European Society of Human Reproduction and Embryology

COURSE 9

Genetics of female reproduction anomalies

Special Interest Group Reproductive Genetics

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Course 9 - Pre-congress course organised by the Special Interest Group Reproductive Genetics

"Genetics of female reproduction anomalies"

PROGRAM

Course co-ordinators: P.H. Vogt (D) and J. P.M. Geraedts (NL)

Course description: The aim of this course is to provide the attendants with recent information in an important field of reproductive genetics. The study of genetic causes of reproductive abnormalities is not only necessary to understand some aspects of infertility. After the course the participants are also supposed to be able to understand some basic mechanisms that are important for the normal development and functioning of the female reproductive organs.Molecular developments have led to important new findings in this area of reproductive genetics. All lecturers in this course have recently published original work in this area and are leaders or members of groups that are leading in this field.

09.00 - 09.30	X-chromosomal disorders impairing folliculogenesis – **J.L. Simpson (USA)**
09.30 - 09.45	Discussion
09.45 - 10.15	Molecular genetics of POF1 locus in Xq26-28 (FMR-1, FMR-2) – **A. Smits (NL)**
10.15 - 10.30	Discussion
10.30 - 11.00	*Coffee break*
11.00 - 11.30	Molecular genetics of POF2 locus in Xq13.3-22 (DACH2; DIAPH2) - **S. Bione (I)**
11.30 - 11.45	Discussion
11.45 - 12.15	Autosomal gene mutations impairing female reproduction (FIGa, FSHR, MATER, NANOS, NOBOX, OBOX) - **A. Rajkovic (USA)**
12.15 - 12.30	Discussion
12.30 - 13.00	FOXL2 expression in folliculogenesis - **L. Crisponi (I)**
13.00 - 13.15	Discussion
13.15 - 14.00	*Lunch*
14.00 - 14.30	Mullerian aplasia -genetic aspects - **K. Aittomaki (FIN)**
14.30 - 14.45	Discussion
14.45 - 15.15	Oocyte derived growth factors and ovarian function (TGFß superfamily: INHa ; BMP15, GDF9) - **S. Galloway (NZ)**
15.15 - 15.30	Discussion
15.30 - 16.00	*Coffee break*
16.00 - 16.30	McKusick-Kaufmann syndrome - **A. Slavotinek (USA)**
16.30 - 16.45	Discussion
16.45 - 17.15	Polycystic Ovarian Syndrome - **J.L. Simpson (USA)**
17.15 - 17.30	Discussion and conclusions
17.30	Business meeting of the SIG Reproductive Genetics

X-Chromosome and Disorders of Folliculogenesis or Ovarian Failures

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Learning Objectives:

1. Describe the scientific basis for assuming that ovarian failure in 45,X women is caused by increase oocyte attention.
2. State which regions on the X chromosome that are relatively more pivotal for ovarian differentiation.
3. List several candidate genes on the X that may be causative for ovarian failure.

Failure of germ cell development or folliculogenesis leads to ovarian failure. Complete and premature ovarian failure (POF) may be different manifestations of the same underlying pathogenic and etiologic processes. Chromosomal abnormalities, mutations of autosomal or X-linked genes, and polygenic/multifactorial factors all play a role. In this contribution, we shall enumerate disorders of ovarian failure caused by perturbations of the X chromosome. For citations alluded to, see reviews cited in the Suggested Reading section.

I. Ovarian differentiation requires only one x (constitutive)

In the absence of the Y chromosome, the indifferent embryonic gonad always develops into an ovary. Germ cells exist in 45,X human fetuses (Jirasek, 1976). Oocytes initially exist even in 46,XY phenotypes females, such as infants with XY gonadal dysgenesis (Cussen and MacMahon, 1979) or the genito-palato-cardiac syndrome (Greenberg *et al.*, 1987). Oocyte development in the presence of a Y chromosome is well documented in other mammalian species. Thus, the pathogenesis of germ cell failure in humans can be deduced to be increased germ cell attrition. If two intact X chromosomes are lacking, ovarian follicles in 45,X individuals usually degenerate by birth. Genes on the second X chromosome are thus necessary for ovarian maintenance, rather than ovarian differentiation per se.

Presumably specific gene product(s) is responsible for primary ovarian differentiation, but identifying them has proved elusive. An erstwhile candidate was AHC (Adrenal Hypoplasia Congenital), the gene that encodes *DAX-1*. Duplication of Xp21 resultes in 46,XY embryos differentiating into females (Bardoni *et al.*, 1994); thus, it was reasoned that this region could play a primary role in ovarian differentiation in 46,XX individuals.
The region contained AHC, a locus that includes or is identical to DAX 1 (Dosage Sensitive Sex Reversal/Adrenal hypoplasia critical region X); the mouse homolog is Ahch. Ahch is upregulated in the XX mouse ovary, as predicted if Ahch (DAX1) were to

play a pivotal role in primary ovarian differentiation. Transgenic XY mice that overexpress Ahch develop as females. However, XX mice lacking Ahch (knockout) show normal ovarian differentiation, ovulation and fertility (Yu *et al.*, 1998), and XY mice mutant for Ahch show testicular germ cell defects. Thus, Ahch cannot be responsible for primary ovarian differentiation in mice, nor presumably could DAX 1 (AHC) in humans.

II. Localizing ovarian maintenance genes to specific regions of the X

The X clearly is pivotal for normal ovarian developmental and folliculogenesis. Determining the specific region is a first step in understanding normal ovarian differentiation and in producing gene products of therapeutic benefit. Until the 1990s, phenotypic-karyotypic correlations to deduce location of gonadal and somatic determinants relied solely on metaphase analysis. Deductions made in this fashion are limited. Prometaphase kayotypes allow 1,200 band analysis (traditional GTG-banding 400 to 500), but each band still contains considerable DNA. More refined analysis can be achieved using polymorphic DNA markers that allow precise resolution far beyond the capacity of light microscopy. This can be followed by sequencing and other molecular approaches to identify pivotal genes.

Progress has been surprisingly slow, especially compared to that achieved in delineating the regions of the Y necessary for testicular differentiation (SRY) or spermatogenesis (DAZ). Several impediments help explain this relative lack of progress. First, the incidence of X-deletions and translocations is low. Thus, the ideal approach of analyzing cases ascertained by population-based methods is impractical. Selection bias also exists. Most del(Xp) or del(Xq) individuals have been identified only because they manifested clinical abnormalities, exceptions being rare familial cases or cases detected in fetuses at the time of prenatal genetic diagnosis Less severely affected individuals may escape detection.

Utilizing X-autosome translocations for analysis is popular but potentially hazardous because of vicissitudes of X-inactivation. Autosomal regions themselves are not devoid of significance for gonadal differentiation.

These pitfalls notwithstanding, Figure 1 shows clinical characteristics associated with terminal deletions. Regions of relatively greater significance can be deduced.

<u>Deletions of X Short Arm</u>

Deletions of the short arm of the X chromosome show variable phenotype, depending upon amount of Xp persisting. The most common breakpoint for terminal deletions is Xp11.2611.4. Approximately half of 46,X,del(Xp)(p11) individuals show primary amenorrhea and gonadal dysgenesis. The other cases menstruated and usually showed breast development. In a 1989 tabulation by the author, 12 of 27 reported del(X)(p11.2611.4) individuals, menstruated spontaneously; however, menstruation was rarely normal (Simpson, 1987). More recent compilations have not altered these general conclusions (Simpson, 1997b;Simpson, 1998;Simpson, 1997a). Deletions characterized by progressively more distal breakpoints have also been reported: Xp21, 22.1, 22.3.

Menstruation occurs more frequently.

X long arm deletions originating at Xq13 are almost always associated with primary amenorrhea, lack of breast development, and complete ovarian failure (Simpson, 1997a). Xq13 thus seems to be an important region for ovarian maintenance. This region has been said to contain the POF2 locus, even while poorly defined. The key loci could lie in proximal Xq21, but not more distal given that del(X)(q21) to (q24) individuals menstruate far more often than del(X)(q13). Del(X)(q21) women who menstruate could have retained a region that contained an ovarian maintenance gene, whereas del(X)(q13 or 21) women with primary amenorrhea might have lost such a locus.

In more distal Xq deletions, the more common phenotype is premature ovarian failure (Krauss *et al.*, 1987;Fitch *et al.*, 1982;Simpson, 1997b;Simpson, 1998). While distal Xq seems less important for ovarian maintenance than proximal Xq, the former must still have regions important for ovarian maintenance.

Although demarcation into discrete regions is not possible, it is
heuristically useful to stratify terminal deletions into those occurring in these regions: Xq 136 21, Xq22-25, Xq 26-28?

Familial Xq terminal or interstitial deletions have been characterized by various break points between Xq25 to Xq28. Break points near or in Xq27 seem most common. The locus for fragile X (FRAXA) also lies in this region, and this will be discussed separately (Section IV).

III. Candidate genes on the X

Although chromosomal regions on Xp (and Xq) are presumed to contain genes pivotal to ovarian germ cell function and folliculogenesis, identifying the actual gene and gene products remain frustratingly unclear. A host of candidate genes are being studied.

X Short Arm

USP9X (ubiquitin specific protease 9): This gene maps to Xp11.4 (Jones *et al.*, 1996). The Drosophila orthologue of *USP9X* is required for eye development and oogenesis. The role USP9X plays in human gonadal development is still unclear. An attractive feature is its location in a region known to have ovarian determinants.

BMP15: Bone Morphogenetic Protein 15: Bone morphogenetic protein 15 (BMP) is a member of the transforming growth factor-beta (TGFβ) superfamily. These genes direct many developmental pathways though binding and activating transmembrane serine/threonine kinase receptors. BMP is involved in folliculogenesis and embryonic development, being expressed in gonads. The BMP 15 gene is located on Xp11.2 and has two exons. Animal studies initially suggested that perturbations of BMP 15 could be important in ovarian development. Heterozygous Inverdale sheep carrying a mutation in the BMP 15 gene show an increased ovulation rate, with twin and triplet births. Primary ovarian failure occurs in homozygotes (Galloway *et al.*, 2000). Bmp15 knockout mutant female mice are subfertile, showing decreased ovulation rates, reduced litter size and

decreased number of litters per lifetime (Dube *et al.*, 1998).

In the humans, (Di Pasquale *et al.*, 2004) reported a heterozygous Y235C missense mutation in the second exon of the BMP15 gene in each of two sisters having ovarian failure. The proband had streak gonads and elevated FSH (80miU/L); the younger sib had only one episode of vaginal spotting and at age 18 years had an FSH of 67 mlU/L. The mother was homozygously normal (Y235) at this allele; the C235 allele was inherited from the father. The authors presented in vitro evidence for a dominant negative mechanism.

Consistent with heterozygous dominant negative effect is that an autosomal TGF family member (GDF-9) has also been implemented in ovarian failure and in these cases the mutation was also heterozygous (Dixit *et al.*, 2004). Given that proteins of TGF family members (BMP-15, GDF-9) may form heterodimers, a single mutation could plausibly generate a dysfunctional gene product.

Region Localized by Quantitative Linkage Analysis: Genome wide linkage analysis has been applied to localize regions influencing age of menopause. One such study has shown association between region Xp11 and age of menopause.

ZFX (Zinc finger X): Mapped to Xp22.1-21.3 Zfx, is a candidate gene for short stature and ovarian failure (Zinn and Ross, 2001). It has attracted attention on the basis of homology to Zfy, once the prime candidate gene for male sex determination. Mice null for *Zfx* are small, less viable, less fertile and characterized by diminished germ cell number in ovaries and testes (Luoh *et al.*, 1997). Their external and internal genitalia are otherwise normal.

X Long Arm

XIST: Xq13 contains the X-inactivation center and XIST. Loss of germ cells may or may not be the result of perturbation of XIST, despite years of speculation that disturbances of X-inactivation per se lead to ovarian failure. The concept of a variably-defined "critical region" inviolate for ovarian development receives less attention than in the past. However, Xq13 is clearly a region rich in pivotal genes.
A Drosphila homologue (dac) exists and is expressed in multiple tissues.

DACH2: Localized on Xq21, this gene has been implicated in POF on the basis of a breakpoint in an X-autosome translocation (Prueitt *et al.*, 2000). DACH2 consists of 13 exons, and does not undergo X-inactivation. Bione *et al.* (2004) studied 257 Italian patients, of whom 19 had primary amenorrhea and 238 secondary amenorrhea either before age 40 (N=212 having traditionally defined POF) or between aged 40 and 45 years (N=26, having "early menopause"). A total of 5 missense mutations (2.7%) were found in evolutionarily conserved amnio acids. These same alleles were also found in controls, but less frequently (0.7%).

DIAPH2: Localized to Xq22, human DIAPH2 is the homologue of Drosophila melanogaster diaphanous *(dia)*. Drosophila *dia* is a member of a gene family whose gene

products help establish cell polarity, govern cytokinesis, and reorganize the actin cytoskeleton. In both males and females perturbations of *dia* cause sterility in flies (Castrillon and Wasserman, 1994). In a human Xq21/autosome translocation, disruption of the last intron of *DIAPH2* was found. (Bione *et al.*, 1998;Bione and Toniolo, 2000;Prueitt *et al.*, 2000)

FMR1: On Xq27 lies the FMR1 locus, perturbation of which causes the fragile X syndrome. About 20% of females with a FMR1 premutation (≥ 60 CGG repeats) develop premature ovarian failure, although paradoxically those with the full mutation do not. Fragile X syndrome is discussed below. This fragile X locus (FMR1) cannot precisely correspond to that which when deleted causes ovarian failure in del(Xq) (2.7 or 2.8).

