations in *LMNA* encoding a nuclear envelope protein includes ovarian failure and premature aging. Malouf syndrome belongs to this condition [72]. Hutchinson-Gilford progeria Syndrome (HGPS) [73], caused by aberrant splicing of the *LMNA* gene and expression of a mutant product called progerin, comprises premature aging and lipodystrophies. Sometimes both syndromes occur together [73].

GAPO syndrome, another form of premature aging and premature follicle depletion, is caused by mutations of the anthrax toxin receptor 1 gene *ANTXR1* [74]. The protein has been involved in cell attachment and migration. Additionally, it allows the interaction of cells and several components of the extracellular matrix by binding extracellular ligands with the actin of the cytoskeleton.

Neurosensory Syndromes

Leukoencephalopathies are also a heterogeneous group of disorders associated with vanishing white matter and are seen in a subset of POI, yielding ovarioleukodystrophy. Mutations of a specific mitochondrial alanine aminoacyl-tRNA synthetase, *AARS2*, have been involved [75,76]. Another group of genes involved is *EIF2B1* to *EIF2B5* which encode the five subunits of the eukaryotic initiation factor 2B [77].

Mutations in *RCBTB1* [78] are present in syndromes including inherited retinal dystrophy and POI. *RCBTB1* is involved in ubiquitination, more specifically as a CUL3 substrate adaptor involved in stress-response to combat oxidative or electrophilic insults.

Mutations of the nuclear gene *POLG* encoding a mitochondrial DNA polymerase gamma can lead to POI with autosomal dominant progressive external ophthalmoplegia [79].

Defects in the respiratory chain or mitochondrial ATP synthase (complex V) result in mitochondrial dysfunction and defective energy production. Mutations of *MT-ATP6/8* encoding two of the subunits of complex V are associated with syndromes including cerebellar ataxia, peripheral neuropathy, diabetes mellitus, and POI [80].

Skeletal Syndromes

POI can occur in some skeletal syndromes, such as Demirhan syndrome, which is caused by mutations in *BMPR1B* [81].

Another condition including proximal symphalangism and POI is caused by mutations in *NOG* [82]. NOG protein is expressed in the ovaries and interacts with BMP, which plays an important role in ovarian function.

Genes Associated With Nonsyndromic POI

See Figures 2 and 3.

Regulation of PF Recruitment

The majority of PF will remain dormant until stimulatory signals or a break from inhibitory signals induces activation. PF recruitment is initiated in mice after birth at postnatal day 4–5, and in humans at 17 weeks of gestation (Figure 1).

Various oocyte-expressed signaling and/or transcription factors have been identified to maintain the quiescent state (Foxo3, Lhx8) or, in contrast, to activate PF growth (Sohlh1, Sohlh2, Nobox) [83] (Figure 2). Interestingly, Foxo3 and Lhx8 are the effectors of the **PI3K/Akt signaling pathway** [84]. Targeted (oocyte-specific) deletion of stimulating factors (Kit, Pdpk1, Rptor, Rps6) of this pathway blocks follicular activation and induces PF apoptosis, whereas loss of the inhibiting factors (Pten, Cdkn1b, Tsc1, Tsc2, Stk11) results in premature and global activation of PF [85] (Figure 2).

A characteristic of PF activation is the transition of squamous pregranulosa cells to cuboidal granulosa cells. Failure thereof results in an arrest at the primordial stage, followed by oocyte death and follicular depletion, as shown in *FoxL2* knockout mice [86]. AMH, expressed during this transition, inhibits PF activation since *Amh* knockout mice display an accelerated exhaustion of the pool [87]. Several additional growth factors have been shown to activate PF recruitment (Figure 2).

