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Molecular and Cellular Endocrinology**Recent advances in the study of genes involved in non-syndromic premature ovarian failure****Paul Laissue^{1,2}, Giovanna Vinci^{1,2}, Reiner. A Veitia^{1,2} and Marc Fellous^{1,2}**

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Abstract

Premature ovarian failure (POF) is a common pathology leading to infertility affecting about 1% of women under 40 years old. In POF patients, the ovarian dysfunction is characterised by the lack of the ovarian response to close a negative feedback loop on the synthesis of pituitary gonadotropins. Although the majority of cases are considered as idiopathic, diverse aetiologies have been associated, including genetic factors. Up to now, the potential genetic causes of non-syndromic POF have been established mainly by genetic linkage analysis of familial cases or by the screening of mutations in candidate genes based on animal models. Here, we review recent advances in the study of candidate genes.

Introduction

Recent developments and interactions between clinical and scientific fields have led to propose diverse and occasionally ambiguous definitions of human infertility (Habbema et al., 2004, Jenkins et al., 2004, Lunenfeld et al., 2004). Clinically, human infertility can be defined as the failure to conceive after one year of unprotected intercourse (Smith et al 2003, Habbema et al., 2004). In 40 to 60 % of infertile couples the aetiology is linked to female reproductive dysfunction, including ovulation disorders, tubal disease and endometriosis (Smith et al., 2003). Among these disorders, Premature Ovarian Failure (POF, OMIM 311360) is a common pathology leading to infertility. This multifactorial disease represents a public health concern since it affects around 1% of women under 40 years old (Coulam et al., 1986, Conway et al., 2000). Depending on the population, this proportion can reach 1.5 % (Luborsky et al., 2003). Classically, POF patients undergo a period of at least 6 months of amenorrhea associated with elevated plasma FSH levels (>40 IU/l). The hypergonadotropic hypogonadism in these patients is caused by the lack of the ovarian response to close a negative feedback loop on the synthesis of pituitary gonadotrophins (FSH and LH). Although the majority of cases are considered as idiopathic, diverse aetiologies have been associated with POF: infectious agents, chemotherapeutic treatments, pelvic surgery, autoimmune diseases, environmental and genetic factors. POF can be observed as a common feature of syndromic pathologies (i.e. Turner and BPES syndromes) or as an isolated condition.

Genetically, ovarian failure is associated with chromosome X and autosomal mutations. Concerning the X chromosome, the pathology is caused by deletions and rearrangements involving the large POF-1, POF-2 and POF-3 loci (Tharapel et al., 1993, Powell et al., 1994, Davison et al., 2000, Marozzi et al., 2000, Lacombe et al., 2006). Thus, genes situated in these regions are obvious candidate genes for POF (Bione et al., 1998, Davison et al., 2000, Prueitt et al., 2000). In Turner Syndrome, ovarian failure is probably due to the haploinsufficiency of genes situated on the critical regions of the X chromosome that escape inactivation (Zinn et al., 1998, Elsheikh et al., 2002, Sybert et al., 2004). In the X Fragile Syndrome, women carrying premutations in *FMR1* (Murray et al., 1998, Allingham-Hawkins et al., 1999) or presenting microdeletions within the *FMR2* gene (Murray et al., 1999) show significant predisposition to develop premature menopause. Furthermore, sequence variants in X-linked genes have been associated with POF. This is the case of *BMPI5*, an oocyte-expressed gene

whose mutations have been related to mammalian ovarian dysfunction (Galloway et al., 2000, 2002, Di Pasquale et al., 2004, 2006, Laissue et al., 2006, Dixit et al., 2006a, Veitia et al., 2006). Mutations in autosomal genes (i.e. *FSHR*, *LHR*, *GDF9*, *INHA*, *GALT* and *FOXL2*) have been also linked to POF pathogenesis in syndromic and non-syndromic cases (Aittomaki et al., 1995, Latronico et al., 1996, Laissue et al., 2006, Dixit et al., 2004, 2005, 2006b, Shelling et al., 2000, Guerrero et al., 2000, Crisponi et al., 2001, Goswami et al., 2005, Christin-Maitre et al., 2006). Up to now, the potential genetic causes of non-syndromic POF have been established mainly by genetic linkage analysis of familial cases or by the screening of mutations in candidate genes based on animal models. Here, we review some recent advances in the research of the genetic causes of POF.

The candidate genes.