IV. Fragile X syndrome *(FMR1)*

A relationship exists between fragile X syndrome, caused by perturbation FMR1, and ovarian failure. Fragile X syndrome is a common form of X-linked mental retardation that is caused by expansion of the FMRI gene, located on Xq27. If more than 200 CGG repeats exist, transcriptional silencing of a RNA-binding protein occurs. In normal males, the normal number of CGG repeats is less than 55. Males or heterozygous females having 55 to 199 repeats are said to have a premutation (Sullivan *et al.*, 2005). During female (but not male) meiosis, the number of triplet repeats may increase (expand). A phenotypically normal woman with a FRAXA premutation may thus have an affected son if the number of CGG repeats on the oocyte of the X she transmits to her male offspring expands during meiosis to greater than 200. Affected males show mental retardation, characteristic facial features, and large testes. Expansion does not occur if there are fewer than 55 CGG repeats. Females may also show mental retardation, but phenotype is less severe than in males.

Approximately 20 to 25% of females with the FRAXA premutation show premature ovarian failure (POF), defined as menopause prior to age 40 years. Schwartz *et al.* (1994) found oligomenorrhea in 38% of premutation carriers versus 6% of controls. Analyzing 1,268 controls, 50 familial POF cases and 244 sporadic POF cases, Allingham-Hawkins *et al.* (1999), reported that 63 of 395 premutation carriers (16%) underwent menopause prior to 40 years of age, versus 0.4% in controls. Sullivan *et al.* (2005) found 12.9% of premutation carriers have POF versus 1.3% of controls. Surprisingly, FSH was increased in premutation carriers aged 30 to 39 years, but not in carriers of other ages. The number of repeats correlates with risk of POF, but only within a specified range. The risk is only slightly increased risk existed at 40 to 79 repeats, but much higher at 80 to 99 repeats. Inexplicabily there is no further increased risk ≥ 100 repeats. The reason for the plateau is not clear. Nonetheless, this observation is consistent with females with the full mutation not showing POF.

It is reasonable to screen for FMRI as part of the evaluation for premature ovarian failure (Foresta *et al.*, 2002). If oocyte or ovarian slice cryopreservation becomes practical, population screening would especially be justified for fertility preservation.

Suggested Reading:

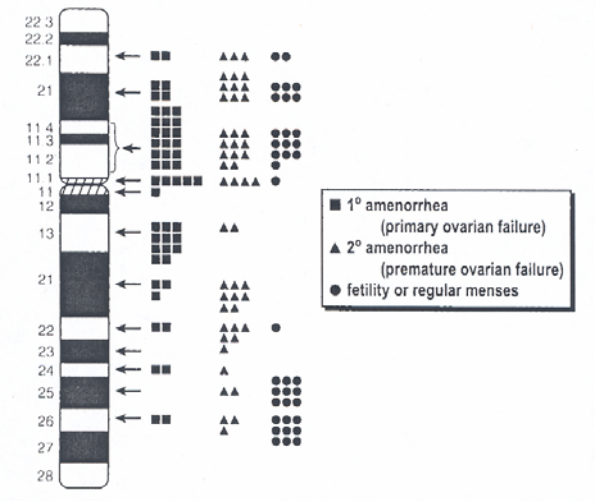
Simpson, J.L., and Elias, S. (2003) Genetics in Obstetrics and Gynecology (3rd Edition). Philadelphia, W.B. Saunders, Chap. 10, pp 243-290.

Simpson, J.L. (2006) Genetic programming in ovarian development and oogenesis. *In*: Treatment of the Postmenopausal Woman: Basic and Clinical Aspects, 3rd Ed. R. Lobo, ed). In Press.

Di Pasquale, E., Beck-Peccoz, P., and Persani, L. (2004) Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. Am J Hum Genet., 75, 106-111.

Figure 1. Schematic diagram of the X chromosome showing ovarian function as a function of nonmosaic terminal deletion. References initially provided by (Simpson, 1981). Non-mosaic cases described since that report include (Naguib *et al.*, 1988); (Massa *et al.*, 1992); (Veneman *et al.*, 1991); (Schwartz *et al.*, 1987), which is a molecular update of (Fitch *et al.*, 1982); (Tharapel *et al.*, 1993); (Zinn *et al.*, 1997); (Zinn *et al.*, 1998); (Ogata and Matsuo, 1995); (Marozzi *et al.*, 1999); (Davison *et al.*, 1998); (James *et al.*, 1998); (Susca *et al.*, 1999). In familial aggregates all affected cases are included because their phenotypes are not always concordant.

In some case, patients are described as having premature ovarian failure, but no information is provided on fertility; in the absence of explicit information it is assumed no pregnancy has occurred. In some younger patients (e.g., $\geq$ 14 years but < 20-25 years), there has been little opportunity to demonstrate pregnancy, nor is there assurance regular menses will continue. Nonetheless, they are designated as having "regular menses/fertility" (Simpson and Rajkovic, 1999).



Molecular genetics of POF2 locus in Xq13.3-22 (DACH2; DIAPH2)

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Learning objectives

- Premature Ovarian Failure (POF: OMIM 311360; 300511)
- genetics of POF
- role of the X chromosome in the etiology of POF
- mapping breakpoints of POF-associated balanced translocations
- candidate POF genes in the POF2 locus
- mutation analysis of POF2 candidate genes
- different mechanisms of POF2 locus involvement in POF

Lecture summary

Premature Ovarian Failure (POF, OMIM 311360-300511) is an heterogeneous disorder defined as cessation of menses before the age of 40 years, in the presence of elevated levels of serum gonadotropins. The age of onset is variable with the median age estimated around 30 years. A later onset form, defined Early Menopause (EM), with amenorrhea occurring between 41 and 45 years of age is often included as it is frequently found in POF families.

Little is known about the pathophysiology of the disorder, that may be due to (i) deficiency in the number of primordial follicles, (ii) accelerated rate of follicular atresia or (iii) dysfunctions in follicular recruitment or maturation. To date no clinical or molecular evidence can distinguish between the different mechanisms.

POF incidence is estimated around 1% of the female population, It affects 1 in 1000 women by age 30 years and 1 in 100 women by age 40 years and it accounts for about 10% of all female sterility.

A POF diagnosis has major implications for endocrine, emotional and reproductive women health. As a consequence of sex hormone deficiency, patients may present with vasomotor symptoms, sleep disturbance, increased risk of osteoporosis, cardiovascular defects and autoimmune thyroid disease.

POF may result as a consequence of external factors such as surgery, infections and chemotherapies, but the common finding of POF familial cases together with the relatively high frequency of associated chromosomal abnormalities suggests a major genetic component. The limited extent of the POF pedigrees hampered the definition of a precise pattern of inheritance and the identification of contributing loci. In few cases, genes responsible for POF inherited as a mendelian disorder have been identified (i.e. FSHR; FOXL2; BMP-15; etc.). Epidemiologic relevance of POF genes

identified is very low, as they account for a very small proportion of cases (Goswami and Conway, 2005)

A relevant role in the etiology of POF was demonstrated for the premutated allele at the FRAXA locus in Xq27.3. The expansion of a CGG triplet exceeding the 200 copies in the 5' UTR of the FMR1 gene is the cause of mental retardation in males. Alleles with a number of triplets between 50 and 200 are defined as premutation and have been recently associated to different disorders: tremor/ataxia in males (FXTAS) and POF in females. An increased occurrence of POF females among premutation carriers (about 15-20% compared with 1% in female population) together with the higher frequency of the premutation allele in POF females (about 5-10% compared with less than 1% in general population), clearly demonstrated the contribution of this genetic variant as a risk-factor (O.R.=20) for POF (Murray, 2000).

X chromosome rearrangements are also a frequent finding in POF. A severe form of gonadal dysgenesis leading to primary amenorrhea and streak ovaries is caused by X monosomy, in Turner syndrome (TS). Partial monosomies and X;autosome balanced translocations have been also observed in association with POF and their description led to the cytogenetic definition of a "critical region" for normal ovarian function on the long arm of the X chromosome, in the Xq13.3-q26 interval. Deeper characterization of the huge critical region identify two distinct loci: the POF1 locus (Xq26-28; OMIM311360) and the POF2 locus (Xq13.3-22; OMIM 300511) differently involved by chromosomal rearrengements. Several hypotheses have been formulated to explain the POF critical region in Xq: (i) the presence of different loci required in double dosage for oocyte development or ovarian function, (ii) position effect of the rearrengements on regulation of flanking genes; (iii) lack of perfect chromosomes pairing leading to diminished number of ovarian follicle by increase of apoptosis. Lack of definitive evidences prevent to choose among these hypothesis.

Fine mapping of balanced X;autosome translocation breakpoints was the tool used by us and by other groups to identify genes involved in POF. In all istances balanced X;autosome translocations induce preferential inactivation of the intact X chromosome, thus limiting their effect to the interruption of specific sequences at the level of the breakpoints. We collected and mapped to genomic intervals smaller than 100 kb, 25 cases of balanced X;autosome translocations: 23 were associated with early onset POF, two were found in normal women. Taking into account data from the literature on 12 additional cases, the distribution of the X chromosome breakpoints showed that most of them (25/37) mapped to a 15 Mb region in Xq21, whereas the remaining 12 were distributed along a larger (25-30 Mb) portion of Xq, between Xq22 and Xq26. The two breakpoints in normal women were close to the POF associated ones and demonstrated that translocations per se were not responsible for POF. The distribution of breakpoints in balanced translocations was compared with that of Xq deletions: the analysis showed that loss of one copy of Xq21 did not greatly affect fertility, whereas rare interstitial deletions distal to Xq21 often interfered with normal ovarian function. These data demonstrated that haploinsufficiency for genes located in the POF2 locus is not responsible for POF, while it is a risk factor for POF when occurring in more distal Xq (Rizzolio et al., 2006).

Transcriptional characterization of breakpoint regions led to the identification of four genes interrupted by the translocations. The DIAPH2 gene in proximal Xq22, was interrupted by the X breakpoint in patient BC, carrying a X;12 balanced translocation inherited from the mother who was also affected. DIAPH2 is one of the human homologues of the Drosophila melanogaster dia gene affecting fruit fly fertility. The

XPNPEP2 gene in Xq25, the DACH2 gene in Xq21.3 and the POF1B gene in Xq21.3 were found interrupted by different POF translocation breakpoints. Three of the genes identified were in the POF2 locus: their role and relevance in the etiology of POF was investigated by extensive mutation analysis in a large cohort of italian POF patients. A collaborative effort involving several gynaecological clinics in Italy led to set up a DNA collection of more than 350 samples of italian POF and EM affected women. Detailed records of clinical and family history was collected in the majority of the cases. A large panel of geographical matched controls was also established by selecting women who experienced menopause at physiological age (>=48). Mutation screening was performed by the mean of DHPLC and sequencing analysis for each exon of the POF1B, DACH2 and DIAPH2 genes. Rare variants affecting the amino acid composition of the encoded proteins were found in all the three genes and in the majority of cases also in the control population leading us to exclude a relevant role in the etiology of the disorder. Rare variants in the DACH2 gene showed an increased frequency in the patients population suggesting that they may represent risk-factor for POF. In conclusion, mutation analysis of the three candidate genes identified in the POF2 locus failed to demonstrate a significant contribution of such genes in the pathogenesis of POF in spite of the wideness of the patient collection was sufficient to account for the genetic heterogeneity of the disorder (Bione et al., 2004).
As genetic evidences on the premutation allele at the FRAXA locus support a multifactorial model of inheritance of the POF condition, we start to investigate if susceptibility variants in the candidate POF2 genes may be associated to the disease. Test for association in a case-control study was undertaken considering common single nucleotide polymorphisms (SNPs) tagging the linkage-disequilibrium (LD) blocks containing the genes. Preliminary results on the DIAPH2 gene suggest positive association of some of the SNPs considered and suggest that a nucleotide variant, outside the coding region of the gene, may represent a risk-factor for POF.
The majority of POF-associated translocation breakpoints in the POF2 locus did not interrupt transcribed sequences. Morover, expression analysis of genes surrounding the breakpoints in Xq21 did not reveal ovary-specific genes. Taken together these results suggest that molecular mechanisms different from X chromosome gene-alteration are responsible for POF, at least in the POF2 critical region. In this regard we investigated the effect of the breakpoints on chromatin organization of X chromosome and autosomal regions involved by the rearrangements. Specific alterations of chromatin modifications were found at the promoters of autosomal genes translocated to the X. Some of these genes also showed expression in granulosa cells and/or in oocytes when analysed by in-situ hybridization in mouse ovaries. Our results suggest that ovary-specific autosomal genes translocated on the derivative X-chromosome may be altered in their correct expression and that this could represent the mechanism leading to POF.
Emerging evidences on different mechanisms acting at the POF2 locus and eventually related to the POF condition will be extensively discussed during the course.