Regulation of Follicle Growth

Gonadotropin-Independent Phase: Role of Ovarian Growth Factors; Early follicle growth up to the large preantral stage is independent of gonadotropins in rodents and relies on intraovarian factors (Figure 2). It requires a coordinated dialog between the oocyte and granulosa cells, in which gap junctions and SMAD and PI3K/Akt pathways are important. The discovery that follicles in ovaries of *Gdf9* knockout mice fail to develop beyond the primary stage was the first of a series showing the importance of factors involving SMAD signaling pathway in follicle development [88]. Furthermore, in the oocyte-knockout of *Furin*, a prohormone convertase responsible for proteolytic cleavage of TGFβ family members, follicle growth is arrested at the secondary stage [89]. Likewise, inhibition of the PI3K/Akt pathway by *Kit* or *Kitlg* deletions leads to the blockage of follicular growth at the primary follicle stage. Using targeted deletion or activation of *Igf1*, Igf*1r*, Irs*2*, *Rictor*, or *Foxo3*, it was shown that the PI3K/Akt signaling pathway not only plays a role in PF activation, but also in follicle survival and development beyond the primary stage [90,91] (Figure 2).

Gonadotropin-Dependent Phase: Role of Gonadotropins; The progression through final stages of follicle development depends on the gonadotropins FSH and **luteinizing hormone (LH)** (Figure 2). The threshold for FSH sensitivity is determined by interplay between various stimulatory and inhibitory growth factors, such as IGF1 and various TGFβ family members tipping the balance to either follicle survival or atresia. Deletion of the *Fshr* yields an enhanced rate of atresia and follicles fail to progress to the antral stage [92]. Targeted deletion of the noncanonical progesterone receptor *Pgrmc1* in granulosa cells suppressed antral follicle development and increased atresia [93]. Finally, LH action is indispensable for ovulation, meiotic resumption of the oocytes, and cumulus expansion. Loss of LH action therefore also results in infertility as follicle development is blocked at the antral stage [94]. In the absence of sex steroid action, the final stages of follicle development show abnormalities leading to follicular arrest, as illustrated in mouse models lacking (cell-specific) androgen or estrogen function [95,96].

Defects in Human Genes in Nonsyndromic POI

Interestingly, there is an overlap between genes involved in the onset of puberty, normal reproductive aging, and POI [97]. We will present only recent data or selected examples of genes that illustrate the precaution that must be taken in the interpretation of genetic data and comparison with animal models.

Genes Involved in Establishment of the PF Pool and Maturation to Primary Follicles;

Heterozygous variants of SOHLH1 and SOHLH2 have been found in POI [98]. Interestingly, two families harboring a homozygous single-base deletion in the coding region or a premature stop codon of SOHLH1 [99] had PA, lack of secondary sex characteristics, and nonvisualized ovaries.

A recessive missense mutation in *Nucleoporin-107* was identified in a consanguineous family of Palestinian origin [100]. NUP107 is a component of the nuclear pore complex, and the NUP107-associated protein SEH1 is required for oogenesis in *Drosophila*. In *Drosophila*, Nup107 knockdown in somatic gonadal cells resulted in female sterility, whereas males were fully fertile. *Nup107* mutations may compromise the meiotic DNA damage response, leading to oocyte death.

A heterozygous stop codon was identified in the eukaryotic translation initiation factor 4E nuclear import factor 1 gene *eIF4ENIF1* in familial POI with dominant inheritance in three

generations [101]. The gene plays an important role in oocyte development in organisms from *Drosophila* to mice.

Heterozygous mutations of the Newborn ovary homeobox (*NOBOX*) transcription factor have been reported in women with sporadic POI [28,102,103]. Contrasting with the knockout mouse model, which displays accelerated postnatal oocyte loss due to a defect in germ cell cyst breakdown [104], patients with *NOBOX* mutations may have PA or SA with follicles detected by histology in the ovaries in adulthood [102]. This may be due to the fact that the human mutations caused only partial loss of function *in vitro*. Functional studies are thus critical before any comparison with animal models and before any conclusion on the human physiological role of a gene can be established. Interestingly, a prevalence of 5.6% and 6.2% of heterozygous mutations has been detected in different cohorts, making this gene potentially one of the most frequent causes of POI in humans, provided that causality of the heterozygous variants is proven. Recently, a homozygous truncated variant of NOBOX has been described [105], with complete loss of function *in vitro* in patients with PA, but with no ovarian phenotype. Fertility of the heterozygous mother excludes a mechanism of haploinsufficiency, as previously proposed.

