HIGHLIGHT

Lessons from studies of complex genetic disorders: identification of FOXL2 – a novel transcription factor on the wing to fertility

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Disturbance of the hypothalamo–pituitary–ovarian axis is present in approximately 10% of women in infertile couples. Thereby, two pathophysiological scenarios can be distinguished. First, hypogonadotropic hypogonadism due to processes directly or indirectly affecting the hypothalamus and/or the pituitary gland and secondly, hypergonadotropic hypogonadism, which results from unresponsiveness of the ovaries to gonadotropins. Primary ovarian malfunction can result from a wide spectrum of medical conditions including infectious, immunological, neoplastic, metabolic and vascular disorders, as well as radiation and chemotherapy. However, little is known so far about the genetic components responsible for ovarian failure (1). Inactivating mutations in the G-protein coupled follicle-stimulating hormone (FSH) receptor have been identified as rare causes of primary hypogonadism (2) with ovaries in the affected women reportedly harboring normal follicles. In contrast, developmental ovarian failure is characterized by absence or reduction in the number of germ cells, resulting in either primary amenorrhea or premature ovarian failure (POF), i.e. onset of menopause before the age of 40. While familial segregation of POF has long been recognized, dissection of molecular events involved is difficult because many ‘mechanistic’ pathophysiological aspects are still not fully understood and not all the genetic players have yet been identified (1).

As a general rule, one way to shed light on a complicated medical issue is to study conditions in which the trait of interest is inherited following a Mendelian pattern. Thus, a widely used technique to proceed from a specific phenotype to genotype analysis comprises systematic, chromosome-wide linkage analysis using inherited, highly polymorphic microsatellite markers to define candidate regions. These are usually several megabases in size and can be further narrowed by similar marker techniques (3). A complementary elegant procedure involves identification and mapping of specific chromosomal translocations in patients with a defined phenotype using fluorescent *in situ* hybridisation (FISH) analysis with the hypothesis that the gene disrupted by the translocation is associated with, if not responsible for, the observed phenotype. These strategies have been successfully applied in positional

cloning of many ‘disease genes’, e.g. in Pendred’s syndrome, multiple endocrine neoplasia type I and polyglandular endocrine syndrome type I.

One well-known genetic disorder associated with ovarian failure is the XO genotype of Turner’s syndrome. In fact, X-chromosomal deletions and translocations have been reported in several cases of ovarian failure without Turner’s syndrome, yet the identification of the involved X-chromosomal genes is still awaited (4). Another heritable disorder, which can be associated with ovarian failure, is blepharophimosis–ptosis–epicanthus inversus syndrome (BPES). The persistent study of this rare and complex condition by several teams of geneticists has recently led to the identification of a novel member of the forkhead winged helix protein family, FOXL2, as the likely underlying genetic cause and a putative new crucial player in ovarian development, and thus fertility (5).

BPES is an autosomal dominantly inherited disorder (MIM 110100), whereby the affected patients exhibit characteristic facial abnormalities, i.e. small palpebral fissures, ptosis and a skinfold running inward and upward from the lower lid. Two forms of BPES exist. In type I, infertility in the form of ovarian failure is an adjunct to the condition and is sex-limited, i.e. only females are affected. In type II, only facial abnormalities are present (6). BPES type II was first mapped to chromosome 3q22–q23 in 1995 followed by the observation that BPES I maps to the same locus, which suggested involvement of a common gene in both syndromes (7, 8). On the basis of three translocational breakpoints ((t3;4), (t3;7) and (t3;21)) identified within the mapped region, several groups attempted to identify the culprit by positional cloning (5, 9–11). However, putative candidate genes involving two retinol-binding proteins and a beta-coatamer protein were either not found to be mutated or were not expressed in the relevant tissues of affected BPES individuals. In another study, breakpoint mapping failed to yield relevant candidate genes (11).