References

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Semen Reprod Med. 18:59-66.
A detailed review on the characteristics of the involvement of the premutation allele in the etiology of POF.

Rizzolio F, Bione S, Sala C, Goegan M, Gentile M, Gregato G, Rossi E, Pramparo T, Zuffardi O, Toniolo D. (2006) Chromosomal rearrangements in Xq and premature ovarian failure: mapping of 25 new cases and review of the literature. Hum Reprod. (in the press)

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Hum Reprod. 19:2759-66.

Autosomal gene mutations impairing female reproduction, implications from animal models.

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Learning Objectives

1. Understand that **ovarian failure is genetically highly heterogenous** with multiple autosomal loci involved.
2. Understand and be able to draw the process of **folliculogenesis** and genes essential for follicular formation
3. Understand and list **oocyte-specific genetic** pathways that play critical role in fertility. Understand the importance of germ cell specific transcriptional regulation in early folliculogenesis
4. Learn about **maternal effect genes** that can cause failure of early embryonic development.

Selected genes that affect fertility by disrupting ovarian development and folliculogenesis

Non-syndromic ovarian failure affects 1–2% of women. Causes of premature ovarian failure include autoimmune oophoritis, chemotherapy and radiation treatment but for most of the cases, the etiology is idiopathic and probably genetic. Premature ovarian failure can result from several different genetic mechanisms including X chromosomal abnormalities, autosomal recessive genes causing various types of XX gonadal dysgenesis and autosomal dominant genes with action restricted to premature ovarian failure. Autosomal recessive inheritance appears to be the major mode of transmission. Follicle stimulating hormone receptor (*FSHR*) is the only well characterized autosomal recessive gene that causes non-syndromic premature ovarian failure (Aittomaki, *et al.*, 1995). A mutation in the oocyte-specific growth factor, *BMP15*, was recently discovered that causes ovarian failure (Di Pasquale, *et al.*, 2004). The above mutations, however, account for a minority of cases with ovarian failure.

A growing list of human diseases is due to mutations affecting transcription factors (Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/omim/). The vast majority of these mutations affects multiple organs and cause syndromes. FOXL2 is an example of a transcription factor involved in syndromic ovarian failure. Mutations in the *FOXL2* gene cause eyelid defects and ovarian failure (Crisponi, *et al.*, 2001). Possibly there may be additional mutations in the *FOXL2* gene that cause only ovarian failure without eyelid defects. For example, a 30 base pair deletion that removes 10 of 14 alanines downstream of the winged helix/forkhead domain of the *FOXL2* gene may be the cause of ovarian failure in a 17-year-old woman with small uterus and small ovaries without visible follicles (Harris, *et al.*, 2002) though she does not appear to have eyelid defects. Functional studies *in vitro* or in mouse models would be necessary to prove whether this deletion is the cause of her ovarian failure and a larger pedigree analysis is necessary to ascertain whether the penetrance of this mutation is variable.

There are currently no other transcription factors implicated in ovarian failure in 46,XX phenotypic females. The search for these genes in humans is hampered by a lack of patients with fortuitous autosomal translocations and small pedigrees to map the loci. In addition, ethical issues surrounding human germ cell research make it difficult to study genes important in ovarian differentiation and development. Ovarian pathologic conditions in transgenic mice closely resemble conditions observed in mutated human homologues, as exemplified in cases involving the FSHβ subunit and FSHR (Kumar, *et al.*, 1998). Therefore, genes that cause ovarian failure in mice are candidate genes for ovarian failure in humans. *Taf4b*, *Figla* and *Nobox*, which were identified and first studied in mice (Choi and Rajkovic, 2006) (Dean, 2002; Rajkovic, *et al.*, 2004), represent excellent candidate genes for non-syndromic primary ovarian failure because of their oocyte-specific expression pattern and restricted ovarian phenotype (Figure 1). In humans, hypomorphic mutations that partially affect *Nobox* and *Figla* function, may cause ovarian failure to occur later in life but before 40 years of age, (i.e. premature ovarian failure). It is also conceivable that ovaries in Turner syndrome lose oocytes because of perturbation in the expression of oocyte-specific transcription factors, and future experiments should examine expression of transcription factors such as *Figla* and *Nobox* in Turner syndrome ovaries. Our recent discovery that Sohlh1 and Lhx8 transcription factors are essential in oogenesis (Figure 2), means that a complex network of regulators exists and more regulators are likely to be uncovered. Another interesting group of genes expressed in the germline and encoding transcription factors include *Nanog*, *Pou5f1* and *Nr6a1*. *Nanog*, *Pou5f1* and *Nr6a1* play important functions early in embryogenesis based on the respective mouse knockouts. Human null mutations in *Nanog*, *Pou5f1* or *Nr6a1* are likely to cause embryonic lethality, as observed in mouse knockouts. However, hypomorphic mutations in *Nanog*, *Pou5f1* and *Nr6a1* could possibly lead to selective loss of the germline and streak gonads.

Transcription factors expressed in the somatic cells of the gonads usually cause syndromes because of their expression and functional importance in multiple tissues. Mutations in the *SF1* gene cause adrenal insufficiency and sex reversal, and it is unclear whether *SF1* mutations in 46,XX females disrupt ovarian function.

Mutations in the *WT1* gene cause multiple congenital anomalies including streak gonads in 46,XX females. *SOX3* resides on the X chromosome and in-frame duplication of 33 base pairs encoding for 11 alanines in a polyalanine tract of the *SOX3* gene was associated with mental retardation and isolated growth hormone deficiency. Duplication of *SOX3* may also cause X-linked hypopituitarism. It is not known whether phenotypic females with an XX karyotype and mutated *SOX3* are infertile. *FOXO3A* is another candidate gene expressed in the somatic cells that may be responsible for premature ovarian failure in women with anaemia. It is also conceivable that specific mutations in transcription factors that are expressed in somatic cells causes only ovarian failure, without any other syndromic features. Transcription factors that are known to disrupt ovarian development in mice should be sequenced in patients with primary and premature ovarian failure.

The size of the germ cell pool, as well as the rate by which the germ cells are lost, determines the time of the menopause. We are far from being able to manipulate menopausal age. Identifying and studying the function and mechanisms of transcription factors that orchestrate germ cell formation and proliferation will be essential if we ever want to manipulate the germ cell

reservoir. Oocyte-specific transcription factors and other oocyte-specific genes (Matzuk and Lamb, 2002) theoretically represent ideal pharmacological targets to regulate fertility, reproductive life-span and infertility in a tissue-specific manner without affecting other organ systems.

Genes that affect fertility by disrupting early embryonic development

Maternal effect genes accumulate RNA or proteins in the egg during oogenesis and play important roles during early embryogenesis. *Mater*, is one such gene, initially discovered as an oocyte antigen in a mouse model for premature ovarian failure(Tong, *et al.*, 2000). *Mater* deficiency does not affect the number of primordial follicles or the overall ovarian architecture. In addition, *Mater*-null females ovulated the same number of eggs as wild-type controls, and resulting zygotes progressed thru the first cleavage normally. However, development of these *Mater*-deficient embryos is arrested at the two-cell stage`. *Mater* contains a leucine-rich domain and a short leucine zipper, which may be important for interaction with other proteins. Further studies are necessary to elucidate proteins which interact with *Mater*. In addition, it would be of interest to know if ooplasmic transfer from wild-type eggs may improve the quality of *Mater* embryos. It is conceivable that human mutations in *Mater* may account for some cases of *in vitro* fertilization failures. Other mammalian maternal effect genes include, *Zar1*, *Dnmt1o*, *Dppa3*, *Npm2*, and *Tcl1*, and perhaps 50 or more may exist. Mechanisms of maternal effect genes actions are currently under investigation. The ovarian phenotype is normal in the absence of maternal effect genes, but early embryogenesis is disrupted. Women, whose fertilized eggs consistently abort early embryonic development, may carry mutations in the maternal effect genes.

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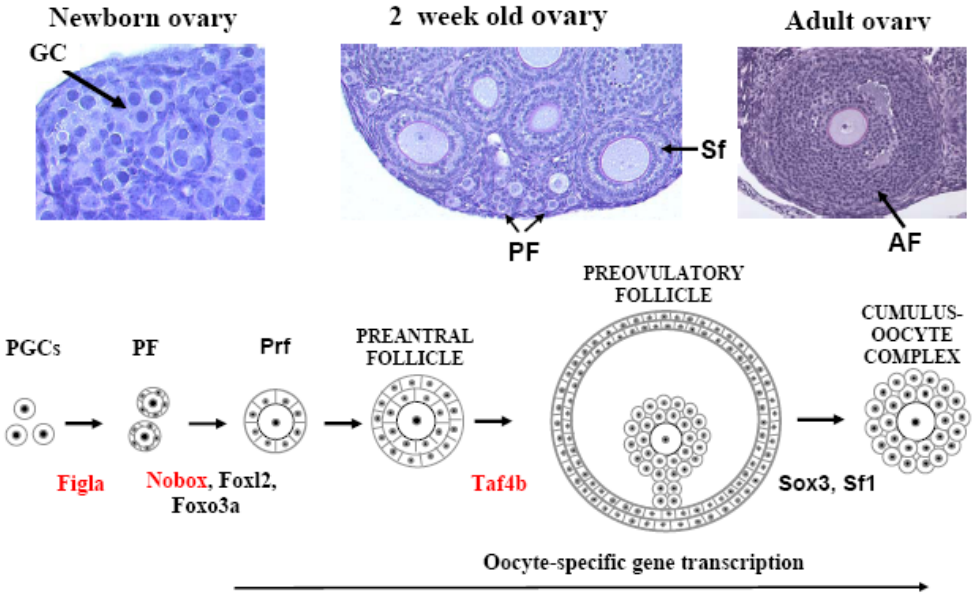
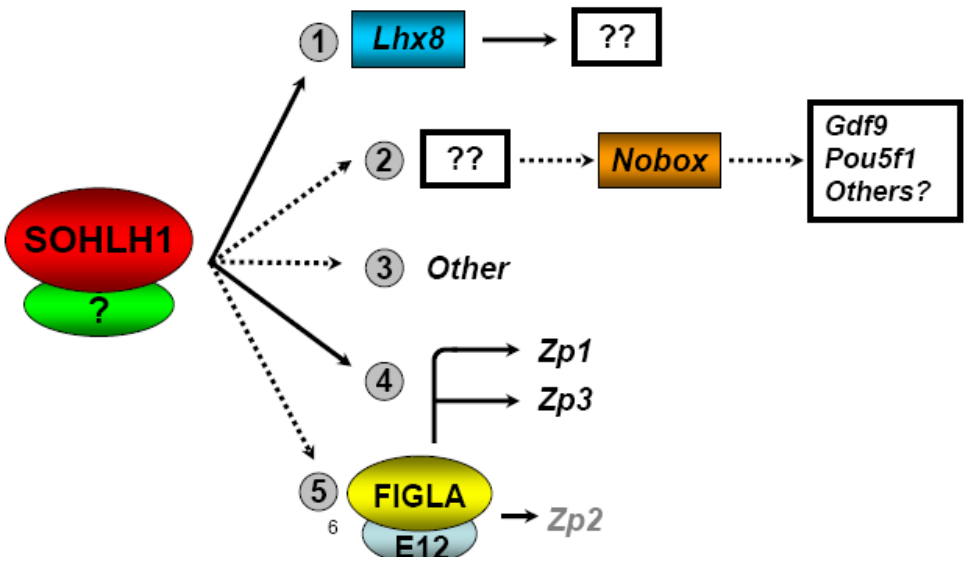


Figure 1. Transcription factors involved in folliculogenesis. Various stages of murine follicular development are shown beginning with primordial germ cells (PGCs), primordial follicle (PF) and primary follicle (Prf). Histology of the murine ovary during postnatal development is also shown and germ cell cysts (GC), PF, secondary follicles (Sf) and early antral follicle (AF) identified by arrows. Anti-Nobox antibodies stain nuclei in germ cells with brown colour in the newborn ovaries. Transcription factors preferentially expressed inthe oocytes and other oocyte-specific genes are shown in red and their importance at particular stages of folliculogenesis inferred from knockout mice.

Fig. 2. Hypothetical model for SOHLH1 role in early folliculogenesis. SOHLH1 regulates a number of genes required for early oocyte development. The SOHLH1 interacting partner is unknown. (**1**) The homeobox gene *Lhx8* is likely a direct transcriptional target of SOHLH1, but genes downstream of LHX8 in the oocyte are unknown. (**2**) *Zp1* and *Zp3* are likely directly regulated by SOHLH1, possibly in conjunction with FIGLA. (**3**) *Figla* is partially downregulated in *Sohlh1* null oocytes but *Zp2*, which is controlled by the FIGLA/E12 complex, is not significantly changed (**4**) The oocyte-specific homeobox gene, *Nobox*, is downstream of SOHLH1. Loss of *Nobox* expression in *Sohlh1* null oocytes also results in loss of genes downstream of the NOBOX pathway, such as *Gdf9* and *Pou5f1*. (**5**) Likely, SOHLH1 has additional target genes. Dashed lines indicate unknown pathways. Solid lines indicate direct transcriptional regulation.



