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Inhibin and premature ovarian failure

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BACKGROUND: Elucidation of the causes of premature ovarian failure (POF) is difficult due to the heterogeneity of the condition. Inhibin is a potential candidate gene for POF based on its dual actions on FSH secretion by the pituitary and gametogenesis in the gonads. A missense mutation in the inhibin α subunit gene (*INHA* G769A) is associated with POF in several populations. However, there is phenotypic heterogeneity in *INHA* G769A mutation carriers.

METHODS: Relevant studies were identified by searching PubMed and mutational frequencies combined for meta-analysis.

RESULTS: Meta-analysis of published studies revealed a risk difference of 0.04 (−0.030 to 0.11). The occurrence of asymptomatic carriers in populations suggests incomplete penetrance and/or a multi-genetic cause of POF. We propose that a decline in inhibin bioactivity caused by the mutation could increase FSH levels; and in a susceptible individual, the heightened sensitivity to gonadotrophins causes POF. Impaired paracrine effects of inhibin could impact folliculogenesis due to reduced antagonism of activin, bone morphogenetic protein 15 and growth differentiation factor 9. Functional studies of this mutation indicate normal production of dimeric inhibin A and B and impaired bioactivity of inhibin B.

CONCLUSIONS: The identification of an autosomal mutation in the inhibin α subunit gene that is significantly linked to POF in certain ethnic populations highlights the role of inhibin in the regulation of ovarian biology and fertility. Although the reduction of inhibin B bioactivity by the *INHA* G769A mutation is clearly not the only cause, evidence suggests that this change may serve as a susceptibility factor, increasing the likelihood of POF.

Key words: inhibin / premature ovarian failure / POF / inhibin A / inhibin B

Introduction

Premature ovarian failure (POF) is characterized by ovarian dysfunction leading to a menopause-like state earlier than 40 years of age. It is considered a common condition as it affects 1% of all women under the age of 40 years. In a population of women 30 years or younger, the prevalence of POF is 0.1% (Coulam *et al.*, 1986). Women with POF may not experience a menstrual cycle at all (primary amenorrhoea) or may experience cessation of ovarian function after a period of menstrual cycling (secondary amenorrhoea).

Patients tend to have elevated serum FSH levels, and this is part of the current clinical assessment criterion for the condition of POF. Serum levels of FSH above 40 IU/l and amenorrhoea for duration of 6 months or more are the clinical parameters defining POF.

Additionally, affected women have very low levels of circulating estrogens. Clinical symptoms observed are similar to those observed with the onset of menopause, such as hot flushes, vaginal dryness, dyspareunia, insomnia, vaginitis and mood swings. In addition, psychological disturbance including depression may result from the realization that fertility is no longer possible and the perception that femininity has been lost. There are elevated risks for cardiovascular disease and low bone density (osteoporosis) due to the low levels of estrogen experienced by these women at an earlier stage in life than normal women. Additionally, women with POF have a nearly 2-fold age-specific increase in mortality rate (Kalantaridou *et al*., 1998).

The etiology of POF is heterogeneous with the majority being idiopathic. Known causes of POF include permanent damage to the ovaries, such as pelvic surgery, chemotherapy or radiotherapy, autoimmune conditions, exposure to environmental toxicants and genetic causes. Genetic abnormalities on the X chromosome regions Xq13.3–q21.3 (Sala *et al*., 1997) and Xq26–28 (Delon *et al*., 1997) give rise to the POF phenotype. Additionally disorders linked to the X chromosome such as Fragile X and Turner's syndrome are associated with POF (Conway *et al*., 1995). POF-associated genes located on the X chromosome include DIAPH2 (Sala *et al*., 1997), FSHPRH1 and LRPR1 (Roberts *et al*., 1996), BMP15 (Di Pasquale *et al*., 2006) and ZFX (Simpson and Rajkovic, 1999). Autosomal mutations in a number of dominantly inherited genes including GDF9, INHA, FOXL2, FSHR and others have been related to POF (Laissue *et al*., 2008; Simpson, 2008).