Crisponi *et al.* (5) started their gene hunting from a balanced translocation (t3;7) on chromosome 3 identified in a male BPES patient and mapped the breakpoint localization within 2 kb of genomic DNA by DNA hybridization with yeast artificial chromosomes (YACs)

to identify a 1 Mb YAC spanning the breakpoint in the first place, followed by hybridization with bacterial and P1 artificial chromosomes to narrow the translocation breakpoint. Prediction of two novel genes localized within the breakpoint region was made through several complementary methodological approaches involving computer assisted-analysis, DNA library hybridization and 5-RACE (rapid amplification of cDNA ends) PCR. One gene, termed C3orf5, was reported to be encoded by 20 exons and was found to be ubiquitously expressed as a 1.4 kb transcript with no obvious similarities to other previously identified genes. In contrast, the second gene was found to be a single exon gene with a 2.7 kb transcript encoding a protein of approximately 376 amino acids. The latter gene shared common features with members of the winged helix/forkhead protein family, and has been named FOXL2. Crisponi *et al.* (5) then succeeded in sequencing both novel genes in a number of BPES type I ($n = 4$) and BPES type II ($n = 2$) families, as well as a male patient with sporadic BPES, and were fortunate to identify genetic modifications in all affected BPES patients but not in the unaffected family members and a healthy control group ($n = 100$). Thereby all mutations were exclusively localized in the FOXL2 gene. Consistent with the clinical phenotype and the molecular genetic findings, mRNA expression studies of the FOXL2 mouse homologue Pfrk/FOXL2 gene demonstrated that the transcript is highly expressed during embryogenesis in the developing eyelids and is also expressed in mice ovary follicular cells. These findings suggest that a defective FOXL2 is the culprit in BPES types I and II. Further Northern Blot analysis of FOXL2 expression in human tissue revealed that the transcript is present in follicular cells surrounding the oocyte which underscores a potential role for FOXL2 in development and maintenance of the follicles.

The novel FOXL2 forkhead protein is the latest sibling in a group of, to date, six different forkhead domain proteins for which genetic defects have been identified in several complex human disorders, i.e. the thyroid transcription factor TTF-2 impaired in the thyroid agenesis, cleft palate and spiky hair syndrome (FOXE1; 9q22 (12)) and the genes implicated in the X-linked polyendocrinopathy, enteropathy and immune dysregulation syndrome (FOXP3; Xp11.23 (13, 14)), the hereditary lymphedema–distichiasis syndrome (FOXC2; 16q24.3 (15)), the anterior segment ocular dysgenesis and cataract syndrome (FOXE3; 1p32 (16)), as well as syndromic congenital glaucoma (FOXC1; 6p25 (17)). These proteins share a characteristic winged helix/fork head motif, i.e. a 100 amino acid DNA binding domain, which was first identified in *Drosophila melanogaster* and is constituted by two wings, three alpha helices and three beta sheets (for detailed topology see ref. 18). Interaction of the winged helix motif with the specific DNA region usually occurs via helix 3 and the respective DNA major groove, while wing 2 interacts with the DNA minor groove. Most of these interactions

are mediated by polar amino acid residues through bridging water molecules and lead to conformational changes of the DNA resulting in specific gene transcription (18). The forkhead protein family currently comprises more than 100 genes identified in different organisms including 20 different human forkhead proteins (18, 19). They play a critical role during organogenesis and have also been implicated in the maintenance of cell differentiation and in tumorigenesis (11, 18, 19).

The exciting findings by Crisponi *et al.* (5) have very recently been confirmed and extended by De Baere and co-workers (10), who have screened 33 probands from families with BPES type I ($n = 3$) and type II ($n = 9$), 14 patients with sporadic occurrence of BPES, seven families with unknown BPES type (due to lack of affected females or pre-pubertal females), as well as 30 women with isolated POE. Besides the impressive number of heterozygous novel mutations ($n = 17$) identified, the study of De Baere *et al.* (10) provides important further insights into a pleiotropic function of the novel forkhead protein. In fact, similarly to Crisponi *et al.* (5), De Baere and co-workers (10) observed that the presence of either BPES type I or II crucially depends on the nature of the FOXL2 alteration (Fig. 1). Thus, a truncated FOXL2 protein is present in BPES type I. FOXL2 truncation can involve complete or incomplete loss of the forkhead domain by mutations upstream ($n = 1$) or within the forkhead domain ($n = 4$), or a protein truncation due to mutations further downstream ($n = 7$, Fig. 1).