Since the implementation of the knockout (KO) technology by homologous recombination (Koller et al., 1989) at least 300 mice models have been shown to display reproductive defects, substantially increasing the number of potential molecular actors involved in human pathology (Matzuk et al., 2002, Roy et al., 2006). Furthermore, in the ovarian context, transgenic mice models have provided keystones in the understanding of the genetic mechanisms concerning the different stages of follicular development and the hormonal regulation of female fertility (Barnett et al., 2006). The study of reproductive characteristics of other mammalian species has also provided hints in the research of POF candidate genes. Moreover, a different and powerful approach has been successfully used to map and identify POF-associated genes. For instance, Aittomaki et al., using a genetic linkage approach in 6 Finnish families, described the first gene whose mutations cause non-syndromic POF (Aittomaki et al., 1995). This study pointed to a locus on the chromosome 2p, containing the *LHR* and *FSHR* genes. This chromosomal region corresponded to the previously described ODG1 locus implicated in a recessive form of ovarian dysgenesis with elevated plasma levels of FSH observed in a geographically isolated subpopulation (Aittomaki et al., 1994). Sequence analysis of the *FSHR* revealed the c.C566>T (p.Ala189Val) missense mutation segregating with the phenotype. The sequence analysis of the exon 11 of the *LHR* in members of two families revealed the first mutation in this gene associated with an ovarian phenotype (Latronico et al. 1996). More recently, using a similar approach in 6 families, Crisponi et al., described mutations in *FOXL2*, a *forkhead* transcription factor as the cause of the BPES syndrome, (Crisponi et al., 2001). This autosomal dominant pathology displays in some cases

an ovarian dysfunction leading to female infertility. Although mutations in the *FSHR* and *LHR* have been identified as aetiological in some non-syndromic POF cases, we do not include in this manuscript extensive comments because these genetic associations have been found more than ten years ago and have already been reviewed in detail.

The TGF- β superfamily of growth factors: BMP15, GDF9 and INHA.

The TGF- β superfamily of growth factors is composed of at least 35 members including, BMP, TGF- β , GDF, activin and inhibin proteins (Massagué et al., 1998, Chang et al., 2002). The common three dimensional structure of these factors, deduced from the crystallographic analysis of the TGF- β -2 monomer, revealed the presence of two β -strands and an α -helix stabilized by three disulfide bonds forming the motif named “cysteine knot” (Daopin et al., 1992, Schlunegger et al., 1992). TGF- β factors are synthesised as inactive precursor factors (preproteins), comprising a signal sequence, a proregion and a mature carboxy-terminal domain (Chang et al., 2002, Lin et al., 2003). The bioactive molecules are released after a post-translational processing consisting in the removal of the signal peptide, dimerisation and specific proteolytic cleavage at the conserved RXXR dibasic site (Massagué et al., 1994). In some factors, such as GDF3, GDF9, BMP15 and Lefty1-2, the cysteine normally implicated in the covalent formation of homo or heterodimers is replaced by a serine (Chang et al., 2002). In these cases the monomers may dimerise due to hydrophobic contacts (Kingsley et al., 1994). The TGF- β signalling cascade is typically initiated with the binding of the ligand to the specific serine/threonine kinase type I and II receptors in order to induce the phosphorylation of Smad transcription factors (Kawabata et al., 1998, Massagué et al., 1998). More precisely, the ligand binds to one of the seven specific Type II receptors (Manning et al., 2002) in order to form a complex with the Type I receptor and to catalyze its phosphorylation (Mathews et al., 1993). The Type I receptor activation leads to an additional phosphorylation of the R-Smad receptor proteins (Wieser 1995, Wrana 1994) and *in fine* to promote or inhibit gene expression after SMAD nuclear localisation (reviewed by Shi et al., 2003, Massagué et al., 2005).

TGF- β proteins are expressed in virtually all tissues of vertebrate species and play crucial roles in numerous developmental processes (Chang et al., 2002, Shimasaki et al., 2004, Juengel et al., 2005). In recent years, the Bone Morphogenetic Proteins (BMP) have been shown to play a key

role in mammalian female reproduction (Shimasaki et al., 2004, Knight et al., 2006). BMP2, BMP4, chordin and NOG (a BMP antagonist) play important roles in the development and function of the mice pituitary gland (Shimasaki et al., 2004). Furthermore, *BMP6*, *BMP7*, *BMP15* and *GDF9* are expressed in the adult pituitary gland suggesting a regulatory role in the synthesis and secretion of FSH (Huang et al., 2001, Otsuka et al., 2002, Shimasaki et al., 2004). Considering the ovarian compartment, the expression study of some members of the BMP signalling cascade during the rat oestrous cycle has provided insights on their physiological role throughout folliculogenesis (Erickson et al., 2003, Shimasaki et al., 2004). In sheep, genes associated with inherited patterns of ovulation rate and prolificacy have been described, such as Inverdale (*FecX^d*), Booroola (*FecB*), Cambridge (*FecC*), Javanese (*FecJ*), Thoka (*FecI*) and Woodlands (*FecX2*) (Davis et al., 1982, 1991, 2001, 2004, 2005, Hanrahan et al., 1985, Bradford et al., 1986). Interestingly, mutations in *BMP15* (*GDF-9B*), the human *FecX* orthologous gene, have been recently associated with non-syndromic POF (Di Pasquale et al., 2004, 2006, Laissue et al., 2006, Dixit et al., 2006a, Veitia et al., 2006). Furthermore, sequence variants of *GDF9*, a *BMP15* paralog, have shown a potential implication in non-syndromic POF pathogenesis and twinning (Laissue et al. 2006, Dixit et al., 2005, Montgomery et al., 2004, Palmer et al. 2006). These two paralog genes, each composed of two exons, are specifically expressed by the oocyte in a similar pattern during folliculogenesis (Laitinen et al., 1998, Dube et al., 1998, Yan et al., 2001, Shimasaki et al., 2004). In rodents, the *BMP15* and *GDF9* spatiotemporal expression is observed since the earliest follicular development and during all stages (Dube et al., 1998, McGrath et al., 1995, Erickson et al., 2003, Shimasaki et al., 2003). Nevertheless, in monoovulatory species, such as ovine, bovine and human, *GDF9* expression is as well observed in primordial follicles (Aaltonen et al., 1999, Bodensteiner et al., 1999, 2000, Erickson et al., 2003, Shimasaki et al., 2003) pointing to the existence of differences among mammalian species. These factors are involved in the regulation of granulosa cell functions, especially mitosis and proliferation (Otsuka et al., 2000, Shimasaki et al., 2004, Moore et al., 2005). Interestingly, *BMP15* and *GDF9* heterozygous and homozygous sheep mutations lead respectively to hyperfertility and infertility, (Galloway et al., 2000, Hanrahan et al., 2004). However, different allele combinations in knock-out (KO) mice display dissimilar reproductive phenotypes. Indeed, although *Gdf9* homozygous inactivation displays an arrest of folliculogenesis at the primary stage (Dong et al., 1996), *Bmp15* *-/-* mice are subfertile showing a reduced ovulation rate and fecundity (Yan et al., 2001).