The importance of inhibin in the regulation of the female reproductive cycle is well established. Inhibin acts principally as an endocrine modulator of pituitary FSH synthesis (Robertson *et al*., 1985). It is also known to act locally in the ovary, the most clearly defined paracrine function being to stimulate androgen biosynthesis in the theca cells (Hillier *et al*., 1991). Other paracrine roles within granulosa cells include antagonism of activin, bone morphogenetic proteins (BMPs-2, -6 and -7) (Wiater and Vale, 2003; Farnworth *et al*., 2006) and growth differentiation factor 9 (GDF9) actions (Wu *et al*., 2004).

Inhibins and activins are members of the transforming growth factor β (TGFβ) superfamily encompassing a vast array of growth and differentiation factors such as TGFβs and BMPs. Inhibin was first isolated from bovine follicular fluid and was shown to suppress FSH release from cultured pituitary cells (Ling *et al*., 1985; Robertson *et al*., 1985). It is recognized that inhibins regulate FSH secretion by inhibiting the stimulatory actions of the structurally related proteins, activins (Vale *et al*., 1986).

Inhibins are heterodimers of an 18 kDa α-subunit disulphide-linked to one of two 14 kDa β-subunits (βA and βB), resulting in inhibin A or inhibin B, respectively. Activins are composed of two β-subunits; βA–βA (activin A), βA–βB (activin AB), βB–βB (activin B). In females, inhibin A and B are expressed in the ovary and are secreted throughout the menstrual cycle in a discordant pattern (Groome *et al*., 1996), with smaller follicles expressing mainly the α- and βB-subunits, whereas dominant follicles and the corpus luteum express α- and βA-subunits (Roberts *et al*., 1993). These individual patterns of expression raise the possibility that inhibin A and B may be functionally distinct (Burger, 1993). In early follicular phase, with rising FSH levels, there is an elevation of plasma levels of inhibins with the increase in inhibin B more pronounced than inhibin A levels (Burger *et al*., 1998). Inhibin B is mainly produced by the granulosa cells of the developing follicles, levels being highest in mid-follicular phase. The dominant follicle selectively produces inhibin A its levels high mid-cycle leading to the suppression of FSH. In the luteal phase, the corpus luteum maintains secretion of inhibin A (Roberts *et al*., 1993).

When the ovarian follicle reserve is depleted as a woman is approaching menopause, a decline in inhibin levels is correlated with increased pituitary FSH secretion (Burger, 1996; Robertson, 1996; 1997). Luteal phase inhibin A and follicular phase inhibin B are correlated inversely with age in perimenopausal women and follicular phase FSH levels (Burger, 1994; Danforth *et al*., 1998; Welt *et al*., 1999; Burger, 2000). Data from women with ovulatory or anovulatory cycles during the perimenopause also support the proposed roles of inhibin and FSH, as women with anovulatory cycles have greater FSH:inhibin ratios compared with the ovulatory group (Landgren *et al*., 2004).

Inhibin and FSH function in the hypothalamic–pituitary–ovarian axis has also been well established with animal models and *in vitro* studies. The biological role of inhibin was first demonstrated using a primary rat pituitary cell culture bioassay (Ling *et al*., 1985; Robertson *et al*., 1985; Farnworth *et al*., 1988; Vale *et al*., 1988). In this *in vitro* bioassay system, treatment with inhibin caused dose-dependent suppression of the FSH content of cultured pituitary cells. Evidence of the suppressive action of inhibin on FSH levels is well documented in rat (DePaolo *et al*., 1981; Sander *et al*., 1986; Fukaya *et al*., 1993), and sheep (Tsonis *et al*., 1986) endocrine systems. The regulatory role of inhibin in folliculogenesis is highlighted with experiments where ewes immunized against inhibin have increased ovulation rates (Findlay, 1993; Forage *et al*., 1987; Findlay *et al*., 2000).