FOXL2 expression in folliculogenesis

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Learning Objectives
At the end of the lecture, participants are expected to gain knowledge in:

- Blepharophimosis/Ptosis/Epicanthus inversus Syndrome (BPES).
- Overview of current knowledge in ovarian folliculogenesis.
- Role of Foxl2 in ovarian histogenesis and reproductive competence of the ovary.
- Overview of current knowledge in female sex determination.
- Possible role of Foxl2 in female sex determination.
- Relevance of Foxl2 studies to human reproduction.

Lecture summary.
Female fertility results from complex molecular events that begin in utero with ovarian differentiation and depend on the supply and maturation of the ovarian germ cells, the oocytes, and the differentiation and proliferation of the ovarian somatic cells, in particular granulosa and thecal cells. Assembly of oocytes and somatic cells into ovarian follicles, a process also called initial folliculogenesis or follicle histogenesis, marks the last step of ovarian differentiation and occurs during fetal life in humans and within the first week of postnatal life in rodents. The perinatal pool of ovarian follicles determines the reproductive lifespan in mammals and it is progressively depleted until menopause, that for most women occurs around 50 years of age. Inadequate formation or maintenance of the pool of ovarian follicles (ovarian dysgenesis) results in premature ovarian failure (POF) with incomplete menarche or early menopause (before age 40), a condition affecting about 1-3% of women. It is currently debated whether the adult mammalian ovary harbours germ stem cells capable of limited self renewal, but several lines of evidence indicate that perinatal formation of the ovarian follicle pool and the ensuing follicle dynamics are the most critical determinants of female fertility. However, the molecular mechanisms necessary for folliculogenesis are largely unknown. Although a number of gene defects lead to ovarian dysgenesis and defects of early follicle differentiation in mice, mutations in only a few of them have been found in human patients. They include genes on the X chromosome, in particular the oocyte factor BMP15. An entry point to the study of follicle formation and its relation to follicle dynamics in humans was provided by our finding that FOXL2 is mutated in patients with Blepharophimosis/Ptosis/Epicanthus inversus Syndrome (BPES), an autosomal dominant disorder associated with POF. We subsequently showed that FOXL2 is selectively expressed in ovarian follicle cells and created mice lacking Foxl2 that recapitulate relevant features of human BPES: males and females are small and show distinctive craniofacial morphology with upper eyelids absent. Furthermore, in mice as in humans sterility is confined to females. Features of Foxl2 null animals point toward a new mechanism of POF with all major somatic cell lineages failing to develop around initially normal oocytes from the time of primordial follicle formation.
In addition to its role in follicle differentiation, a possible role for *FOXL2* in repressing male sex determination was hinted by findings in goats in which the orthologous locus is associated with both lack of horns (thought to be homologous to the eyelid anomalies in BPES) and XX maleness (polled intersex syndrome, PIS). We demonstrated that mouse XX gonads lacking *Foxl2* form meiotic prophase oocytes but then activate the genetic program for somatic testis determination. This suggest that Foxl2 may function as a conserved repressor of the male gene pathway, raising the possibility that the sex determination pathway continues to be involved throughout ovarian

development and female reproductive life. Further characterization of the gene, along with the identification of direct downstream target genes and interacting proteins, will be useful to understand the genetic pathways controlled by Foxl2. These studies should help to dissect the earliest steps of folliculogenesis and thus have a fundamental relevance for human reproduction.

Blepharophimosis/Ptosis/Epicanthus inversus Syndrome (BPES) (MIM #110100).
BPES type I is an autosomal dominant disorder in which POF is co-inherited with abnormalities of the eyelids. The infertility is sexually dimorphic. Males remain fertile and transmit the trait to the next generation. Both female and male affected individuals show typical facies with characteristic eyelid dysplasia, namely small palpebral fissures (blepharophimosis), drooping eyelids (ptosis) and a tiny skin fold running inward and upward from the lower lid (epicanthus inversus). Similar craniofacial abnormalities have been observed in other families where both affected males and females are fertile. This disorder is also inherited as an autosomal dominant trait and has been named BPES type II. In 2001 we used a positional cloning approach to identify a novel winged helix/forkhead transcription factor gene, *FOXL2*, that is mutated in both types of BPES. Genotype/phenotype correlations suggest that haploinsufficency caused by loss-of-function mutations in one *FOXL2* allele is associated with the full phenotype (type I), whereas milder mutations or abnormally longer proteins are associated with a hypomorphic phenotype (type II). To elucidate anomalies associated with *Foxl2* deficiency, we created a mouse knockout model by ablating the complete coding region of *Foxl2*. Mice lacking *Foxl2* recapitulate relevant features of human BPES: males and females are small and show distinctive craniofacial morphology with upper eyelids absent. Furthermore in mice as in humans, sterility is confined to females. We provided evidences that complete individual follicles never form and that concomitant with the earliest defects detected in follicle formation, *Foxl2* expression in granulosa cells is required to repress the testis determination gene pathway in the postnatal ovary.

Overview of current knowledge in ovarian folliculogenesis.
Folliculogenesis
Folliculogenesis refers, strictly speaking, to the formation of ovarian follicles, but the meaning of this term is commonly extended to include follicle maturation. The ovarian follicle is a densely-packed shell of somatic cells that contains an immature oocyte and is one of the most intricately regulated developmental processes in biology. Early ovarian folliculogenesis begins with the breakdown of germ cell clusters and formation of primordial follicles. These smallest ovarian follicle units are continuously recruited to grow into primary and more advanced ovarian follicles that enter the menstrual cycle. Contrary to male spermatogenesis which can last indefinitely, oogenesis ends when the limited pool of follicles in the ovaries runs out signalling the beginning of the menopause.
PGCs
Primordial germ cells (PGCs) are the earliest recognised precursors of gametes. A founder population of approximately 45 primordial germ cells (PGCs) at embryonic 7 dpc (E7.5), gives rise in mouse, to the germ cell lineage. PGCs migration starts at E9.5 until E11.5 and progresses from the hindgut epithelium to developing genital ridge. Around E12.5, mitotic division of primordial germ cells results in clusters of oogonia which breakdown shortly after birth forming primordial follicles. Circa E13.5 female germ cells begin entry into the meiotic prophase I and remain arrested in the diplotene stage until the time of ovulation. In the developing ovary, the proliferation of germ cells or oogonia (GC) and epithelial cells (pregranulosa, PC) lead to the rapid enlargement of the genital ridges, which progressively organize into epithelial structures, called ovigerous cords (OC), continuous with the surface epithelium of the ovary. They are delimited by a thin basement membrane (BM), which is itself bordered by mesenchymal cells (MC). The differentiating ovary is, thus, organized into two distinct compartments: OCs and the interstitial tissue. During OC breakdown at birth, PCs progressively rearrange around GCs, the pre-existing BM is destroyed, and

a new one is synthesized, leading eventually to the formation of primordial follicles, containing a single GC in meiotic prophase I surrounded by a layer of flattened granulosa cells, delimited by a continuous BM. In mice, primary follicles grow rapidly in the days following their formation and constitute the initial waves of growing follicles that ensure the first ovulations of reproductive life.

Phases of development

Regularly and until exhaustion, starting shortly after birth in mice, a subset of primordial follicles is selected from a resting pool of quiescent follicles to undergo the follicular activation, characterized by the oocyte growth and the transition of squamous to cuboidal granulosa cells. The rate of entry into the phase of follicle growth is largely independent from growing follicles, but rather correlates with the size of the pool of resting oocytes. Thus, follicle formation is a critical developmental step with long-lasting consequences for follicle dynamics. Primordial follicles occupy the periphery of the ovary underneath the epithelial surface with more advanced follicles located centrally. The number of germ cell clusters declines rapidly after birth with few clusters remaining beyond postnatal day 7. By postnatal day 3, some primordial follicles becomes primary follicles and the oocytes grow beyond 20µm. The transcription of numerous oocyte-specific genes is initiated during these early stages of the primordial to primary follicle transition. Primary follicles grow into larger secondary follicles and ultimately become antral follicles where oocytes reach a diameter of approximately 70µm and are surrounded by highly differentiated granulosa cells and thecal cells. In preovulatory follicles, the oocyte resumes meiosis and becomes arrested again in metaphase II. Physiologically, ovarian follicles can be divided into two broad groups, each being characterized by the presence or absence of a morphological antrum (cavity). Follicles prior to the formation of an antrum are independent of the pituitary gonadotropins for growth and survival. In contrast, around the period of antrum formation, oocytes regain meiotic competence and oocytes in early antral follicles depend on pituitary follicle stimulating hormone (FSH) for survival and growth. The oocyte is then ovulated in the oviduct, and can thus be fertilized. Subsequently, theca and granulosa cells differentiate into luteal cells and form the corpora lutea, which produce progesterone. Throughout the ovarian reproductive lifespan, a dramatic number of follicles is eliminated by the process of follicular atresia. During this process, oocytes and granulosa cells degenerate while thecal cells persist, becoming hypertrophied and subsequently forming interstitial glands that display steroidogenic capacities. Although recent studies have claimed that female germ cell renewal occurs after birth in mice and possibly in humans, the challenge remains to understand the progressive decline in follicle number that inevitably leads to the arrest of the reproductive function.

Normal ovarian development and function require specific transcriptional regulation.

Transcriptional regulation is a fundamental process enabling a cell to differentiate from others containing the same genetic information, through the activation and repression of specific genes, and it involves proteins known as transcription factors. An organ is comprised of a number of differentiated cell lines that provide its functional characteristics. Transcription factors play key roles in the temporal and spatial activation or silencing of genes during ovary formation and folliculogenesis and control both oocyte development and somatic cell function. Ovarian function has evolved as a specific interplay of these differentiated cells to prepare the oocytes for ovulation and fertilization.

Role of Foxl2 in ovarian histogenesis and reproductive competence of the ovary.

Foxl2

Foxl2 is a member of the forkhead gene family of transcription factor. Forkhead genes encode a subgroup of the helix-turn-helix class of proteins. The arrangements of loops (or wings) connecting the beta strands that flank one of the three alpha helices gives rise to a butterfly like appearance hence their alternative name of “winged-helix” transcription factors.

The nomenclature of the chordate forkhead transcription factors have recently been revised and termed Fox (Forkhead box): they are divided into 17 subclasses (A to Q) according to the aminoacid sequence of their conserved forkhead DNA binding domain. Fox genes have vital roles

in the formation of a diverse range of organs in development and are involved in a wide range of processes. We found that mutations in the *FOXL2* gene cause the BPES syndrome type I and II.

Creation of Foxl2 mouse model

To elucidate anomalies associated with *Foxl2* deficiency, we created a mouse with the complete coding region ablated. We showed that:

1. *Foxl2*$^{-/-}$ mice exhibit craniofacial dysmorphic features and female sterility, comparable to BPES in humans thereby providing an entry point to study major features of this human condition.
2. this mouse model can be relevant for human POF in general because it controls a developmental process (final histogenesis, i.e. follicle formation) that is pivotal to ovarian reproductive competence.
3. this model provides evidences that Foxl2, expressed in pre-granulosa cells, regulates the fate of all major ovarian cell lineages including oocytes and stroma.
4. and that Foxl2 expression in granulosa cells is required to repress the testis determination gene pathway in the postnatal ovary, and possibly at earlier stages.

Role of Foxl2 in ovarian histogenesis and reproductive competence of the ovary **.**

Macroscopically, anomalies in *Foxl2*$^{-/-}$ female genitalia and gonads were evident by 2 weeks. Ovary size was reduced and tubes were hypotrophic with incomplete glandular cytodifferentiation consistent with hypoestrogenism. Earlier defects were manifest in histologic sections of 1 week ovaries. Ovaries of *Foxl2* null mice contain oocytes surrounded by flat granulosa cells but no follicular structures are present: in particular a basal lamina showed highly variable thickness and delineated anastomotic cords that contained multiple oocytes. There is no transition from flat to cuboidal granulosa cells. Furthermore, in the absence of *Foxl2,* several cell lineages were not induced, including steroidogenic theca cells and interstitial glands. These data indicate the persistence of primitive stroma which is seemingly "frozen" in a newborn state in the *Foxl2*$^{-/-}$ ovaries. In spite of these anomalies in the somatic cells, oocyte differentiation was only partly affected with an initially reduced rate of growth and, in some cases, massive oocyte growth occurring relatively later. Expression of major oocyte regulators, *Figla, Gdf9* and *c-Kit* are not affected by the lack of *Foxl2* as well as oocyte count is unaffected at birth. Further work should determine conclusively whether oocytes in *Foxl2* null mice are fully competent. A specific polyclonal antibody detected Foxl2 in somatic cells of the fetal ovary and at decreasing levels in granulosa cells of adult follicles from the primordial to the preantral stage.

Taken together our data indicate that ovarian failure associated with BPES results primarily from the failure of granulosa cells in follicle formation leading to deregulated oogenesis by an unknown mechanism.

Overview of current knowledge in female sex determination.