Genes Involved in the Maturation and Growth from Primary to Ovulatory Follicles;

The two steroid hormone receptors, for estrogens (ESR1) and androgens (AR), are positive regulators of follicular maturation. Two families with homozygous mutations of *ESR1* have been described. The probands had PA without breast development, very high estrogen plasma concentrations, and multicystic ovaries [106,107]. Functional studies reveal altered estrogen signaling.

Interestingly, a continuum of phenotypes is associated with *FSHR* mutations, varying from absence of pubertal maturation to normal breast development with SA, according to severity of the receptor inactivation [108–111]. The first mutation described in the Finnish population was associated with the existence of preantral or rare antral follicles in the ovaries [108]. However, functional studies have shown that it was a partial loss of function mutation [112]. A complete loss of function mutation of the *FSHR* has also been described, causing PA and complete block of follicular maturation after the primary stage [113]. Remarkably, there was an increased density of small follicles when compared with an age-matched woman. Thus, the gonadotropin-dependent growth phase in humans starts at the primary follicle stage, contrary to rodents, in which preantral follicles are observed in ovaries of mice deficient in *Fshb* or *Fshr* (see above). Thus caution must be taken before extrapolating data from mouse models to humans. Partial mutations of the *FSHR* are associated with SA and the presence of different sized antral follicles, depending on severity of the mutation [114]. Of note, there is a correlation between the phenotype of the patients and the molecular studies. Because of the existence of follicles in the ovaries, *in vitro* maturation may be obtained and fertility restored ([115] and see below).

Mutations in the other gonadotropin receptor gene, *LHCGR*, cause POI with SA, anovulation, and recurrent cyst formation. In the affected families, disorders of sex differentiation are found in male relatives with hypogonadism due to Leydig cell hypoplasia [116,117].

The first involvement of *BMP15* in POI was reported in an Italian family with 46,XX ovarian dysgenesis [118]. Since then, several heterozygous and one homozygous BMP15 variant have been associated with PA or SA, but streak ovaries without follicles have been found using US, which was interpreted as premature depletion of the OR [118]. Functional studies suggest impaired production of the mature protein or, in some cases, a dominant negative effect [118].

However, most of the variants detected occur in the heterozygous state, and *BMP15* haploinsufficiency was proposed to have a predisposing impact for POI. It was also proposed that reduced BMP15 dosage would contribute to the ovarian phenotype of Turner syndrome patients [119]. These conclusions were challenged by a very recent work on a family with a *BMP15* knockout-like effect [120], with both parents bearing deletions in the proregion of the BMP15 precursor. The heterozygous mother conceived normally and had three children. Thus, it seems that haploinsufficiency is not involved in humans. Most of the mutations of *BMP15* described were heterozygous and a mechanism of haploinsufficiency or a dominant negative effect was suspected but most often not demonstrated, making it impossible to implicate the corresponding gene as the unique cause of POI. Additional genetic mutations in an oligogenic mode of inheritance and/or environmental factors must be involved. Despite streak ovaries, AMH was initially detectable in the two POI sisters bearing both deletions of BMP15, supporting the presence of an OR [120]. Five years later, however, AMH was not detected in both sisters, probably because of exhaustion of the PF pool, and one sister had received an egg donation.

In case POI is due to a block in follicular maturation, urgent fertility preservation is needed to avoid follicular atresia.

A recent study showed a homozygous single-base deletion in the coding region of *GDF9* in POI with PA [121], confirming the causative role of this gene.

Innovative Treatments for POI

The most frequent therapeutic approach of infertility of POI patients is embryo transfer from donated oocytes. Given the complexity of this therapeutic approach, couples requiring oocyte donation should discuss its medical, ethical, legal, and psychological aspects with medical experts. Recently, a new innovative fertility treatment has been developed for POI (Figure 4).