In addition, extension of FOXL2 is present in BPES type II. FOXL2 extension can result from in-frame duplication within the forkhead domain, e.g. by addition of five hydrophobic amino acids ($n = 1$), but more frequently appears to occur within the polyalanine region downstream of the forkhead domain ($n = 4$). Within this region with putative transcriptional repressive activity, addition of up to ten alanine residues has been described in BPES type II individuals. Moreover, three different insertion or deletion mutations further downstream of the polyalanine region have been identified to result in an extended FOXL2 protein. A similar pleiotropic effect has also been observed with mutations in other forkhead proteins, most notably FOXC1, which is implicated in several eye developmental defects (17).

Although De Baere *et al.* (10) reported FOXL2 mutations in 100% of their BPES type I families, 'only' 67% of BPES type II families, 71% of BPES families of unknown type and 57% of sporadic BPES patients were found to harbor FOXL2 mutations. Thus, involvement of other 'BPES genes' has been proposed with previous linkage analyses suggesting a second BPES candidate locus at 7p21–p13 (20). Alternatively, a position effect due to chromosomal rearrangement outside the coding region of the disease gene has been suggested. Position effects, believed to result in disruption of distant regulatory element and/or the minimal

Figure 1 Diagram of the novel FOXL2 gene and mutations identified in patients with blepharophimosis-ptosis-epicanthus inversus syndrome (BPES) type I and II or patients with BPES of yet unknown type (italics).

promoter, have been described in a number of rare human genetic disorders (21). Interestingly, absence of a FOXL2 mutation was also observed in the BPES patient with the balanced translocation (t3;7) from which Crisponi *et al.* (5) succeeded in cloning the BPES culprit.

Finally, no FOXL2 mutations were detected in a group of 30 women with non-syndromic POF. In view of the potentially high promise of FOXL2 for elucidating ovarian failure, this is the most disappointing finding of the De Baere study. However, as discussed above, a position effect and thus FOXL2 involvement in non-syndromic ovarian failure cannot (yet) be excluded.

Many other open questions remain to be answered with respect to FOXL2 transcriptional regulation, protein conformation, interaction partners and signaling pathways and ultimately protein function. Although highly speculative, an anti-apoptotic role of FOXL2 has been suggested because 'physiologic' atresia in ovarian follicles is notably enhanced in patients with POF (1). Moreover, a transforming growth factor- β (TGF- β) signaling cascade has been proposed, since members of the TGF- β family including the highly homologous forkhead transcription factor FAST-1 are implicated in ovarian development (22). In addition, the polled/intersex syndrome goat, which lacks horns

and displays recessive XX sex reversal, has been proposed as a useful animal model for investigating FOXL2 function, after a candidate gene region homologous to the human FOXL2 locus has been identified in the goat model through linkage analysis (23).

Future detailed studies of functional FOXL2 impairment through the different mutations may contribute not only to clarify the highly variable morphological presentation of POF in BPES patients ranging from seemingly normal ovaries to streak gonads, but may also explain the interesting observation that female patients with likely BPES type II can exhibit some mild degree of fertility problems (10). This finding suggests a phenotypic overlap in the two forms of BPES. Furthermore, it is currently unclear how a heterozygous mutation in a putative transcriptional repressor can be sufficient for the BPES phenotype. Both a dominant negative mutational effect, or haplotype insufficiency, in which the truncated FOXL2 in BPES type I would represent a null allele and the extended FOXL2 protein in BPES type II a hypomorphic allele, have been hypothesized (5, 10).

With many open questions remaining to be answered after the identification of the sixth forkhead protein implicated in human disease, it will be exciting to discover more about the molecular and functional

features of the pleiotropic FOXL2 and whether it is 'foxed' only in syndromic ovarian failure (5) or may indeed provide a 'wing' for novel insights into fertility and infertility.

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