Further evidence for the importance of inhibin in reproductive physiology is evident with the homozygous deletion of the inhibin α subunit gene (*Inha)* in mice (Matzuk *et al*., 1992). The *Inha*$^{-/-}$ mice develop gonadal sex cord tumours within 6 weeks, causing death in males and females in 12 and 17 weeks, respectively. These tumours develop with 100% penetrance; manifesting as Sertoli cell tumours (in males) and granulosa cell tumours (in females) that progress rapidly, resulting in haemorrhaging within the ovary. The development of cachexia, as is generally observed in cancer, occurred in these mice. Indices of cachexia included cellular necrosis and inflammation of hepatic cells, atrophy of mucosal cells in the stomach, development of anaemia and severe weight loss. These indices are thought to occur due to the lack of inhibins and the concomitant elevation of activins; with a 13- and 20-fold increase observed in the male and female, respectively. When the knockout mice were gonadectomized, life expectancy increased. Interestingly, these mice developed adrenal tumours which caused death at 36 and 33 weeks in males and females, respectively. The tissue-specific responses observed in cell growth characteristic of the adrenal and gonads highlight tissue-specific functions of inhibins and activins.

Although it is difficult to determine which factors produce the different effects observed in the *Inha*$^{-/-}$ mice, several experiments demonstrate that it is inhibin which possesses the anti-tumourigenic properties. The liver pathology and cachexia can be attributed to the over-expression of activins in the *Inha*$^{-/-}$ mouse, as this phenotype is also observed in mice given an excess of recombinant activin A (Matzuk *et al*., 1994). However, the additional deletion of the activin type II receptor (ActRIIA) gene in the *Inha*$^{-/-}$ mice does not prevent tumour development but does prevent cachexia. Additionally the transplant of a wild-type ovary into the *Inha*$^{-/-}$ mouse demonstrated the reduction of development of ovarian tumours, suggesting

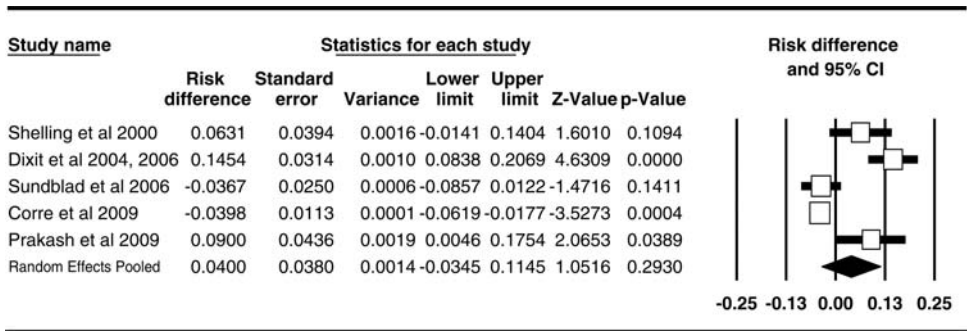

Figure 1 Meta-analysis of *INH G769A* gene variants in POF and controls.

Random effects risk difference (RD) with 95% confidence interval (CI) for the risk of POF in *INHG769A* carriers compared with controls of published studies, were calculated, using the Meta-Analysis V2 software. The RD of each study is marked with a square (□) and the CI displayed as a horizontal line. The pooled RD of all studies is represented with a black diamond (◆), its width marking the range of the 95% CI.

that local activin production is not sufficient for tumour development, but the presence of circulating inhibin (from the transplanted wild-type ovary) is sufficient to stop tumour development (Matzuk *et al.*, 1996). Furthermore, the simultaneous deletion of the inhibin α and FSHβ subunits in mice causes the development of ovarian tumours despite activin levels being lowered (Kumar *et al.*, 1996).

Over-expression of the inhibin α subunit gene in the rat also indicates that inhibin is required for normal ovarian function (Cho *et al.*, 2001). In rats with elevated inhibins, there was a decrease in litter size by 52% and a greater interval between pregnancies observed when compared with the wild-type animal. Although no impairment in fertility was observed in males, in the females, the reduction in fertility was caused by a decreased rate of folliculogenesis reflected by a smaller population of antral follicles, corpora lutea and a 54% decrease in the number of oocytes released/ovulated. These animal studies have confirmed the role of inhibin and FSH in the regulation of follicle growth and development, and can be used to investigate molecular interactions. However, care should be taken not to over-interpret interspecies data.

Hence, the endocrine role of inhibin on the pituitary and its paracrine roles within the ovary support the concept that inhibin plays an important role in the regulation of ovarian function and folliculogenesis, and also raise the possibility that it may be considered to be a potential candidate gene for the development of POF.