Ovarian soma development including the identification of putative gene(s) responsible for female sex determination has remained poorly understood. During embryonic and fetal development, sexually dimorphic features of the mammalian ovary include mitotic amplification of germ cells, followed by their massive entry into meiosis. In many mammals, development of somatic cells is morphologically less conspicuous until the time of follicle formation. Although many somatic genes show sexually dimorphic expression patterns in embryo-fetal gonads, only a few of them are considered candidates for a role in female sex determination. Ablation of *Wnt4,* encoding the secreted factor, in mice leads to ectopic steroidogenesis in fetal gonads, followed by oocyte loss and neonatal ovary-to-testis sex reversal. Thus, *Wnt4* is required for relatively early steps of ovarian differentiation. Another candidate, *Dax1*, located in a chromosomal region duplicated in patients with 46,XY sex reversal (termed DSS, for dosage sensitive sex reversal), has demonstrated an anti-testis effect in some mouse genetic backgrounds but its deletion does not obviously affect ovary differentiation. Early development of the somatic component of the ovary culminates with follicle formation, which occurs concomitant with the progression of oocytes through the last stages of meiotic prophase.

Possible role of Foxl2 in female sex determination.

In addition to its role in follicle dynamics, a possible role for FOXL2 in repressing male sex determination was previously hinted by findings in goats in which down-regulation of *Foxl2* because of a deletion in the upstream flanking regions is associated with both lack of horns (thought to be homologous to the eyelid anomalies in BPES) and XX maleness (PIS). We demonstrated that a male differentiation program is similarly initiated in *Foxl2*$^{-/-}$ female mice, indicating that Foxl2 may function as a conserved repressor of the genetic program for somatic testis determination in mammals. However, in XX mice lacking *Foxl2*, gonadal sex reversal occurs perinatally, thus genitalia remain female. Future studies will determine the causes of the divergent sex reversal phenotypes in goat and mouse. The results raise the possibility that the sex determination pathway continues to be involved throughout ovarian development and at subsequent stages of ovarian differentiation and function. The fetal like somatic expression profile associated with persistent cords in *Foxl2*$^{-/-}$ mouse ovaries could represent a pure developmental arrest but we have investigated whether they also reflect partial gonadal sex reversal. A critical test examined the expression of *Sox9* which is necessary for testis determination in humans and mice and it is itself sufficient to induce ovary to testis sex reversal in transgenic mice. We found that *Sox9* mRNA expression was sharply upregulated in *Foxl2*$^{-/-}$ ovaries between birth and 1 week postnatum. Concomitant with *Sox9*, *Foxl2*$^{-/-}$ supporting cells also upregulated other genes that are required for male sex determination or are considered strong candidates for such a role (*Fgf9, Dhh, Dmrt1, Wt1* and *Gata4*). *Foxl2* null ovaries contain cords delimited by the basal lamina that show some male Sertoli-like specific features detectable by electron microscopy. Interestingly, we observed that *Sox9* activation correlated with selective *Foxl2* down-regulation in a complementary pattern. Sertoli like cells are a defining feature of a fraction of human ovarian tumors and are reported in some mouse models of ovarian dysfunction. However we found no evidence for tumoral formations in *Foxl2*$^{-/-}$ mutants at all ages tested (ovary size was consistently small – over 90% reduced at 3-4 months) and staining for the proliferation marker Ki67 was inconspicuous in all ovaries. Thus neoplastic transformation if any cannot be considered a direct result of *Foxl2* deficiency in mice, consistent with absent report in BPES patients. Occasionally we found epithelial cells with a Sertoli like morphology expressing Sox9 in wild type ovaries. Interestingly we observed that this Sox9 activation correlated with Foxl2 down-regulation, indicating that mammalian female sex determination is labile and may require *Foxl2* activity in the gonadal soma throughout ovary development and maturation. Other cases of postnatal sex reversal have been reported. However in those instances oocyte loss was observed: oocytes are thus considered necessary though not sufficient for maintaing the female sex postnatally. In contrast, in the phenotype observed on *Foxl2* null mice several lines of evidence argue against a role of oocyte loss:

1. somatic male differentiation was initiated in the presence of a full complement of meiotic prophase oocytes.
2. oocytes maintained a normal pattern of *m*RNA and protein expression for all differentiation markers tested and progressed through meiotic prophase with no timing or morphological anomalies.
3. many postnatal oocytes, although with delay compared with wild type, expressed genes required for oocyte growth, attained large size and formed a thick zona pellucida.
4. the rate of oocyte loss was not increased when compared with wild type during the first week after birth.

Although oocyte degeneration is unlikely to be a critical feature of *Foxl2*$^{-/-}$ sex reversal, it is possible that support cells lacking *Foxl2* are unresponsive to specific female promoting signals originated from oocytes. In fact, considerable evidence indicates that oocytes are required to maintain female somatic differentiation. For example, ovaries of aged rats and some mouse models of POF, show a progressively larger fraction of follicles that trans-differentiate into testis like tubules and usually contain either atretic or no oocytes. However, we and other have found that

Foxl2 expression is initiated in the absence of oocytes. Thus, although an interaction between *Foxl2* and oocyte genes cannot be excluded, oocytes do not control the induction of *Foxl2* expression. It is possible that oocytes repress male differentiation independently of *Foxl2*. This would rationalize the incomplete ovary to testis sex reversal observed in *Foxl2*$^{-/-}$ mice and the enhanced testis tubule like morphology in germ less epithelial cords.
Foxl2 is the first female specific gene that affects postnatal sex determination without directly involving oocyte survival or differentiation.

Relevance of Foxl2 studies to human reproduction.
Infertility is a common disorder, affecting 1 in 10 couples worldwide. Intraovarian factors are thought to determine the duration of female reproductive competence. In particular, the size of the germ cell pool as well as the rate by which the germ cells are lost, determine the time of the menopause. We are far from being able to manipulate menopausal age. Identifying and studying the function and mechanisms of transcription factors that orchestrate the formation of the initial follicle pool will be essential if we ever want to manipulate the germ cell reservoir. Transcription factors represent theoretically powerful pharmacological targets to regulate fertility, and reproductive lifespan, in a tissue specific manner without affecting other organ systems.
Foxl2 provides a first wedge into the physiology of the specification of follicles and the knockout mouse provides the first model directly relevant to ovarian gonadal dysgenesis in humans along with a route to genes selectively involved in the determination of the initial follicle pool. The investigation of the gene, along with the identification of direct downstream target genes and interacting proteins would be useful for understanding the genetic pathways that *Foxl2* controls. Such genes should include candidates for mutation in instances of POF without extra-ovarian anomalies where affected genes have been difficult to identify. In the long run they may provide targets for therapeutic intervention and for the control of female reproductive lifespan.

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may function as a conserved repressor of the genetic program for somatic testis determination in mammals.

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Müllerian aplasia – genetic aspects

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Learning objects

To understand the development of female genital tract
To understand the pathogenesis of Müllerian aplasia
To recognize the most common forms of Müllerian aplasia, particularly Mayer-Rokitansky-Küster-Hauser syndrome
To understand the genetics of Müllerian aplasia

Müllerian aplasia – genetic aspects

Müllerian aplasia is a congenital malformation of the female genital tract, which has a frequency of 1 in 4000-5000 new-born girls. It is caused by a disruption of female sexual differentiation during early embryogenesis, but the exact molecular mechanisms that lead to this malformation are unknown in most patients.

1. The background of Müllerian aplasia in sex determination

Human sex determination occurs essentially in two phases; primary sex determination, the differentiation of the gonads, and secondary sex determination, the development of the male and female phenotype. In humans there is no "default state", the development of both testes and ovaries is an active process controlled by relevant genes. Gonadal differentiation is determined by genetic sex, namely by the sex chromosome constitution of an individual, XX leading to female and XY to male development. After gonadal differentiation, the hormones produced by the gonad determine the phenotype (Table 1). During this process the two duct systems, mesonephric or Wolffian ducts and paramesonephric or Müllerian ducts undergo development into male or female inner genitalia or regress.

Primary sex determination, that is the differentiation of the bipotential gonad to a testis or an ovary begins on the 7th gestational week. Prior to that the gonadal development seems to be fairly similar in males and females. *SRY*, the testis determining factor in humans resides on the short arm of the Y chromosome. Although *SRY* is capable of initiating testicular development many other genes, such as SOX9, are also necessary. In males the secondary sex determination is due to androgens secreted by the Leydig cells and anti- Müllerian hormone (AMH) secreted by the Sertoli cells. As the result, vas deferens, epididymis, and seminal vesicles are formed from the Wolffian ducts, while the Müllerian ducts regress mainly due to AMH action mediated via the AMH receptor (AMHR).

Less is known about the pivotal genes for female development. In 1999 *WNT4* was suggested as one. *WNT4* is secreted by the Müllerian duct epithelium and induces the

development of Müllerian mesenchyme. In mice inactivation of *WNT4* cause failure of the formation of Müllerian duct derivatives. Another function of *WNT4* is to suppress the production of androgens thereby preventing the virilization of a female fetus and maintaining the right hormonal milieu for female development. Recently, a human mutation has also been reported as explained below. In females the Müllerian ducts develop into Fallopian tubes, uterus, cervix and upper part of the vagina, while Wolffian ducts regress. Thus, Müllerian aplasia is a disruption of this development.

Table 1. Sex determination

	Gonad			Müllerian ducts	Wolffian ducts
XY	TESTIS	Sertoli cells Leydig cells	AMH Androgens	Regression	Vas deferens Epididymis Seminal vesicle
Bipotential gonad					
XX	OVARY	Granulosa cells Theca cells	Estrogens	Fallopian tubes Uterus&cervix Upper vagina	Regression

2. Mayer-Rokitansky-Küster-Hauser syndrome (MRKHS)

During female differentiation, the Müllerian ducts develop into Fallopian tubes, uterus, cervix, and upper vagina. Müllerian aplasia in general refers to any condition with absence of these Müllerian derivatives. However, the term is also used for Mayer-Rokitansky-Küster –Hauser syndrome (MRKHS), which is the most common form of Müllerian aplasia (MA).

Women with MRKHS have normal female chromosome constitution (46,XX), but have congenital absence of the uterus and vagina (also named CAUV). A shortened vagina (often 1-2cm) derived from the urogenital sinus is present, while the upper part is absent. The vagina is usually insufficient for sexual intercourse without treatment. Most patients with MRKHS have normal functioning ovaries. The condition is usually diagnosed at puberty, around the age of 16-17 years, because of primary amenorrhea. The pubertal development, due to functioning ovaries, is otherwise normal.

In laparoscopy, the uterus is usually replaced by a fibrous strand connecting small bulbs of uterine horns with normal looking or hypoplastic Fallopian tubes. In some patients all Müllerian derivatives are lacking. In our experience the latter is much less common than the classic MRKHS phenotype. Furthermore, although most patients have two normally functioning ovaries, some patients also have ovarian abnormalities, typically one ovary is missing. The frequency of ovarian abnormalities seems to be more common in total aplasia than MRKHS. The significance of these phenotypic differences is not presently known, that is, it is not known whether total

Müllerian aplasia and the MRKHS phenotype (with Fallopian tubes) have different etiology. Furthermore, it is also not known, if patients with ovarian abnormalities are an etiologically distinct group.

Associated anomalies

Certain anomalies are known to be associated with MA. Particularly renal and skeletal anomalies are seen and this condition is called MURCS (MUllerian aplasia, Renal agenesis/ectopy and Cervicothoracic Somite anomalies) association. Renal anomalies include aplasia of one kidney, horseshoe kidney or pelvic kidney but others may also occur. The most common skeletal malformations are fusion anomalies of the cervical spine, often resembling Klippel-Feil syndrome. Other less common features include small stature, limb defects and hearing deficit.

The frequency of associated anomalies differs in different reports and is clearly dependent on how thoroughly they have been sought for. In a recent review 32% of patients had renal anomalies and 12% changes in the skeletal system. More recently Pittock et al. found vertebral anomalies in 40% of their patients. In our own series skeletal anomalies were found in 46% of patients and urinary tract anomalies in 24%.

Genetics of Müllerian aplasia

Inheritance of Müllerian aplasia
Although most cases of Müllerian aplasia are sporadic, familial occurrence has also been reported. Based on familial cases autosomal recessive inheritance was suggested. That is, however, unlikely as a major cause for MA given the small number of affected sib pairs and discordant monozygotic twins, which have also been identified. Another suggested option is autosomal dominant inheritance with a new mutation in patient or with a mutation inherited from the patient's father. The fact that daughters born to MA patients from surrogate pregnancies have not had MA suggests that in these families autosomal dominant inheritance is unlikely. Taken all this together, the commonly held view is that MA is a complex trait and as such may have a heterogeneous etiology. This is actually supported by the low number of affected sibs, which seems to be in the same order (2-4%) as in many multifactorial disorders.

Molecular genetic studies in MA
As familial occurrence of MA is rare, there have not been enough multiplex families for genetic mapping studies. The molecular genetic analysis has therefore based on candidate gene studies. These include known genes associated with infertility or Müllerian anomalies, and developmental genes. The latter have usually been identified in animal models.

Given the role of AMH in regression of Müllerian ducts in males, the AMH pathway is a natural candidate for genes causing MA. Mutations in these genes have not yet been identified, although two patients have been found to carry translocation with break points close to AMHRII. Other genes, which have been analyzed with negative results include CFTR (cystic fibrosis conductance regulator) and GALT (galactose-1-phophate uridyl transeferase) genes as well as developmental genes WT1 and PAX2. Also mutations in HNF-1β have been suggested to cause MA wihtout conclusive evidence.