Premature activation of PFs caused by chemotherapy, particularly cyclophosphamide, is a significant cause of the disappearance of follicles from the ovaries. Fertility preservation through tissue cryopreservation before chemotherapy is therefore an important method for preventing POI [122]. Post-treatment, the tissue can be autotransplanted. Infants have been born as a result of the technique.

For cancer patients at high risk of reintroduction of the malignancy, such as leukemia, *in vitro* maturation of follicles all the way to metaphase II oocytes is a much needed therapy that still remains to be developed.

In early stages of ovarian insufficiency there are PFs left in the ovaries. Hence, cryopreservation of ovarian tissue as fertility preservation should be carried out as soon as the risk of follicular decrease has been identified. Although these PFs are inactive, Hovatta *et al.* [123] showed that human ovarian follicles can be activated when ovarian tissue is cut into small pieces and placed in organ culture. Recently, Hippo signaling was identified as the regulatory factor in this activation [5,124]. When residual follicles in ovarian tissue from POI patients were stimulated by cutting the tissue into small pieces, and subsequently exposed to phosphatase and tensin homolog (PTEN) inhibitors and protein kinase B (Akt) activators prior to transplantation, full oocyte maturation can be achieved [125] (Figure 4). Of note, PTEN is also an important tumor suppressor, and therefore its inactivation *in vivo* might be risky. After IVA, the follicles have to be stimulated to grow, and FSH stimulation is used in a similar manner as in ovulation induction or before *in vitro* fertilization treatments. The ovarian tissue has been transplanted back to patients after IVA and healthy infants have been born (Figure 4). This IVA method is useful for those