The hypothesis that in POF a decline in circulating inhibin levels could result in raised FSH concentrations, increased follicle recruitment and hence an increased rate of follicle depletion is well supported. Indeed, a decline in serum inhibin levels occurs when the stores of ovarian follicles begins to diminish prior to menopause (MacNaughton *et al.*, 1992). This increase in FSH secretion coincides with an enhanced rate of follicular loss during the menopausal transition (Richardson *et al.*, 1987). The hormonal patterns of POF patients also implicate inhibin as important for ovarian function, as women with idiopathic POF have low serum levels of inhibin A and inhibin B, compared with age-matched fertile women (Munz *et al.*, 2004).

Due to the heterogeneity of the condition, diagnosis is difficult and it is important to identify genes that impact ovarian function that can be linked to idiopathic POF. Also it is becoming increasingly important to begin to understand the molecular basis of the disease, so that we might be able to be able to diagnose the disease earlier. In addition, by understanding the molecules involved in the development of the disease, we can then determine more defined avenues for improved treatment for each woman in whom a cause can be found.

Methods

We conducted a MEDLINE search for all reports conducting population analysis of the incidence of the inhibin α subunit mutation (*INHA* G769A) in women with POF. Five studies were included in the analysis of women with primary and secondary amenorrhea and controls (summarized in Fig. 1). Random effects meta-analysis to elucidate an association between the *INHA* G769A mutation and POF was conducted with Comprehensive Meta-Analysis Version 2 software (www.Meta-Analysis.com). The results are presented as risk difference between the two comparison groups.

Results

A genetic link between inhibin and POF

As described above, there is strong evidence that inhibin is important in regulating ovarian function. Hence it is a strong candidate gene for mutational studies in humans. A functional mutation in any one of the three inhibin genes could lead to a decrease in the amount of bioactive inhibin. This loss could remove the negative feedback on the pituitary, cause an increase in FSH levels contributing to premature depletion of follicles and hence result in POF.

The first evidence of a genetic link between inhibin and POF was established with the cytogenetic analysis of a POF patient who had a chromosomal translocation between chromosomes 2 and 15: 46,XX,t(2;15) (q32.3;q13.3) (Burton *et al.*, 2000). As the inhibin α subunit locus is 2q33–36 (Burton *et al.*, 2000), it was important to determine whether inhibin might play a role in the development of POF. Southern blot analysis using an inhibin α subunit gene probe did not indicate the disruption of the gene by the translocation (Shelling *et al.*, unpublished data), however, the possibility still exists that this translocation could disrupt upstream or downstream regulatory elements (as is seen for translocations in the FOXL2 gene which causes POF).

Further investigation was aimed at mutational screening of the inhibin α subunit gene (*INHA*), focusing on the region encoding the inhibin mature peptide (αC), which after translation is proteolytically cleaved from the larger precursor peptide (ProαNαC). A missense mutation *INHA* G769A was identified causing an amino acid transition from alanine to a threonine. In a New Zealand population, a heterozygous change was identified, significantly correlated to the condition (7% in POF, $n = 38$ compared with 0.07% in controls, $n = 150$; $P = 0.011$) (Shelling *et al*., 2000). Significant mutations were not found in the inhibin βA or βB subunit genes (Shelling *et al*., 2000), although a silent transition 1032C>T variant was observed in the *INHβA* gene.

Incidence of the *INH G769A* in multi-ethnic populations

To date six studies have been performed to investigate the association of the *INHA* G769A mutation and POF across various ethnic populations. In this review, we combined the published studies to assess the risk difference between POF and control women carrying the *INHA* G769A mutation. Using random effects modelling, the combined risk difference between the two comparison groups across the studies is 0.04 (95% Confidence Interval (CI) −0.03 to 0.11) (Fig. 1). Two populations demonstrate no association between the *INHA* G769A mutation and the risk of POF (Sundblad *et al*., 2006; Corre *et al*., 2009). However, in the New Zealand and Indian populations there is a positive risk difference due to the *INHA* G769A mutation between POF and control subjects (Fig. 1). Another meta-analysis of this data found a cumulative odds ratio of 1.38 (95% CI 0.48–3.94), and a significant odds ratio of 8.10 (95% CI 1.27–51.6) in the Asian Indian populations (Zintzara, 2009).