Furthermore, the ovarian effect of the *Bmp15*^{-/-} *Gdf9*^{+/-} double KO mice is more severe than the ovarian phenotype produced by the single KO (*Bmp15*^{-/-} or *Gdf9*^{+/-}). In addition, the double KO mice do not show significant differences in terms of fertility compared with *Gdf9*^{-/-} KO mice (Yan et al., 2001). In order to accurately assess the effect of sheep point mutations, *in vitro* studies have shown a deleterious effect only when the mutant BMP15 is coexpressed with GDF9 (Liao et al. 2003, 2004). Furthermore, the impaired processing of the BMP15 proteins containing the mouse GDF9 proregion is observed even when GDF9 was not coexpressed, suggesting specie-specific differences (Hashimoto et al., 2005).

Up to now, several hypotheses have been proposed to clarify the different complex biological behaviour of these proteins between mono and polyovulatory species (Yan et al., 2001, Liao et al., 2003, 2004, Hashimoto et al., 2005, Yoshino et al., 2006) but the complete mechanism has not been yet elucidated. However, it is clear that the ability of these proteins to form homo and heterodimers is a critical feature in terms of post-translational processing and bioactivity properties (Liao et al., 2003, 2004, Yoshino et al., 2006). Recently, Di Pasquale et al., reported the first human mutation of *BMP15* related to an ovarian phenotype in two sisters affected with primary amenorrhea (Di Pasquale et al., 2004). These patients carry the *BMP15* heterozygous point mutation 704A>G (p.Tyr235Cys) inherited from their father. The variant is located in the proregion. In this study, functional assays showed a decrease of the granulosa cell proliferation caused by a dominant negative effect of the mutant protein. These findings suggested the potential implication of *BMP15* (and perhaps of *GDF9*) as a major gene in the aetiology of POF. Subsequently, in order to further assess their implication in POF pathogenesis, large panels of patients were analyzed in various populations (Di Pasquale et al., 2004, 2006, Laissue et al., 2006, Dixit et al., 2005, 2006a, Chand et al., 2006, Kovanci et al., 2007, Takebayashi et al., 2000, Veitia et al., 2006). Surprisingly, despite the extensive number of patients screened for mutations in the coding sequences of *BMP15* (n=626) and *GDF9* (n=512), a small number of potentially deleterious variants have been described (Table 1 and figure 1).

Among the variants described in human *BMP15*, the c.704A>G (Tyr235Cys), c.631C>T (p.Glu211X), c.202C>T (p.Arg61Trp), c.443T>C (p.Leu148Pro) and c.181C>T (p.Arg61Trp), could be linked to the phenotype as they were absent in the control populations. Indeed, these mutations are located at conserved amino acid positions in mammalian species and are suggested to produce deleterious effects

by *in silico* analyses (Di Pasquale et al., 2004, Dixit et al., 2006a, Laissue et al., 2006). Although all human *BMP15* mutations are heterozygous, a homozygous mutation, c.631C>T (p.Glu211X, located in the propeptide) has been observed in an Indian patient. The protein lacks the C-terminal region which contains the mature region (Dixit et al., 2006a). The effect of this drastic variant may explain the severity of the phenotype, mainly characterised by primary amenorrhea. Interestingly, subsequent studies of women from the same cohort revealed two patients carrying concomitantly *BMP15* and *INHA* mutations. Thus, the patient presenting the BMP15-p.Glu211X mutation carries also the *INHA* c.275G>A (p.Ser92Asn) mutation. Similarly, a woman showed the BMP15-c.182G>A (p.Arg61Glu) and *INHA*-c.769G>A (p.Ala257Thr) mutation (Dixit et al., 2006a, 2006b, see below).