Two groups of genes have raised interest as candidate genes for MA; the HOX and WNT gene families. The homeobox gene family is a large family of 39 genes with important roles in embryogenesis. HOX genes include a DNA binding homeodomain thereby controlling the transcription of target genes. Several members of the HOXA subgroup are expressed in the developing Müllerian ducts and were therefore candidate genes for MA. Specifically, HOXA10, HOXA11, and HOXA13 knock-out mice have shown Müllerian anomalies, but human mutations have not been identified so far.

The WNT gene family codes for secreted glycoproteins, which act locally controlling the transcriptional activation of a number of genes via Frizzled receptor and intracellular signalling. Wnt4 is one of the few genes shown to function in ovarian differentiation. Wnt4 knock-out mice have defects in kidney development and adrenal function in addition to disturbances of gonadal development and steroidogenic function, which are found only in females. Recently the first human mutation was identified in a female with Müllerian and unilateral renal agenesis and androgen excess without virilization. Later studies on patients with MRKHS have not identified other mutations.

Syndromes with Müllerian aplasia

In addition to MA without any other manifestations and MURCS association, MA occurs in several syndromes. These include disturbances of sexual differentiation and rare malformation syndromes.

Disturbances of Sexual Differentiation (DSD)
It is important to note that sex is not determined by chromosomes, it is determined by active genes and there are several instances of XX males and XY females. The most common of the previous is caused by a translocation of the *SRY* gene to the X chromosome, when the patients are otherwise normal but infertile males. However, mutations of the *SRY* explain only 10-15% of XY females, who in the complete form have normal Müllerian structures, but streak gonads. The most common cause for XY female is androgen insensitivity caused by mutations in androgen receptor (AR) gene. These individuals are phenotypic females with Müllerian aplasia, since the gonads produce AMH as normal males. Many other rare conditions also include MA, such as homozygous mutations in the LH receptor, which also result in XY females.

Malformation syndromes
Different types of Müllerian anomalies are found in association with unbalanced chromosome anomalies. These patients may not always be thoroughly studied for these as many also have severe mental retardation. Many other malformation syndromes also include Müllerian defects. Most of these are different types of Müllerian fusion defects. Müllerian aplasia has been found in patients with TAR (thrombocytopenia absent radius), Fascio-auriculo-vertebral or Goldenhar syndrome, Al Awardi syndrome, and Roberts syndrome.

Conclusions

Müllerian aplasia is a congenital anomaly, which has its origin in early fetal life. The genetic etiology of MA in most patients is not presently known. Considering the present data, MA is likely to have heterogeneous genetic etiology. At the moment, the many transcription factors known to operate during embryogenesis are the most promising candidate genes.

Müllerian aplasia is an abnormality, which crucially influences the life of an affected female, and it truly deserves the attention of the research community to enlighten the underlying molecular events.

Suggested reading

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Oocyte-derived growth factors and ovarian function (TGFβ superfamily: INHα, BMP15, GDF9)

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Learning Objectives
Give an overview of the TGFβ superfamily members structure and signalling.
Understand the importance of TGFβ family members in the developing ovary.
List the known mutations in GDF9, BMP15 and INHA associated with effects on fertility.
Describe the contribution of large animal models in the identification of naturally occurring variants, and in the elucidation of the action of genes involved in fertility.
Gain skills to assess the practical relevance of new mutations as they are reported.

Lecture Summary
The TGFβ superfamily
The TGFβ superfamily is a large group of signalling proteins with a very similar structure, but a diverse range of effects. The approximately 40 members of the family are found in a broad range of species, tissues and developmental stages, and appear to be derived from a very early (evolutionarily) signalling molecule by gene duplication. The superfamily members have a variety of names, initially related to their discovery, comprising three main nomenclature groups: transforming growth factor-β (TGFβ), growth and differentiation factors (GDF), and bone morphogenetic factors (BMP), but also including the inhibins, activins, AMH and a number of others (1). Despite their rather confusing nomenclature, the essential features of these proteins are simple. The genes and their proteins are relatively small (900-1500 coding nucleotides). The coding region is usually two exons interrupted by only one intron. The pre-pro-protein comprises a signal peptide for transportation, a pro-region, which is eventually cleaved off, and the remaining mature fragment, which contains the biological activity. Relative sizes of the genes and proteins for inhibin α , GDF9 and BMP15 are shown in Table 1. Most of the superfamily members are biologically active as disulphide-linked dimers of the mature fragment.

Common to members of this family are six conserved cysteine residues in the mature fragment which combine to form three intrachain disulphide bonds producing a characteristic cysteine knot structure. Almost all members of the family have a 7th conserved cysteine which forms a covalent interchain disulphide bond in the biologically active dimer.

Signalling occurs when the dimeric mature fragment interacts with two trans-membrane glycoprotein receptors (Type I and Type II), triggering phosphorylation of the receptors, and the subsequent phosphorylation and activation of intracellular SMAD proteins. Activated SMAD proteins enter the nucleus and regulate gene expression. Receptors for the TGFβ superfamily also have a variety of names related

to their initial discovery. The seven Type I receptors are approximately 55kDa and are now named based on their activin-like kinase activity (ALK). They are listed here with both names: ALK1 (ACTVRL1), ALK2 (ActR1A, ACVR1)), ALK3 (BMPR1A), ALK4 (ActR1B), ALK5 (TβR1), ALK6 (BMPR1B) and ALK7. The five Type II receptors are approximately 70 kDa transmembrane and are also known by a variety of names: ACVR2 (ActRIIA), ACVR2B (ActRIIB), AMHR2 (MISRII), BMPR2 (BMPRII) and TGFBR2 (TβR2).

Regulation and diversity of signalling from this highly related group of proteins is achieved by a combination of tissue specificity, homo- and hetero-dimerisation, receptor combinations, interactions with the cleaved pro-region, and other binding proteins.

Effects on fertility

Several members of the family have specific effects on fertility (2, 3). The inhibins and activins are expressed by granulosa cells of the ovarian follicle, while GDF9, BMP15 and BMP6 are expressed by the oocyte. AMH is another member of the family which has reproductive effects in both males and females. This talk will concentrate particularly on GDF9, BMP15 and inhibin α, all three of which have naturally-occurring mutations associated with infertility in females.

GDF9 and BMP15 are closely related proteins expressed almost exclusively in the oocyte. They are unusual among the TGFβ superfamily in that neither has the 7th conserved cysteine residue, and therefore they lack the interchain covalent link via a disulphide bond. Whether they act as homodimers or heterodimers with each other (Table 1) is still to be clearly elucidated. GDF9 maps to HSA5, and BMP15 is X-linked in humans and sheep. Knockout studies in mice showed that female mice deficient in GDF9 are infertile due to the arrest of follicles at the primordial stage of development. Female mice deficient in BMP15 are fertile, but produce fewer litters and fewer offspring per litter (1). Our group has been studying sheep carrying major genes for ovulation rate. In two distinct pedigrees, animals carrying two copies of an X-linked gene were infertile with a similar phenotype to the GDF9 knockout mouse, but single copy carriers had an increased ovulation rate and produced twins and triplets (4). We have identified two single point mutations in the BMP15 gene in these animals which result in changes to the BMP15 protein (Q239Ter, V299D) and have shown that BMP15 is essential for follicle development in sheep, highlighting differences between rodents and sheep. Three further mutations causing amino acid changes in BMP15 have now been identified in sheep (Q291Ter, C325Y, S371I) bringing to five the number of BMP15 mutations known to cause infertility in homozygous carriers (Figure 1). In addition, a point mutation in sheep GDF9 (S395F) also causes infertility in homozygous carriers (5) (Figure 1). It now seems that selection of domestic sheep for increased ovulation rate has selected for mutations in BMP15 and GDF9.

The name ‘inhibin’ was given to a non-steroidal protein from the gonads that suppressed FSH , and ‘activin’ to another protein antagonist of inhibin which stimulated pituitary FSH release. It soon became clear that the regulation of FSH was occurring by a complex combination of related protein subunits which combined to produce the inhibins and activins, and which were the products of several distinct genes. Each of these proteins are members of the TGFβ superfamily. The inhibin α

subunit precursor is coded for by INHA (on HSA2q) (Table 1), inhibin β_A by INHBA (on HSA7p), and inhibin β_B by INHBB (on HSA2q). The biologically active inhibin and activin proteins are dimeric combinations of the mature fragments of the above gene products (Inhibin A = Inhα + Inhβ_A, Inhibin B = Inhα + Inhβ_B, Activin A = Inhβ_A + Inhβ_A, Activin B = Inhβ_B + Inhβ_B, and Activin AB = Inhβ_A + Inhβ_B). Knockout studies in mice shows that absence of the inhibin α subunit (which effectively removes both Inhibin A and Inhibin B) produces mice with increased FSH and with granulosa cell and sertoli cell tumours.

Fertility effects in humans

Although BMP15 and/or GDF9 are essential for fertility in sheep and rodents, and the inhibins have a pivotal role in FSH regulation, progress has been slow in clearly identifying variants of these genes with a major role in human infertility.

Four factors contribute to this difficulty. Firstly the likelihood of being able to generate (in animals), or readily identify (in humans) segregating pedigrees for infertility is very low. Second, the use of gene knockouts does not necessarily reflect a natural situation, where point mutations can alter the function of a gene rather than lead to its complete absence. Thirdly, species differences in the relative role of these genes means that even with good animal models, the same phenotypic effects may not be matched in humans. And lastly, perturbations in these genes are likely to only make up a very small percentage of the causes of infertility in the population as a whole.

A few studies have reported searches for variants in these genes in women with premature ovarian failure (POF), primary amenorrhea (PA), secondary amenorrhea (SA), polycystic ovary syndrome (PCOS), and dizygotic twinning (DZ).

A Japanese study of 15 women with POF, 38 women with PCOS and 3 fertile controls found no missense mutations in exons of BMP15 or GDF9 (Takebayashi *et al*., 2000 Fertility and Sterility 74: 976-979). The first report of a BMP15 gene effect in humans was of two sisters with primary amenorrhea due to ovarian dysgensis with BMP15 Y235C amino acid change (Figure 1) which appears to give rise to abnormal protein processing (Di Pasquale *et al*., 2004 Am. J. Hum Genet. 75: 106-111). A larger study to look at the prevalence of BMP15 variants in POF (166 Italian POF samples and 181 female controls) found a A180T amino acid change in five non-familial SA samples. (Di Pasquale *et al*., 2006 J. Clin. Endocrin. Metab Epub Feb 7 doi:10.1210/JC.2005-2650). Two amino acid changes were reported in the pro-region of GDF9 in 195 Indian women with ovarian failure and 220 controls, one in 5 women (K67E: Figure 1) and another (V216M: not shown here) in 2 women with ovarian failure (Dixit *et al*., 2005 Menopause 12: 749-754). Interestingly, although a study in dizygotic twinning found no association between common polymorphisms in the GDF9 gene and DZ twinning in over 600 DZ twin families, they did identify one rare four base-pair deletion leading to a premature stop codon in GDF9 which appears associated with twinning in two heterozygous carriers, but not in 429 controls (Figure 1) (Montgomery et al., 2004 Twin Research 7: 548-555).

In 2000 Shelling *et al*. (6) reported an amino acid change (A257T) in the inhibin α subunit in three out of 43 women with POF, a variant which also occurred in 1 of 150 controls (Figure 1). This was also reported in 7/157 Italian POF samples but not in

100 controls (Marozzi *et al*., 2002 Human reproduction 17: 1741-1745), and in 14/114 Indian samples (POF, PA, and SA) but not in 100 controls (Dixit *et al*., 2004 Human Reproduction 19: 1760-1764). However the same mutation was not detected in 84 Korean POF samples and 100 controls (Jeong et al., 2004 Yonsei Medical Journal 3: 479-482), and a study of women in Argentina was unable to detect a significant association between this mutation and ovarian failure (Sundblad *et al*., 2006 Human Reproduction Epub Jan 5 doi:10.1093/humrep/dei452).

Studies in animal models and *in vitro* functional studies suggest that variants of these proteins are very likely to have a role in human infertility. Individual studies in humans to date provide tantalising support for this. But fully characterising the extent to which variants in the TGFβ genes contribute to human infertility would require sequencing of the coding and regulatory regions of these genes in very large numbers of affected individuals with clearly defined phenotypes, and in similarly large numbers of (preferably) related, unaffected individuals.

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Figure 1: Naturally occuring amino acid changes in TGFβ members ssociated with effects on fertility

Pictorial representation of TGFβ superfamily proteins BMP15, GDF9 and inhibin α (INHA) showing naturally-occurring amino acid variants in sheep (above each diagram) and/or human (below each diagram). Positions of the signal peptide (SP), pro-region (pro) and the mature fragment are indicated. Lengths of each proteins (in amino acids (aa)) is shown for the full-length proteins (after the gene name) and the mature fragments (under the diagrams). Notation for the variants is: normal amino acid followed by the amino acid residue number that has changed followed by changed amino acid (eg V299D). Alanine (A), cysteine (C), aspartate (D), glutamate (E), phenylalanine (F), isoleucine (I), lysine (K), glutamine (Q), serine (S), threonine (T), valine (V), tyrosine (Y), deletion (Del), termination or stop codon (Ter).