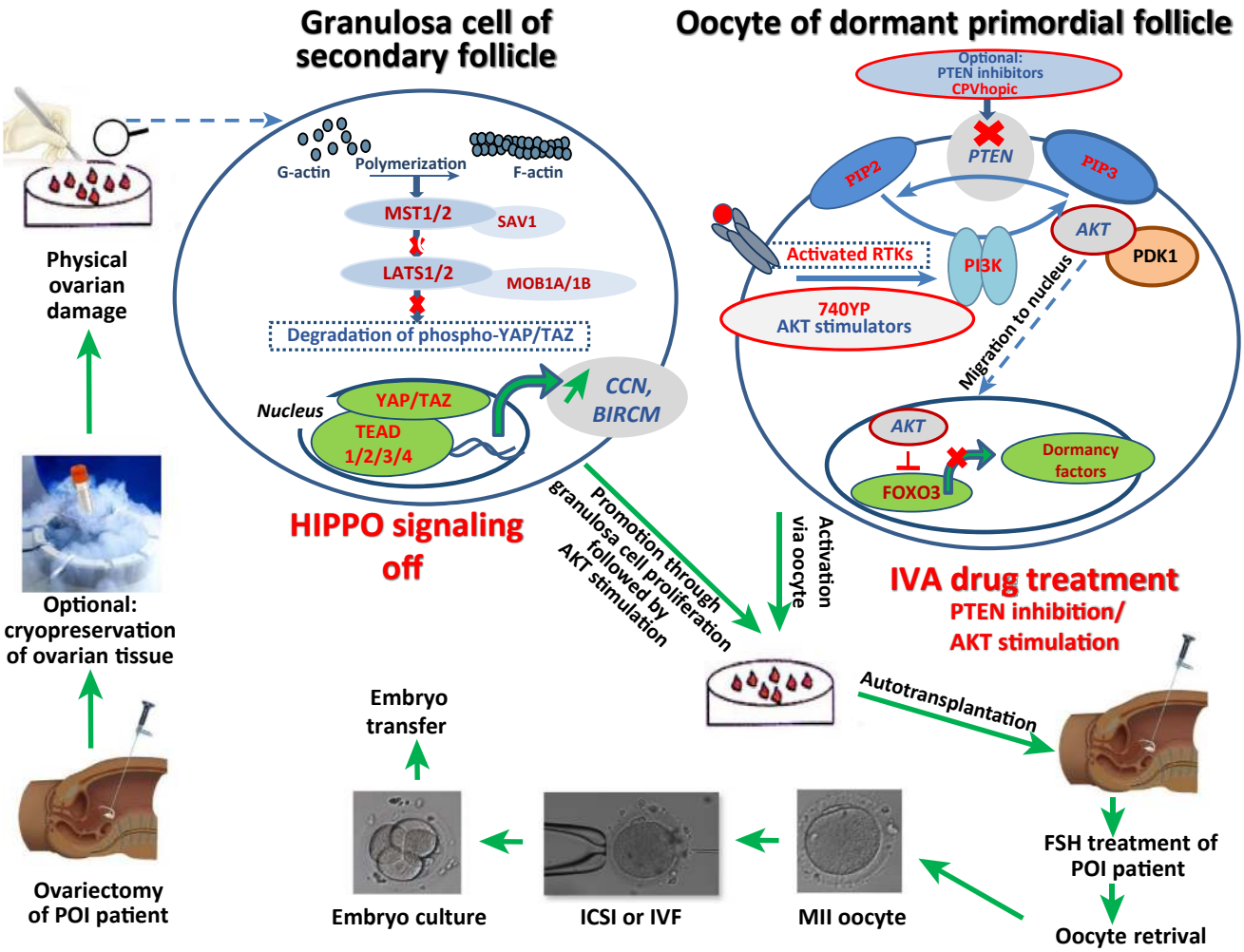

Figure 4. *In Vitro* Activation of Dormant Follicles. Ovarian cortical tissue is laparoscopically biopsied from the woman undergoing primary ovarian insufficiency (POI). The tissue is cut into slices for activation of Hippo signaling and initiation of follicular growth. Sliced tissue is then activated using a PTEN inhibitor or AKT stimulator *in vitro* for 24 hours. Thereafter, the tissue pieces can be transplanted back to the ovary of the donor woman. The activated follicles are stimulated using human recombinant follicle-stimulating hormone (FSH) for 6–10 days, until 15–17-mm sized antral follicles are seen by ultrasonography. The woman will be given human recombinant luteinizing hormone to induce the final maturation of the oocytes. The oocytes are collected 36 hours later using transvaginal ultrasound-guided needle aspiration. They are injected by intracytoplasmic sperm injection (ICSI) with sperm from the partner. The embryos are cultured for 3–5 days and the morphologically best embryo will be transferred to the intravaginal progesterone-treated female partner's womb. The rest of the embryos are cryo-stored for future transfers. IVA, *In vitro* activation; IVF, *in vitro* fertilization.

patients who may have residual follicles left in their ovaries (see flow chart in Figure 5 for POI diagnosis and treatment).

Ultimately, recent technological developments with induced pluripotent stem cells allow the reconstitution of complete oogenesis [126]. Currently this has only been achieved in the mouse, but advances with human cells now make it conceivable for modeling POI and would prove invaluable for supporting genetic diagnosis. In specific cases (e.g., altered follicle recruitment or growth), such models may prove useful for drug screening and selecting the most appropriate treatment.