Despite the fact that the homozygous mutation encoding the p.Glu211X truncated protein may induce the ovarian dysfunction, the heterozygous sequence variants, affecting the proregion, could cause a pathological dominant phenotype. These mutations could produce an abnormal protein folding associated with an impaired dimerization and secretion of the mature region. In this sense, the TGF- β proregion has demonstrated to play a crucial role in these posttranslational processes (Kingsley et al., 1994, Chang et al., 2002). Recent results reported a new variant, the c.202C>T (BMP15-p.Arg68Trp) (Di Pasquale et al., 2006 and Veitia et al., 2006). Interestingly, the authors concluded opposite considerations about its potential deleterious effect. In fact, Di Pasquale et al., suggested that this variant could be associated with the phenotype due to the obvious biochemical differences stemming from the amino acid change (Di Pasquale et al., 2006). Conversely, Veitia et al., believe that its association with the phenotype is unlikely, since the mutation was present in the unaffected mother and one affected daughter but it was absent in the second POF daughter (Veitia et al., 2006). Thus, to settle the issue, it would be interesting to analyze the segregation of the variant in the family described by Di Pasquale et al.

Concerning *GDF9*, human mutations described up to now in POF patients affect exclusively the proregion. Two *GDF9* mutations might be related with POF phenotype: c.557C>A (p.Ser186Tyr) and c.646G>A (p.Val216Met) (Laissue et al., 2006, Dixit et al., 2005). The deleterious impact of these mutant proteins could be similar to that of BMP15 due to their similarity in sequence, structure and function. Furthermore, the study of the effect of BMP15 and *GDF9* mutations is particularly delicate as these proteins can form homo and heterodimers and a differential biological behaviour between

mammalian species has been described. Interestingly, human *GDF9* missense and nonsense mutations have been also observed with a significant increased frequency in mothers of dizygotic twins, suggesting that some variants may be linked to a polyovulatory phenotype (Montgomery et al., 2004, Palmer et al., 2006). Among these, three missense mutations, c.307C>T (Pro103Ser), c.1121C>T (Pro374Leu) and c.1360C>T (Arg454Lys) occur in conserved amino acid positions and are related to an increased ovulation rate (Palmer et al., 2006). Furthermore, this study revealed two novel insertions/deletions (c.392-393insT and c.1268-1269delAA) resulting in truncated proteins.

In all cases, the potential deleterious effect of *BMP15* and *GDF9* mutations described in POF patients deserve further functional *in vitro* studies. These assays are particularly challenging since different studies using recombinant *GDF9* lead to contradicting results in terms of cumulus cell expansion and regulation of gene expression (Pangas et al., 2005).

Inhibins, like *BMP15* and *GDF9*, belong to the TGF- β family of growth factors and represent molecular actors potentially implicated in POF pathogenesis. Inhibin A ($\alpha\beta$ -A) and B ($\alpha\beta$ -B) are two $\alpha\beta$ heterodimeric glycoproteins differing by the β subunit (Ling et al., 1985, Miyamoto et al., 1985, Rivier et al., 1985, Robertson et al., 1985). Inhibins are synthesised by the gonads in response to FSH and they reduce the effects of activins at the pituitary level. Functional studies have suggested a model of antagonism in which the interaction of inhibin with betaglycan facilitates the recruitment of ActRII or ActRIIB into a complex and sequesters them away from activins (Bilezikjian, et al., 2006, Lewis et al., 2000, Wiater et al., 2003).

The inhibin subunits are encoded by independent genes: *INHA* (2q33-qter), *INHBA* (7p15-p13) and *INHBB* (2cen-q13) (Barton et al., 1989) and are specifically expressed in the granulosa cells since early stages of follicular development (Drummond et al., 1996). It has been postulated that *INHA* mutations could be related to POF pathogenesis due to the physiological role of both, *INHA* and *INHBB*, in the ovarian negative feedback on the pituitary FSH production. A series of knock-out models (*Inha*, *Activin β A*, *Activin β B*, *ActRII* and *Follistatin*) created by Matzuk and co-workers offered crucial information on the negative effect of this loop on the FSH synthesis and secretion (Matzuk et al., 1992, 1995a, 1995b, 1995c, Vassali et al., 1994). Specifically, the inhibin alpha KO

female mice displayed increased plasma FSH levels in addition to the development of gonadal tumours and infertility (Matzuk et al., 1992).