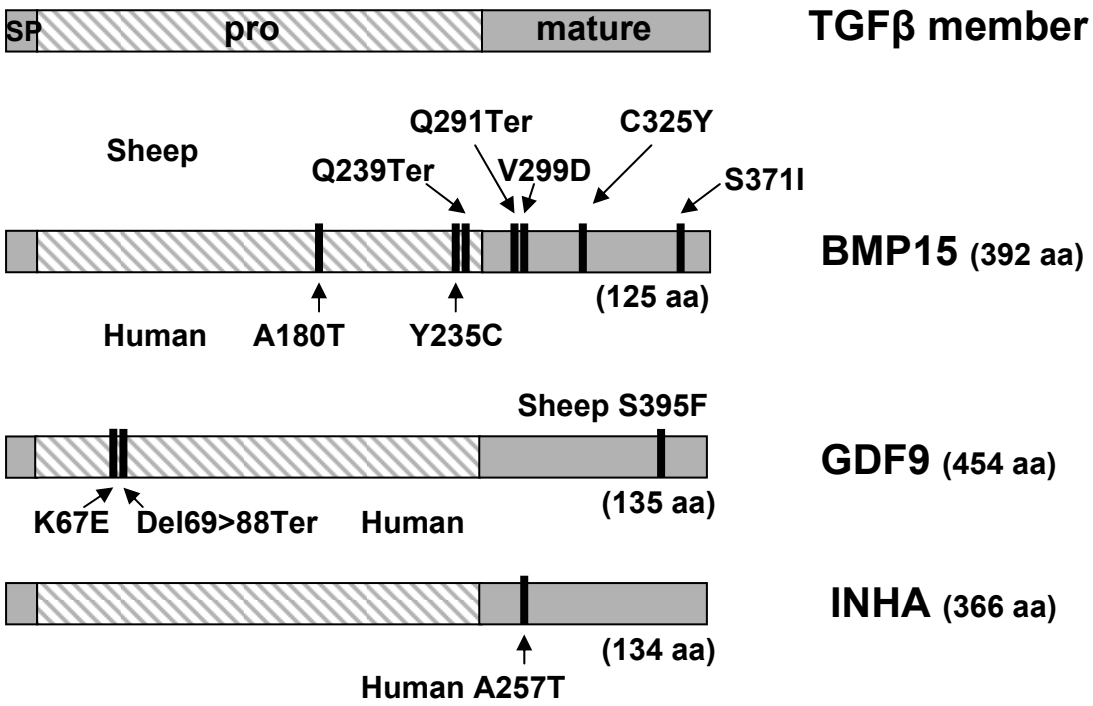

Table 1: Gene and protein details for human inhibin α, GDF9 and BMP15

Gene	Known as:	coding sequence	intron length	pre-pro-protein	mature fragment	subunit of:
INHA	INHα, inhibin alpha (subunit)	1098 bp	2051 bp	366 aa	134 aa	Inhibin A (Inhα + $Inh\beta_A$) Inhibin B (Inhα + $Inh\beta_B$)
GDF9	GDF9, growth differentiation factor 9	1365 bp	1580 bp	454 aa	135 aa	GDF9 + GDF9? GDF9 +BMP15?
BMP15	BMP15, bone morphogenetic factor 15 GDF9B, growth differentiation factor 9b	1179	4645 bp	392 aa	125 aa	BMP15 + BMP15? BMP15 + GDF9?

McKusick-Kaufman syndrome

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Learning Objectives

1) To define the diagnostic triad and commonest clinical features of McKusick-Kaufman syndrome
2) To be able to differentiate McKusick-Kaufman syndrome from Bardet-Biedl syndrome, an allelic condition with more severe clinical features
3) To manage infants suspected to have McKusick-Kaufman syndrome appropriately
4) To understand that mutations in the *MKKS* gene cause McKusick-Kaufman syndrome

Lecture Summary

Overview

McKusick-Kaufman syndrome (MKS) is a rare, panethnic multiple congenital anomaly syndrome characterized by a diagnostic triad of hydrometrocolpos (HMC), postaxial polydactyly (PAP) and congenital heart disease (CHD). MKS was first described in the Amish population and the Amish phenotype consists solely of the MKS diagnostic triad. More recently, close clinical overlap between MKS and an allelic condition, Bardet-Biedl syndrome (BBS), has been described, complicating both the diagnosis and the management of individuals previously considered to have MKS because of HMC and PAP in the neonatal period. MKS is an autosomal recessive condition and mutations in the *MKKS* gene on chromosome 20p12 have been identified both in MKS and BBS patients. Testing is currently available on a research basis only.

Clinical Features Of McKusick-Kaufman syndrome

Hydrometrocolpos (HMC)

HMC in infancy is defined as a dilatation of the vagina and female reproductive tract caused by the accumulation of cervical secretions as a result of maternal hormonal stimulation. HMC can be caused by agenesis of the vagina, a transverse vaginal membrane or imperforate hymen. HMC commonly presents at birth or in the neonatal period with a cystic abdominal mass that can be large enough to cause obstruction to the intestines, ureteric outflow tract and kidneys and inferior vena cava. A clinical suspicion of HMC should be confirmed by abdominal ultrasound.

Postaxial polydactyly (PAP)
Polydactyly refers to an additional digit (finger or toe) and postaxial describes the anatomical site of the supernumerary digit (ulnar side of the hand or fibular side of the foot). In MKS, the additional digit can be fully formed or a rudimentary skin tag.

Congenital heart disease (CHD)
The incidence of individual heart defects in MKS is unknown, but reported anomalies have included atrioventricular septal defects, atrial and ventricular septal defects, hypoplasia of the aorta and left ventricle, Tetralogy of Fallot and patent ductus arteriosus.

Other clinical features of McKusick-Kaufman syndrome
The most common associated clinical features in MKS were reviewed in 49 published patients (Slavotinek and Biesecker, 2000) and have been summarized in Table 1:

Table 1. Clinical features of McKusick-Kaufman syndrome

Clinical feature	Incidence	Percentage
Diagnostic triad		
Hydrometrocolpos	42/44	95%
Postaxial polydactyly	48/49	98%
Congenital heart disease	7/49	14%
Genitourinary anomalies		
Vaginal agenesis	26/44	59%
Urogenital sinus	16/44	36%
Ectopic urethra	8/44	18%
Digital anomalies		
Syndactyly	12/49	24%
Metacarpal/tarsal anomalies	8/49	16%
Brachydactyly	3/49	6%
Renal anomalies		
Hydronephrosis	31/49	63%
Hydroureter	12/49	24%
Pelvicalyceal dilatation	7/49	14%
Gastrointestinal anomalies		
Hirschsprung disease	6/49	12%
Imperforate anus	4/49	8%
Other		
Developmental delay	3/37	14%

Normal growth, development and fertility have been described for some MKS patients, but data on adults with this condition is scarce. If a child with HMC and PAP does not develop features of BBS, then prognosis is generally considered to be good.

Diagnostic criteria for McKusick-Kaufman Syndrome

There are no published diagnostic criteria for MKS, but occurrence of the diagnostic triad (HMC, PAP, CHD) or HMC and PAP in a female without evidence of an overlapping syndrome have been used as a guideline. It is very important to note that all three features of the triad can occur in female patients with Bardet-Biedl syndrome and that the diagnosis of MKS must therefore be delayed until the clinician is certain that the age-dependent features of BBS, such as rod-cone dystrophy, obesity and developmental disability, are not apparent. In males with one or more features of MKS, diagnosis is difficult without an affected female relative.

Differential Diagnosis

The condition that is most commonly misdiagnosed as MKS is Bardet-Biedl syndrome (BBS; for review of the clinical features in BBS, see Beales et al., 1999). BBS has six major diagnostic criteria including rod-cone dystrophy, polydactyly, obesity in early childhood, cognitive impairment, male hypogonadism and renal anomalies. HMC has been described in BBS, and females with HMC and PAP in the neonatal period can have either MKS or BBS (David et al., 1999). Differentiation depends on the later development of BBS features that establish a child as having BBS rather than MKS. Clinical and research testing for mutations in the BBS genes is also available (www.geneclinics.org).

Ellis-van Creveld syndrome has chondrodysplasia, short stature, PAP, ectodermal dysplasia and CHD as cardinal features. HMC has also been reported in rare individuals with EvC, leading to diagnostic confusion. However, EvC is clearly distinguishable from MKS because of the associated skeletal anomalies; molecular genetic testing for EvC is available (www.geneclinics.org) as part of research.

Diagnostic Evaluations

A renal ultrasound scan or renal imaging studies should be performed at diagnosis to evaluate affected individuals for hydronephrosis, renal cystic dysplasia, and other renal anomalies.

A pelvic ultrasound should be undertaken to detect genitourinary malformations.

A chest radiograph, electrocardiogram and echocardiogram should be performed in additional to cardiac auscultation to evaluate for cardiac defects.

Skeletal radiographs should be undertaken to determine if polydactyly is postaxial or mesoaxial and to examine if there is involvement of the underlying bone; radiographs may also detect syndactyly (fused digits).

Measurement of growth parameters should be done using an appropriate growth chart.

In patients suspected to have BBS, ophthamological examination with an electroretinogram (ERG) should be performed.

Management

Surgical intervention for the HMC is appropriate and prompt treatment is necessary when obstructive symptoms are present. Management of PAP, CHD and other phenotypic manifestations of MKS is the same as for other clinical situations in which these anomalies occur. Developmental delay can be assisted by early intervention services.

For surveillance, checking blood pressure and renal function in those with renal anomalies is prudent and growth and development should also be followed. Other considerations include the possibility of recurrent urinary tract infections and re-stenosis of the vaginal orifice in those who have had surgery.

Children suspected of having MKS should have periodic evaluations for features of BBS at least annually until the diagnosis of either MKS or BBS can be substantiated. Serial growth measurements to follow height and weight, developmental assessments and regular ophthalmological examinations are the most important measures. However, monitoring for rare complications of BBS with hearing and dental evaluations, electroencephalograms for seizures and thyroid function tests may also be employed.

Molecular Genetic Etiology of McKusick-Kaufman Syndrome

MKS is inherited as an autosomal recessive syndrome. In this pattern of inheritance, both parents of an affected child are obligate carriers and are asymptomatic. Both parents carry one mutated allele and one normal allele. The recurrence risk to both parents of a further affected child is 25% or 1 in 4 in each pregnancy and both males and females can be affected. The children of an individual with MKS will also be asymptomatic obligate carriers. Consanguinity has been described in up to 29% of the parents of individuals with MKS.

The causative gene for MKS, *MKKS*, is located on chromosome 20p12 and was isolated using positional cloning methods in a large Amish family (Stone et al., 2000). In the Amish family, two mutations were identified, p.H84Y, and p.A242S, and affected individuals on the Amish pedigree were homozygous for both of these mutations. In a non-Amish female thought to have MKS, two further mutations were identified, p.Y37C and C.1215_1216delGG that were not present in control chromosomes, implicating the *MKKS* gene in the pathogenesis of MKS. Mutations in *MKKS* have subsequently been described in other patients with both the MKS and BBS phenotypes (Slavotinek et al., 2000). A high frequency of “single” mutations in *MKKS* have also been described, and it has been hypothesized that these single sequence alterations imply triallelic inheritance or a modifying locus or that cryptic mutations have not been identified (Slavotinek et al., 2002).

The *MKKS* gene encodes a 570 amino acid protein with similarity to the group II chaperonin protein family. Mutations in *MKKS* have been identified in all of the coding exons of the gene and no mutation "hot spots" have been identified.

Currently, molecular genetic testing for MKS by sequencing of the MKKS gene is not available at a clinical laboratory in Europe or in the United States. Research testing (a result for the family is not guaranteed) is available (see www.geneclinics.org). Prenatal testing may be possible by ultrasound scans or by genetic testing in families in whom the disease-causing mutations has previously been identified.

Triallelic inheritance or modifier genes have been postulated for MKS, but as yet there has been no demonstrations of triallelism for this phenotype and no modifiers have been isolated (Nakane and Biesecker, 2005).

Interestingly, the *MKKS* gene has also been sequenced in Danish men with juvenile-onset obesity, and it was concluded that it is unlikely that sequence variants in this gene are major determinants of non-syndromic obesity. However, the variant p.A242S was found to segregate with obesity in two families (Andersen et al., 2005).

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Polycystic Ovarian Syndrome (PCOS)

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Learning Objectives:

1. State the recurrence risk and ostensible mode of inheritance in PCOS.
2. Estimate the approximate number of PCOS cases known to be caused by perturbation of an identified mutation.
3. List up to 5 classes of candidate genes being elevated as causative for PCOS, and list the currently most promising chromosomal region.

Polycystic ovarian syndrome (PCOS) clearly has a genetic component. Familial clustering has long been recognized. Yet few causative genes have been identified and etiology is still fundamentally unknown. Progress has been hampered by lack of crisp diagnostic criteria. Because PCOS is associated with infertility, relatively few large pedigrees for linkage analysis are infrequent. In this communication we shall review selected family studies and systematically consider candidate genes. For citations alluded to, see reviews cited in the Suggested Reading section.