There is a higher incidence of mutations in the *INHA* gene in the Asian Indian population (Dixit *et al*., 2004, 2006; Prakash *et al*., 2009). Dixit and co-workers reported 10.5% of the sporadic POF cases ($n = 133$) compared with 0.005% controls carried the *INHA* G769A mutation (Dixit *et al*., 2004, 2006). Of this population, one patient possessed a homozygous mutation. The circulating FSH levels in this patient were the highest recorded within the group studied (three separate measurements of 100, 88 and 85 IU/l). This patient reached menopause at the age of 24 years. Of the 60 women with primary amenorrhoea screened separately for the inhibin α subunit mutation, six were found to be carriers (10%). Shelling *et al*. (2000) report one mutation carrier to have primary amenorrhoea.

Another such study in the Indian population has revealed the presence of the *INHA* G769A and an additional three novel new missense mutations (Prakash *et al*., 2009). The presence of the *INHA* G769A mutation was significantly greater in women with POF although two of the controls were also carriers (Prakash *et al*., 2009). The presence of the *INHA* G769A mutation was associated with early onset of POF in this population, similar to observations of Dixit *et al*. (2004, 2006) and Shelling *et al*. (2000).

The initial study carried out in POF women in the Italian population showed a significant correlation between the *INHA* G769A mutation and POF (sporadic POF 4.5%, $n = 157$) and primary amenorrhoea (25%, $n = 12$) POF women (Marozzi *et al*., 2002). The study also demonstrated a significant link to the mutation in familial POF cases (7.7%). Recently, the study was extended to include a larger cohort of Italian and German subjects and no difference in mutation frequency was observed between in POF and control subjects (Corre *et al*., 2009). Interestingly, the *INHA* G769A mutation is rare in the Korean population where neither POF patients ($n = 84$) or controls ($n = 100$) were identified as carriers (Jeong *et al*., 2004).

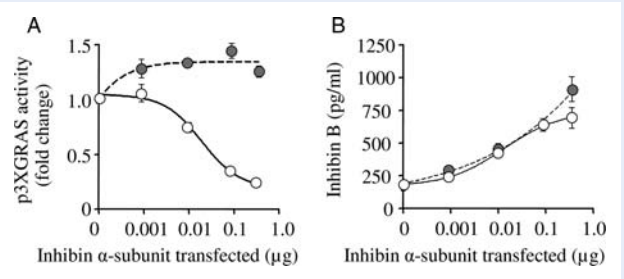

Figure 2 Difference in inhibin bioactivities between wild type and *INH G769A* (A257T) mutant inhibin B (data modified from Chand *et al*. (2007)).

Mouse gonadotroph LβT2 cells were transfected with increasing amounts of wild type or A257T inhibin α subunit expression vector and p3XGRAS-PRL-Luc reporter construct. Transfected cells were treated with 0.5 nM activin. (**A**) Effect of wild type or A257T inhibin on p3XGRAS-PRL-Luc activity, presented as fold change relative to basal reporter activity. (**B**) Dimeric inhibin B levels in conditioned culture media following overexpression of wild-type (○) or mutant (●) inhibin α subunits.

Larger cohort studies with appropriate controls are required to further understand the relationship between the genetic variants in inhibin alpha subunit and POF. The current published studies indicate that in a susceptible population the *INHA* G769A mutation is associated with the early onset of POF.

Functional characterization of the *INHA* G769A mutation *in vitro*

Functional studies of this mutation include over-expression of wild-type or mutant inhibins in gonadotroph and ovarian granulosa cell lines with an activin-sensitive luciferase reporter construct (p3xGRAS), inhibin ELISA and betaglycan binding assays. We discuss these findings in the next two sections.