In humans, it has been demonstrated that decreased levels of Inhibins are related to the increase of plasma FSH levels during the ovarian aging process (Petraglia et al., 1998, Danforth et al., 1998, Shahara et al., 1998, Welt et al., 1999). Furthermore, the altered INHA subunit could modify its binding affinity for the betaglycan protein, enhancing the pituitary physiological role of activins on FSH production. Up to now, in order to determine whether the *INHA* mutations are a common cause of non-syndromic POF, three independent studies have explored large panels of patients. The sequence analysis of the two *INHA* exons from a total of 447 non-syndromic POF patients (Shelling et al., 2000, Marozzi et al., 2002, Dixit et al., 2004, 2006b), revealed a significant association between the c.769G>A (p.Ala257Thr) mutation and the phenotype. In addition, three other missense variants recently reported, c.275G>A (p.Ser92Asn), c.525C>G (p.His175Gln) and c.545C>A (p.Ala182Asp), could be linked to the phenotype as they were absent in 200 control individuals and concern highly conserved positions among mammalian species. Among these, c.275G>A and c.525C>G were heterozygous whereas c.545C>A was homozygous. Interestingly, the *INHA* p.Ser92Asn mutation observed in a familial POF case is carried by the same individual presenting the *BMP15* p.Glu211X mutation. The *INHA* 5' untranslated region has been also screened for variants, showing an increased prevalence of the C allele of the single nucleotide polymorphism 16C>T (Marozzi et al., 2002, Harris et al., 2005), enhancing the hypothesis about a potential relationship between regulatory variants of this gene and ovarian dysfunction.

As described above, the BMP actions in the ovarian physiology are critical in female mammalian reproduction. For instance, *BMP4* and *BMP7* are expressed by theca cells in some mammalian species and modulate FSH signalling, promoting estradiol production and inhibiting progesterone biosynthesis (Shimasaki et al., 2004). Furthermore, *BMP-2* enhances the FSH-induced estradiol and the *INHA* production in sheep granulosa cells (Souza et al., et al., 2002). This factor also stimulates the transcription of the mRNA encoding the *INHb* subunit and the expression and secretion of its active forms. In this context, proteins possessing BMP antagonistic properties display particular interest in the elucidation of POF molecular aetiology. This is the case of *NOG*, a secreted polypeptide that binds to some TGF β superfamily members (i.e. *BMP2*, 4, 7, 14 and *GDF5*) to antagonize their biological

effect (Zimmerman et al., 1996, Merino et al., 1999, Groppe et al., 2002, Shimasaki et al., 2004). *NOG* mutations are related to proximal symphalangism (SYM1), an autosomal dominant disorder, typically characterized by the ankylosis of the proximal interphalangeal joints. Kosaki et al., described a SYM1 patient carrying a *NOG* mutation and affected with POF (Kosaki et al., 2004). These findings suggested a potential implication of this gene in POF. Very recently, Laissue et al., sequenced the entire *NOG* open reading frame in a panel of 100 POF patients (Laissue et al., 2007). Although the results only revealed the polymorphism c.275G>A (p.Gly92Glu) located in a polyglycine loop, probably unrelated with the phenotype, it is not unlikely that regulatory variants could have pathogenic effects. Thus, the *NOG* sequence analysis in large cohorts of patients from different ethnic origins would be useful to further evaluate the association of this gene with POF. Altogether, the strong implication of the TGF- β factors in female mammalian fertility justifies the interest in the study of pathological aspects of ovarian function.

The *forkhead* transcriptions factors: FOXL2, FOXO3a, and FOXO1a.

The family of *forkhead* transcriptions factors involves over 100 members present extensively in mammalian and non-mammalian species (Kaufman et al., 1996, Kaestner et al., 2000, Lehmann et al., 2003). The *forkhead* proteins, also know as “winged helix” proteins, contain a highly conserved characteristic 110 amino acid DNA-binding domain. Crystallographic analysis has revealed a helix-turn-helix core formed by 3 α -helices flanked by two loops connecting the β -strands (Clark et al., 1993). FOX proteins are involved in several developmental processes (Carlson and Mahlapuu, 2002, Lehmann et al., 2003), including an important role in mediating TGF- β superfamily signals by binding to members of the Smad family proteins (Attisano et al., 2001).

It has been demonstrated that *FOXL2* mutations are responsible for BPES (MIM-110100), an autosomal dominant disease (Crisponi et. al 2001). This pathology is characterised by mild craniofacial anomalies including eyelid dysplasia (Blepharophimosis-Ptosis-Epicanthus inversus) and POF (Zlotogora, 1983). It is clinically classified in two types: in BPES type I patients present both the eyelid and ovarian phenotypes, whereas in BPES type II, patients have a normal ovarian function and fertility. At the molecular level, *FOXL2* contains the characteristic *forkhead* domain and a polyalanine tract (PolyAla domain), formed by 14 alanines strictly conserved in mammals. The exact role of this

domain remains unknown. Gene mutations leading to truncated proteins tend to be responsible for BPES type I, whereas elongated proteins mainly lead to BPES type II. Nevertheless, exceptions to this rule make genetic counselling delicate (De Baere et al., 2003). Expansions in the PolyAla tract, from 14 to 24 residues, account for 30% of mutations in the *FOXL2* open reading frame (ORF), producing cytoplasmatic and nuclear aggregation of the mutant protein (Caburet et al., 2004). *FOXL2* is mainly expressed in the craniofacial region, the pituitary gland and the ovary. In the developing eyelids, *FOXL2* expression is found in a wide region of the primordial mesenchyma (Cocquet et al. at 2002). During pituitary organogenesis, *FOXL2* is expressed in an invagination of the oral ectoderm in the mouse primordial adenohypophysis, named Rathke's pouch (Treier et al., 1998). Thus, the effect of mutations at pituitary level might perturb the hypothalamus-hypophysis-gonadal axis and somehow play a role in ovarian failure. In the human ovary, *FOXL2* is expressed in a population of cells of the female genital ridge before sex determination. Since this period and until adulthood, it is highly expressed in granulosa cells and less in theca and stromal cells (Cocquet et al., 2002, 2003).