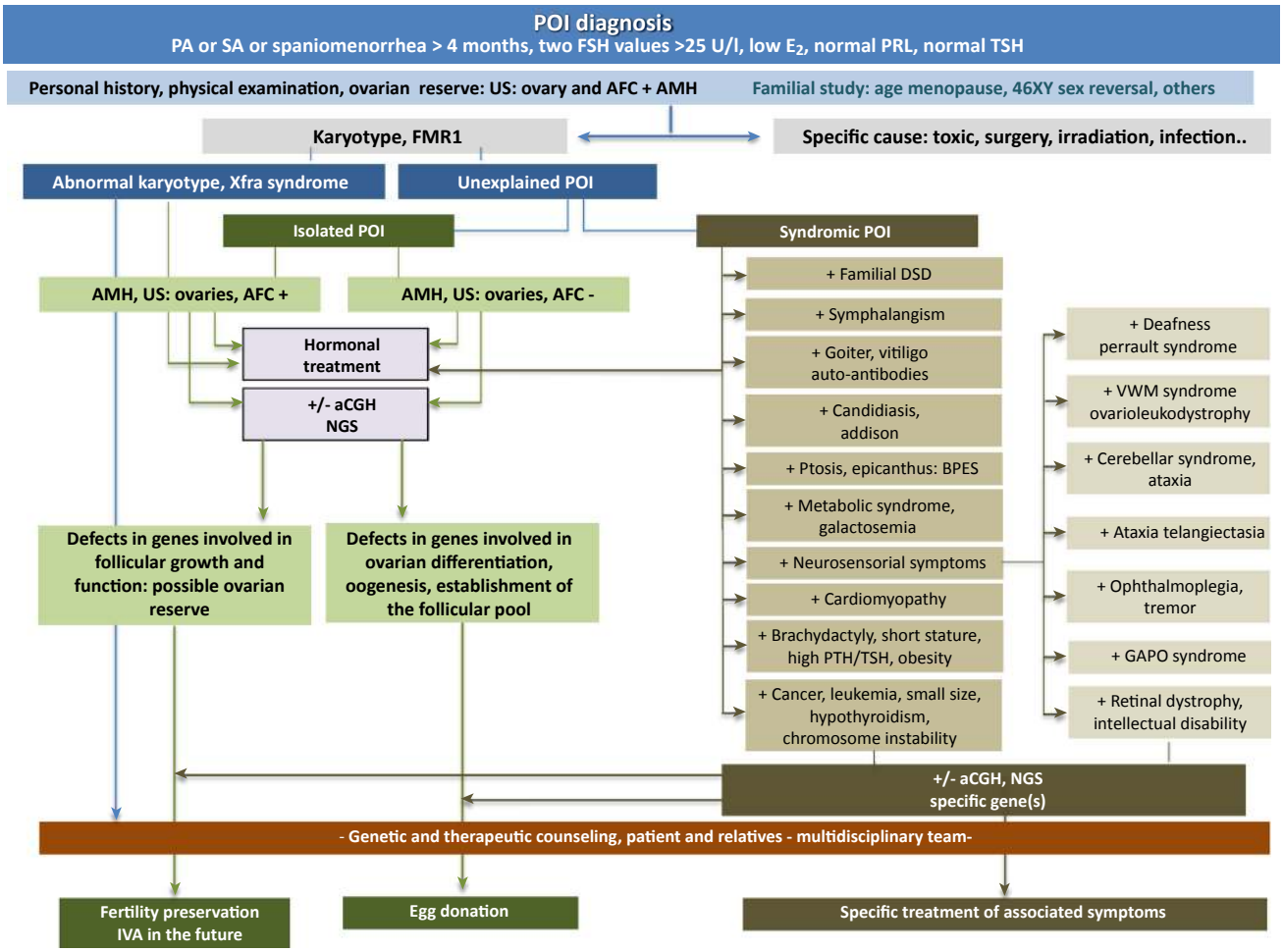

Figure 5. Flow Chart for Primary Ovarian Insufficiency Diagnosis and Treatment. After the initial diagnosis of primary ovarian insufficiency (POI), family investigation and evaluation of the follicular reserve by anti-Müllerian hormone (AMH) assay and antral follicular count (AFC) are performed. Specific causes are eliminated. In the case of unexplained POI, karyotype and FMR1 study are performed. In all cases hormonal treatment has to be started. In isolated POI, array comparative genomic hybridization (aCGH) or next generation sequencing (NGS) can highlight defects in genes involved in ovarian differentiation or in the establishment of the follicular pool. This, together with the undetectable ovarian reserve, will lead to genetic counseling in the patient and relatives and therapeutic counseling for the patient's infertility by a multidisciplinary team. If there is a wish to conceive, egg donation will be performed. In the case of a detectable ovarian reserve and/or a defect in genes involved in follicular maturation, genetic counselling will be performed in the patient and relatives and therapeutic counseling will lead to fertility preservation. In the future, *in vitro* activation (IVA) of small follicles might be performed. In syndromic POI, NGS of specific genes will be performed according to the clinical phenotype of the patient. Specific treatment of associated symptoms is needed. AFC antral follicle count; DSD, disorder of sexual differentiation; FSH, follicle-stimulating hormone; PA, primary amenorrhea; PRL, prolactin; PTH, parathyroid hormone; SA, secondary amenorrhea; TSH, thyroid-stimulating hormone; US,ultrasonography; VWM, vanishing white matter.

Other technological developments, such as tissue engineering to generate ovarian implants, are promising leads that may help restore fertility [127].

Concluding Remarks and Future Perspectives

Taken together, the vast technological advancements have provided valuable new information on the molecular pathophysiology of POI and its new diagnosis and treatment opportunities. Information derived from recent genetic studies has improved the accuracy of POI diagnosis and may reveal new targets for the treatment of infertility or for contraception in the future.

Because of its increased nonreproductive morbidity and mortality (e.g., autoimmunity and tumors) POI should be followed by a multidisciplinary team. The very recent identification of a link between POI and tumor susceptibility makes the genetic diagnosis of all isolated cases of unexplained POI necessary. Also, POI as a genetic disorder becomes amenable to innovative therapies, unlike most other genetic diseases. This obviously necessitates the presence of remnant OR that has to be evaluated besides conventional methods by genetic studies (Figure 5). Indeed, the key question is: what is the state of the follicular pool in the POI patient? The mutated gene may provide important information on the OR, depending on its level of action during either establishment and/or maintenance of the follicular pool, or follicular growth. This belongs to the questions that will need to be answered in the future (see Outstanding Questions).