A gonadotroph cell line, LβT2 was co-transfected with an activin-sensitive luciferase reporter construct (pGRAS-Luc) and increasing amounts of either wild-type or mutant α-subunit. Transfection efficiency was assessed by the measurement of secreted inhibin B (LβT2 cells express inhibin β_B but not β_A) (Fig. 2B). The over-expression of wild-type inhibin α subunit resulted in a dose-dependent decrease in p3xGRAS-Luc activity (Fig. 2A). In contrast, activin-induced p3xGRAS-Luc activity was unaffected by transfection of increasing doses of α mutant DNA, indicating that the A257T inhibin B has compromised biological activity (Chand *et al*., 2007). Subsequent transfection studies in the ovarian tumour cell line COV434 have suggested that the A257T α-subunit mutation has significantly less effect on the inhibin A response. Together, these results suggest that A257T and/or juxtaposed residues (Fig. 3A) are important for inhibin B activity and highlight mechanistic differences between the inhibin isoforms.

How the *INHA* 769G>A mutation, which causes an amino acid substitution at position 257 (position 25 in the mature protein αC), impairs the bioactivity of inhibin B remains to be determined. In the characterization of mutant inhibin A and B *in vitro*, detection using a

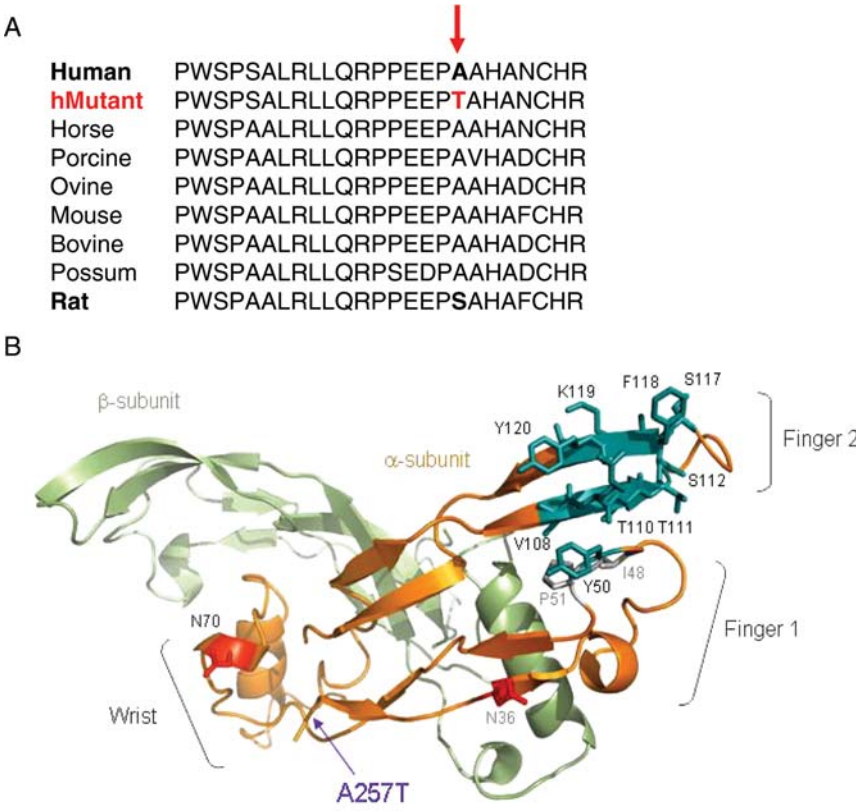

Figure 3 Homology modelling of inhibin A and location of A257T amino acid substitution.

(**A**) Inhibin α amino acid sequence alignment across the various species of region containing the POF inhibin α mutation (Shelling *et al*., 2000). (**B**) Ribbon schematic of a homology model of the inhibin heterodimers (Modified from Makanji *et al*., 2007, used with permission from the American Society for Biochemistry and Molecular Biology.). The inhibin α-subunit is coloured orange whereas the inhibin βA-subunit is green. The position of the A257T α-subunit mutation is indicated relative to: (i) residues that play a role in binding to betaglycan (*cyan*); and (ii) asparagine residues of key glycosylation sites (*red*).

2-site specific ELISA confirms that Thr^{257} is not crucial for the dimerization of the inhibin α and βA or βB subunits. This is to be expected as Thr^{257} is located in the extended N-terminus of the α subunit, well removed from the αβ dimer interface (Fig. 3B).

Is there a physiological consequence of the *INHA* G769A mutation?