POF may be caused by either a decreased number of follicles forming during ovarian development or by an increased rate of follicle loss. *Foxl2*^{-/-} KO mice display an impairment of the granulosa cell transition from squamous to cuboidal state during follicular maturation (Uda et al., 2004, Schmidt et al., 2004). In these models, the infertility is produced after a premature massive follicle recruitment leading to accelerated follicular pool depletion (Uda et al., 2004, Schmidt et al., 2004). Thus, *Foxl2* appears to be essential for granulosa cell differentiation and maintenance (Schmidt et al., 2004). Four independent studies described *Foxl2* target genes (*GnRHR*, *STAR*, *CYP19/aromatase* and *Cga*) (Ellsworth et al., 2003, 2006, Pisarska et al., 2004, Pannetier et al., 2006). Specifically, Pisarska et al., have demonstrated that *FOXL2* is a transcriptional repressor of the *STAR* gene, a marker of granulosa cell differentiation and a key actor in the rate-limiting step during steroidogenesis (Pisarska et al., 2004). Furthermore, it has been shown that *FOXL2* is involved in steroid production in rainbow trout, chicken and goat (Baron et al., 2004, Hudson et al., 2005, Pannetier et al., 2006). Aromatase inhibition reduces *Foxl2* expression in both the chicken and the rainbow trout, suggesting that estrogens somehow stimulate *Foxl2* expression (Baron et al., 2004). In mammals, chromatin immunoprecipitation (ChIP) assays have shown the interaction between *FOXL2* and the promoter region of *CYP19/aromatase in vivo* (Pannetier et al., 2006). Moreover, *FOXL2* up-regulates the aromatase production in heterologous transfected cells. More recently, a genome-wide approach used

to identify potential FOXL2 ovarian targets, has shown its involvement in crucial processes such as cholesterol and steroid biosynthesis, reactive oxygen species detoxification, apoptosis and inflammation (Batista et al., 2007).

These studies underline the biological importance of *FOXL2* in ovarian function suggesting its potential implication in the pathogenesis of non-syndromic POF. Surprisingly, sequence analysis of the entire *FOXL2* ORF in a total of 290 non-syndromic POF patients from five different studies (De Baere et al. 2001, 2002, Harris et al., 2002, Bodega et al., 2004, Gersak et al., 2004), revealed only one potential deleterious mutation (c. 898–927del) producing a deletion of 10 alanines from the PolyAla tract. This heterozygous mutation was found in a POF patient with primary amenorrhea (Harris et al., 2002, Gersak et al., 2004). Nevertheless, it has been shown that extragenic *FOXL2* deletions in BPES patients affecting potentially long-range cis-regulatory elements, can perturb *FOXL2* expression (Crisponi et al., 2004, Beysen et al., 2005.). Thus, it is possible that *FOXL2* regulatory regions could be implicated in the pathogenesis of non-syndromic POF.

Similarly, to *FOXL2* other members of the *forkhead* family have been shown a key role in ovarian function. This is the case of FOXO, a small subfamily of transcription factors consisting of FOXO3a (FKHRL1), FOXO1a (FKHR), and FOXO4 (AFX). These proteins are the mammalian homologs of the *Caenorhabditis elegans* DAF-16, a transcription factor which is a nuclear target of signaling induced by the activation of the insulin-like receptor Daf-2 (Braeckman et al., 2001). In both *C. elegans* and mammalian cells, DAF-16 and FoxOs are downstream effectors of a signaling pathway including the lipid kinase phosphoinositide 3-kinase (PtdIns-3k) and the serine/threonine protein kinase B (PKB, also known as c-Akt). Both DAF-16 and FOXO can be phosphorylated at three conserved residues, resulting in their translocation from the nucleus to the cytosol (Burgering et al., 2002). The activation of PtdIns-3K/PKB thus results in inhibition of DAF-16/FoxO transcriptional activity. In *C. elegans* this insulin-like signaling pathway is involved in development, longevity and fertility. In mammals, FOXO factors are implicated in a wide variety of biological responses, including metabolism, cellular oxidative stress responses and aging (Kops et al., 2002).