Outstanding Questions

Does investigation of the genetic causes of POI improve our understanding of the regulation of meiosis in mammals? The vast majority of meiotic genes are shared by rodents and primates.

What is the interplay between DNA repair in cancer and infertility? Several genes involved in genomic stability are also associated with infertility. Based on the massive generation of hundreds of DSBs that need repair in each meiotic cell, this might not appear as a surprise. We may even consider that this extraordinary requirement of DNA repair could explain why genetic variants that only mildly impact genetic stability in somatic cells are unraveled in the case of infertility. In this case, a broader consideration of cancer predisposition in families with cases of infertility is likely a wise option.

Can we demonstrate systematically the genetic etiology of POI? It has been proposed that POI might frequently be a multigenic disease. Additionally, the functional demonstration of the pathogenicity of variants can be tedious and also depends on the genetic background. Therefore, induced pluripotent cells could offer a fantastic possibility to ascertain the genetic diagnosis.

Although mouse knockout studies in particular have identified several pathways that play a crucial role in PF recruitment, we still lack full understanding of the mechanisms that control gradual recruitment, as loss of function of the majority of these factors causes global activation of PFs.

How efficient is the *in vitro* activation of dormant follicles on a larger scale? We may reach the answer in the future, when more centers apply and test this. A question has been raised: how do we distinguish such pregnancies from the extremely rare, but not impossible, spontaneous pregnancies among the women with resumptive POI?

Can genetics provide information on the existence of a persistent follicular pool that is small, or comprises only PFs? Recent studies using near-infrared imaging of FSH receptors allowed the monitoring of undetectable

Acknowledgements

Apologies to those whose related publications were not cited due to space limitations. We thank Alain Gougeon for providing photographs of the different steps of follicular maturation.

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secondary follicles using US [128]. Identification of the genetic cause could be a possible predictor of an OR if a gene involved in follicular growth is identified. On the contrary, mutations in meiosis or DNA repair genes will most often exclude such a possibility (see flow chart in Figure 5 for diagnosis and treatment of POI).

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