I. Family studies

Most family studies indicate autosomal dominant inheritance of low penetrance and variable expressivity (Table 1). However, one should counsel a first-degree relative of an affected family member that her risk of clinical PCOS is only 5-10%. This is far less than if only a single dominant genes were responsible, indicating complex genetics. Polygenic factors, phenocopies, or heterogeneity are considerations.

II. Candidate genes

A. *CYP11A (Cytochrome P450 Side-Chain Cleavage Enzyme)*

Adult onset 11β-hydroxylase deficiency has been found in women with PCOS and hirsutism, mostly in Arab populations. CYP11A is thus, an attractive gene in which to search for polymorphism associated with traditional (non-adrenal) PCOS.

Gharani *et al.* (1997) found weak linkage between the CYP11A gene, and hyperandrogenemia in PCOS women. This association study involved 97 women with PCOS, and demonstrated strong association between a CYP11A 5' UTR (untranslated region) pentanucleotide repeat polymorphism with total serum testosterone levels. The value of this study

is limited because of failure to make appropriate statistical adjustments for multiple testing.

In another study, association was found between a pentanucleotide repeat at -528 and PCOS (Diamanti-Kandarakis *et al.*, 2000). However, this has not been confirmed (Urbanek *et al.*, 1999;San Millan *et al.*, 2001).

Several groups have pursued digenic hypothesis, encompassing 11β-hydroxylase polymorphisms and hexose-6-phosphate dehydrogenase polymorphisms. The hypothesis is that alleles are either locus cumulatively cause PCOS. However, recent studies show no association (San Millan *et al.*, 2005;White, 2005).

B. ***CYP17 (Cytochrome P450 17-Hydroxylase/17, 20-Desmolase)***

Initial studies suggested an association between CYP17, which encodes 17-hydroxylase/17,20-lyase, and PCOS. However, subsequent reports failed to confirm this finding (Carey *et al.*, 1994;Gharani *et al.*, 1996;Witchel *et al.*, 1998). On the other hand, Qin *et al.* (2006) recently reported an association between an activating polymorphism (HSD 17B5) in the promoter region and hyperandrogenism.

Occasionally hirsutism and PCOS are observed in adult onset 17α-hydroxylase deficiency. This tract is most common in Japanese.

C. ***CYP19 (Aromatase)***

In a study of 25 PCOS cases and 50 controls all exons of the aromatase gene were sequenced. No mutations were detected (Soderlund *et al.*, 2005).

D. ***CYP21 (Cytochrome P450 21-Hydroxylase) and Non-Classical Adrenal Hyperplasia (NCAH)***

CYP21 encodes 21-hydroxylase, the enzyme responsible for most cases of congenital adrenal hyperplasia (CAH). A relationship to some forms of PCOS is long established because women with non-classical adrenal hyperplasia (NCAH) (21-hydroxylace deficiency) may present with a PCOS phenotype. NCAH is most common in Ashkenazi Jews and most commonly due to homozygous or compound heterozygous mutation for Val 281 Leu (68%) and Pro 458 Ser (7%). NCAH accounts for perhaps 3-5% of cases having the physical findings of PCOS. In the non-Ashkenazim NCAH is rare and, hence, an uncommon explanation for PCOS overall. Some studies have found a significant frequency of CYP21 mutations in PCOS women who do not have NCAH. That is, they have normal 17-hydroxyprogesterone response to ACTH stimulation (Escobar-Morreale *et al.*, 1999;Witchel and Aston, 2000). Overall, however, most PCOS cases are not associated with CYP21 polymorphisms.

D. ***Androgen Receptor***

A trinucleotide (CAG) repeat polymorphism in the X-linked androgen receptor gene has been shown to be inversely associated with androgen levels (Mifsud *et al.*, 2000). Although a relationship to PCOS is biologically attractive, linkage studies involving 150 families had previously failed to reveal an association (Urbanek *et al.*,1999).

After these initial contradictions, a study of 122 women with PCOS demonstrated significantly greater frequency of longer CAG alleles (>22 repeats) in a polymorphism in exon 1 of the androgen receptor gene (Hickey *et al.*, 2002). Ibanez *et al.* (2003) found an association between the shorter androgen receptor gene CAG repeat polymorphism and precocious pubarche. Jaaskelainen *et al.* (2005) studied 106 PCOS and 112 non hirsute controls. They concluded the CAG polymorphism is not a major factor, even while noting that all 5 women with CAG <15 repeats had PCOS.

In conclusion, an association of PCOS with AR is still unproved.

E. ***Sex Hormone-Binding Globulin***

In 4 of 482 women with PCOS, hirsutism or ovarian dysfunction, Hogeveen *et al.* (2002) identified a polymorphism in the coding region of SHBG that encodes a missense mutation, P156L. If the mutation is causative at all, it explains very few cases.

Xita *et al.* (2003) showed an association with a $(TAAAA)^n$ polymorphism in the SHBG. Women with PCOS were found to have a significantly greater frequency of longer $(TAAAA)^n$ alleles (> 8 repeats); normal women showed shorter alleles (< 8 repeats) in higher frequency. A later study by the same group (Xita *et al.*, 2005) showed an association between late age of menarche and TAAAA > 8 repeats.

F. ***Insulin Gene***

In 1997, Waterworth *et al.* (1997) reported an association between PCOS and a VNTR polymorphism 5' to the insulin gene. The much larger study by Urbanek *et al.* (1999) found no evidence for linkage of the insulin gene and PCOS; there was also no association between the class III allele of the insulin VNTR and hyperandrogenemia. Vankova *et al.* (2002) and Powell *et al.* (2005) also found no association between the INS VNTR polymorphism and PCOS. The latter study consisted of 255 parent offspring trios, 185 other U.K. PCOS cases, and 1,599 Finnish cases.

G. ***Insulin Receptor (IR) and Insulin Receptor Substract (IRS)***

A general relationship between PCOS and insulin receptivity is accepted. Dunaif *et al.* (1995) found that 50% of women with PCOS showed increased insulin receptor and serine phosphorylation in skeletal muscle cells and fibroblasts. Logically, studies have sought major gene mutations

in the insulin receptor gene, located on chromosome 19p13.3. All have been negative.

Tyrosine autophosphorylation of the insulin receptor has also been found to be decreased in ovaries of women with PCOS (Moran *et al.*, 2001). Siegel *et al.*, (2002) showed an association between PCOS and a C/T single nucleotide polymorphism at the tyrosine kinase domain of the insulin receptor.

Several studies have reported linkage or association between a marker (D19S884) located near the insulin receptor gene and PCOS (Urbanek *et al.*, 1999;Tucci *et al.*, 2001), using both sib-pair analysis and transmission/disequilibrium tests. Evidence for linkage and association has been found for chromosome region 19p13.3, where the 15R is located.

The Heritage Family study found evidence for linkage between chromosome 19p13.3 and androgen levels in Caucasians (Ukkola *et al.*, 2002). In their latest report, Urbanek *et al.* (2005) studied 217 families, concentrating on a 13-Mb sequence of the INSR gene in which D19S884 is located. Nominal significance was found between this region and PCOS.

A putative PCOS gene on 19p remains attractive and has biological plausibility. However, statistical significance is barely achieved and is far from that expected for an unassailable causative gene. Perhaps the perturbation involves fundamental signal transduction mechanisms, which might lead to altered expression of a family of genes each involved in steroidogenesis and/or insulin action. Consistent with this would be the finding of Ho *et al.* (2005) that theca cells of PCOS cases show increased levels of GATA6, a transcription factor that regulates several steroidogenic genes.

H. ***Insulin Receptor Substrate Proteins***

El Mkadem *et al.* (2001) reported an association between IRS-1 insulin resistance in women with PCOS and both Gly972Arg variant and the IRS-2 Gly1057Asp variant. Dilek *et al.* (2005) reached a similar conclusion in a study of 60 Turkish women and a like number of controls; androgen levels did not differ, although Gly972 Arg patients were more hirsute and had increased fasting insulin levels.

On the other hand, Ehrmann *et al.* (2002) found no association of IRS-1 with PCOS in a U.S. (Illinois) sample. The IRS-2 Gly/Gly polymorphism was associated with higher blood glucose levels in non-diabetic Caucasian and African-American women with PCOS. Studying a Spanish sample,

Villuendas *et al.* (2005) found no association between PCOS and either the Gly972Arg in IRS-1 or Gly1057 Asp in IRS-2.

Ibanez *et al.* (2002) found an increased frequency of the G972R variant of the IRS-1 gene among girls with a history of precocious puberty.

I. ***Follistatin***

Their pilot study of 39 affected sibling pairs to the contrary (Milner *et al.*, 1999), Urbanek *et al.* (2000) concluded that the follistatin gene is not linked to PCOS.

J. ***Microarray Analysis***

Microarray analysis can identify many genes worthy of study. However, interpreting and applying microarray in results is not simple. Overexpressed in PCOS theca cells compared to normal theca cells are aldehyde dehydrogenase 6, retinol dehydrogenase 2, and the transcription factor GATA6 (Wood *et al.*, 2003).

K. ***Calpain-10***

Calpain-10 is a cysteine protease that has been shown to be associated with susceptibility to type II diabetes. The 112/121-haplotype of calpain-10 proved associated with higher insulin levels in African-Americans, and also with an increase risk for PCOS in both African-Americans and Whites (Ehrmann *et al.*, 2002).

In a Spanish sample, Gonzalez *et al.* (2002; 2003) showed an association between the CAPN10 UCSNP-44 allele and PCOS. However, Haddad *et al.* (2002) reporting no association between CAPN10 gene variants and PCOS.

L. ***Leptin and Leptin Receptor***

Oksanen *et al.* (2000) found no abnormalities in either of these genes, studying 38 PCOS women.

M. ***Peroxisome Proliferator-Activated Receptor-gamma (PPARγ)***

PPARγ polymorphism was found to be associated with PCOS by Orio, Jr. *et al.* (2003) and Korhonen *et al.* (2003). Hahn *et al.* (2005) found that 23% German PCOS subjects had no wild type PPAR gamma allele (Pro/Pro), but rather at least one allele coding for Ala. These women (-/Ala) were more insulin sensitive.

N. ***Genes Related to Inflammation (TNF, TNF-R, IL-6)***

Other studies have sought associations with selected genes that play a role in inflammation.

Polymorphisms involving the Tumor Necrosis Factor Receptor gene (TNF-R) were reported not to be associated with PCOS by Peral *et al.* (2002) and Fernandez-Real *et al.* (2002). Similarly, negative studies were obtained for interleukin-6 (IL-6).

Suggested Reading:

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Table 1. PCOS in female relatives of women with PCOS.

	Diagnostic Criteria	Specific Outcome Trait	Sisters (%)	Mothers (%)	All First-Degree Female Relatives (%)
Cooper *et al.* (1968)	Oligomenorrhea, hirsutism, polycystic ovaries (culdoscopy, gynecography, or wedge resection)	Oligomenorrhea	9/19 (47%)	4/13 (31%)	
		Hirsutism	14/24 (58%)	4/13 (31%)	
		Elevated 24-hour urinary 17-kerosteroids	12/19 (63%)	2/7 (29%)	
		Enlarged ovaries	10/19 (53%)	0/7 (0%)	
Wilroy, Jr. *et al.* (1975)	Oligomenorrhea, hirsutism, and polycystic ovaries (exam and surgery)	Oligomenorrhea			16/67 (24%)
		Hirsutism			28/54 (52%)
Ferriman and Purdie, (1979)	Hirsutism and/or oligomenorrhea anatomica diagnosis by "air contrast gynecography"	Hirsutism	30/337 (9%)	32/284 (5%)	
		Oligomennorhea	32/337 (9%)	24/284 (8%)	
Lunde *et al.* (1989)	Menstrual dysfunction, hyperandrogenism, obesity, infertility and polycystic ovaries anatomic diagnosis by "transabdominal ultrasound"	Hirsutism	8/129 (6%)	17/132 (13%)	
		Oligomenorrhea	19/129 (15%)	16/132 (12%)	
Hague *et al.* (1988)	Menstrual irregularities, hirsutism, infertility, obesity and multicystic ovaries anatomic diagnosis by "wedge reseaction"	Hirsutism			28/107 (26%)
		Oligomenorrhea			19/107 (18%)
Carey *et al.* (1993)	Polycystic ovaries anatomic diagnosis by "transabdominal ultrasound"	Polycystic ovaries (ultrasound)			37/50 (74%)
		Elevated testosterone			16/50 (32%)
Norman *et al.* (1996)	Elevated androgens, decreased SHBG, polycystic ovaries anatomic diagnosis by "ultrasound"	Polycystic ovaries (ultrasound)	11/15 (73%)	1/1 (100%)	
		Increased testosterone or androstenedione	13/15 (87%)	1/5 (20%)	
		Hyperinsulinemia	10/15 (66%)	5/5 (100%)	
Legro *et al.* (1998)	Elevated testosterone and oligomenorrhea	Hyperadrogenemia	36/80 (45%)		
Govind *et al.* (1999)	Polycystic ovaries anatomic diagnosis by "ultrasound"	Polycystic ovaries	35/53 (66%)	15/29 (52%)	50/82 (61%)
Kahsar-Miller *et al.* (2001)	Oligomenorrhea and either hirsutism or elevated testosterone	PCOS as defined	16/50 (32%)	19/78 (24%)	