Animal models have been created to investigate the FOXO3a function. Mice bearing a null mutation in the *Foxo3a* locus (*Foxo3a*^{-/-}) are viable, but females exhibit a marked age-dependent decline in reproductive fitness and were sterile by 15 weeks of age (Castrillon et al., 2003). The ovarian phenotype consists of massive activation of primordial follicles which results in the progression of increased numbers of follicles to more advanced stages of follicular development, leading to oocyte death and depletion of functional follicles at an earlier age than wild type mice and secondary infertility. Follicular activation is characterized by oocyte growth, while the surrounding granulosa cells become cuboidal and proliferative. Follicular activation is independent of pituitary gonadotropins. These animals also show significant elevation in both FSH and LH levels, exhibiting classic signs of hypergonadotropic hypogonadism secondary to POF, indicating a normal pituitary response to follicular depletion. This model shows that the transcription factor Foxo3a plays a crucial regulatory role as suppressor of follicular activation. The loss of Foxo3a also causes signs of premature aging. Furthermore, these mice exhibit other abnormalities, including a mild compensated anaemia with reticulocytosis and decreased rate of glucose uptake in glucose tolerance tests. This suggests that Foxo3a plays different physiologic roles.

To determine the role of intra-oocyte Foxo3a in the follicular development and female fertility, a transgenic mouse model has been generated, in which the expression of Foxo3a is constitutively active in oocytes in primary and more-developed follicles (Liu et al., 2007). In this study, the expression of Foxo3a in the wild type mice is localized in the nuclei of oocytes in primordial and early primary follicles, and is dramatically downregulated in oocytes of primary and more-developed follicles. Thus, Foxo3a in oocytes may be a factor that restrains primordial follicles from being activated, and that downregulation of Foxo3a expression in oocytes may be a prerequisite for oocyte growth and follicular development. In contrast, the constitutive expression of Foxo3a in the oocytes of transgenic mice led to retardation of oocyte growth and follicular development, and also to anovulation and luteinization of unruptured follicles, causing infertility. Furthermore, the constitutively active Foxo3a in oocytes caused a dramatic reduction in the expression of Bmp15, suggesting a Foxo3a role as an upstream regulator of Bmp15 in oocytes. These transgenic mice also exhibit a dramatic reduction in the expression of connexin 37 and connexin 43, which play an important role in the establishment of paracrine and gap junction

communications in follicles. The negative regulation of both connexin 37 and connexin 43 by the *Foxo3a* transgene may be one of the factors leading to retardation of follicular development in the transgenic mice.

The POF phenotype of both *Foxo3a*^{-/-} mice and *Foxo3a* transgenic mice resembles the human POF phenotype, thus suggesting that *FOXO3a* is an excellent candidate gene for the development of POF in women. The coding region of the human *FOXO3a* gene consists of two exons which encode a protein of 673 aminoacids. Mutation screening in POF patients have revealed eight variants in *FOXO3A* gene, absent in controls (Watkins et al., 2006). In particular, one variant involves a nonconservative amino-acid change from serine to leucine at position 421 (p. S421L), resulting from the heterozygous nucleotide substitution c.1262C>T. Serine residues are sites of phosphorylation in the FOXO proteins: the change to a leucine may induce a conformational change in the protein, with effects on DNA binding and nuclear localization. The second variant resulted in a conservative change at amino-acid position 506 from arginine to histidine (p. R506H ; c.1517G>A). However, further investigations, focused on the screening of larger cohorts of patients and on the development of cellular models, will give insights on the role of FOXO3A mutations and their link with POF in women.

FoxO1a is also a candidate gene potentially associated with non-syndromic POF. Among the diverse functions of FOXO proteins, these factors have been found to be molecular actors implicated in cell proliferation, including granulosa cells, (Cunningham et al., 2004) via p27kip1 modulation (Medema et al., 2000, Stahl et al., 2002). Watkins et al., have recently sequenced the *FOXO1a* gene in a group of 90 non-syndromic POF patients (Watkins et al., 2006). Although their results showed no association between the sequence variants and the phenotype as they are present in the control population, it is necessary to extend the study of this gene to larger panels of POF patients.

Conclusions:

Although during the last years there have been important advances in the diagnosis and treatment of human infertility, the physiopathological and molecular processes leading to this frequent clinical condition, are still poorly understood. Animal models displaying reproductive dysfunction, especially

mice knock-out animals, have provided insights in the genetic mechanisms potentially implicated in human disorders. In the female context, the research of molecular actors involved in POF pathogenesis presents a special challenge as the majority of cases are considered as idiopathic. The delicate regulation of the bidirectional communication between the oocytes and their surrounding granulosa cells is a crucial requirement in maintaining the follicular unit in order to ensure normal ovulation. Thus, the BMP15, GDF9, INHA and NOG proteins, as well as members of the *forkhead* family of transcription factors, have demonstrated a key role in mammalian folliculogenesis and ovulation rate, constituting potential actors of POF pathogenesis. Finally, even though mutations in the coding regions of these genes explain a small proportion of non-syndromic POF cases, it is necessary to consider the study of their regulatory regions and the dissection of the upstream and downstream molecular pathways.

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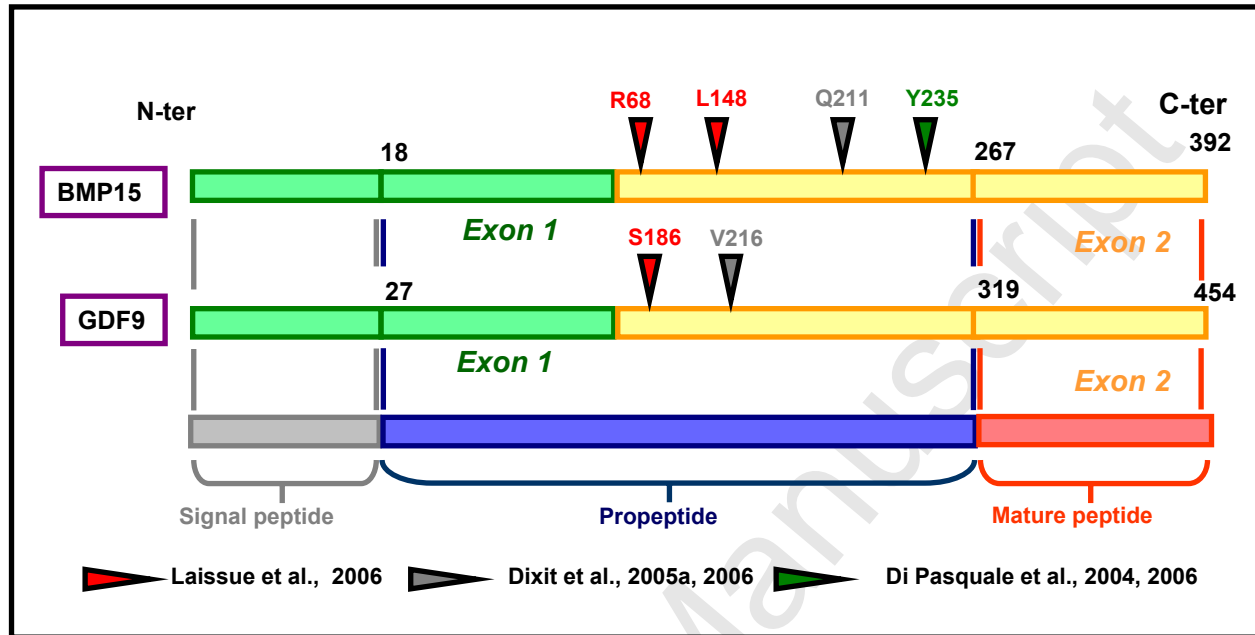
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Figures

**Figure 1:**

Schematic representation of human BMP15 and GDF9 mutations potentially associated with non-syndromic POF. Green and yellow cases represent the gene exons (1 and 2 respectively) encoding the signal peptide (gray), the propeptide (blue) and the mature peptide (red) domains. Color arrows represent the mutations at protein level described by different researchers. Only 5 mutations are included as they were not found in the control population and are located in conserved positions among mammalian species.

Table 1: Human *BMP15*, *GDF9* and *INH α* mutations potentially associated with POF phenotype since they were not observed in the control population and are located at conserved positions among mammalian species.

Gene	Nucleotide change	Allelic status	Amino acid change	Phenotype	Reference
<i>BMP15</i>	c.704A>G	Heterozygous	p.Tyr235Cys	PA	Di Pasquale et al., 2004, 2006
	c.443T>C	Heterozygous	p.Leu148Pro	SA	Di Pasquale et al., 2004, Laissue et al., 2006
	c.202C>T	Heterozygous	p.Arg68Trp	SA	Di Pasquale et al., 2004, Laissue et al., 2006
	c.181C>T	Heterozygous	p.Arg61Trp	SA	Dixit et al., 2006a
	c.631C>T	Homozygous	p.Glu211X	PA	Dixit et al., 2006a
<i>GDF9</i>	c.557C>A	Heterozygous	p.Ser186Tyr	SA	Laissue et al., 2005
	c.646G>A	Heterozygous?	p.Val216Met	SA	Dixit et al., 2005
<i>INHα</i>	c.769G>A	Heterozygous	p.Ala257Thr	PA,SA	Shelling et al., 2000, Marozzi et al., 2002, Dixit et al., 2005, 2006b
	c.275G>A	Heterozygous	p.Ser92Asn	SA	Dixit et.al 2006b
	c.525C>G	Heterozygous	p.His175Gln	SA	Dixit et.al 2006b
	c.545C>A	Homozygous	p.Ala182Asp	PA	Dixit et.al 2006b

List of abbreviations

POF	Premature Ovarian Failure
FSH	Follicle-Stimulating Hormone
LH	Luteinizing Hormone
FMR	Fragile X Mental Retardation
BMP15 (GDF9-B)	Bone Morphogenetic Protein 15
FSHR	Follicle-Stimulating Hormone Receptor
LHR	Luteinizing Hormone Receptor
INH	Inhibin
GALT	Galactose-1-phosphate uridylyltransferase
FOX	Forkhead Box
ODG	Ovarian Dysgenesis
BPES	Blepharophimosis, Ptosis, Epicanthus inversus Syndrome
TGF-B	Transforming Growth Factor-Beta
GDF	Growth Differentiation Factor
Lefty	Left-Right Determination Factor
ActRII	Activin Receptor, Type II