As the *INHA* G769A mutation is commonly a heterozygous mutation, it is likely that any consequence this change may have on inhibin biological function would not be a complete loss of function. It is suggested that in mutation carriers, a reduction in inhibin function by 50%, would have effects at two stages: (i) fetal gonadal development; and (ii) in the regulation of normal folliculogenesis and ovulation. A reduction in inhibin biological potency could possibly hinder the normal development of the fetal ovary, continuing to affect ovarian development and function after birth. The elevation of circulating FSH levels as a result of a reduction of the endocrine effect of inhibin may cause ovarian dysregulation, primarily at the follicular development stage. As inhibin has important paracrine effects on the action of activin, GDF9 and BMP15 within the follicles, impairment in inhibin bioactivity could contribute towards aberrant folliculogenesis, maturation and atresia.

Alignment of amino acid sequences across various mammalian species indicates that the *INHA* G769A mutation causes an amino acid substitution in a highly conserved region (Fig. 3A), suggesting this region may encode an important regulatory function of the protein. Currently the three dimensional structures of inhibin α subunit alone and as a dimer with a β subunit have not been produced, however, recently extensive functional analysis has been performed by generating numerous inhibin α subunit mutants. Inhibin mediates its effect principally by binding to betaglycan, this complex then associates with the activin type II receptor to prevent activin binding and suppresses the SMAD signalling pathway.

By generating mutant α subunit proteins, the interaction of inhibin A with its receptor betaglycan has been characterized (Makanji *et al*., 2008). In this study mutations were produced in areas of α subunit protein which when glycosylated reduced its affinity for betaglycan. Residues Val^{108} and Tyr^{120} were found to be crucial for betaglycan interactions and mutation of these residues to alanine reduced affinity by 6- and 8-fold, respectively (Makanji *et al*., 2008). Furthermore these two residues are part of the essential core residues that form the outer convex surface of the protruding, 'finger' motif of the protein including Arg^{109}, Thr^{110}, Thr^{111}, Ser^{112}, Ser^{117}, Phe^{118}, Lys^{119} and Tyr^{120} (Fig. 3B) (Makanji *et al*., 2008).

The consequence of the alanine to threonine substitution due to the *INH*A G769A mutation, does not include a disruption to inhibin dimer formation (Chand *et al*., 2007). We now know that this amino acid substitution occurs in a region that remains exposed after the dimerization of the inhibin α subunit to the inhibin/activin β subunit and does not impact betaglycan interaction (Makanji *et al*., 2007). This raises an interesting question on how else inhibin may be acting. Is the mutation containing region important for an interaction with an inhibin-specific receptor other than betaglycan? The three novel α subunit mutations ($A^{245}D$, $P^{252}H$ and $H^{259}Q$) are in close proximity to Ala^{257}, highlighting the importance of this region for inhibin biological activity.

Impact on the paracrine actions of inhibin

The severity of the POF phenotype in mutation carriers could be compounded by the impairment of the paracrine actions of inhibin within the ovary including steroidogenesis, possibly with the dysregulation of androgen production. Within ovarian theca-interstitial cells, inhibin stimulates LH-dependent androgen production hence promoting follicular recruitment (Hsueh *et al*., 1987; Hillier and Miro, 1993). Furthermore inhibin treatment of ovarian follicles *in vitro* increases estradiol secretion (Hsueh *et al*., 1987; Smyth *et al*., 1994; Garrett *et al*., 2000). This biological effect of inhibin is mediated with its potent antagonism of activin actions, the net consequence being that inhibin is a regulating factor in ovarian steroidogenesis (Hutchinson *et al*., 1987; Hillier, 1991). Other paracrine roles of activin which are opposed by inhibin include granulosa cell proliferation and the potentiating effects of activin and FSH in the ovary to increase FSH receptor expression.

The action of inhibin as an activin antagonist is mediated via competition for the ActRIIs, facilitated by betaglycan (Lewis *et al*., 2000). Betaglycan or TGFβ type III receptor (TGFβRIII), binds to TGFβ1 and TGFβ2 via its endoglin-related domain (Lopez-Casillas *et al*., 1993; Ethier *et al*., 2002) and inhibin binds to the uromodulin-related domain with high affinity (Esparza-Lopez *et al*., 2001). Similar to mechanisms for activin antagonism, the betaglycan–inhibin complex binds to BMP receptor II (BMPRII) competing for BMP binding (Wiater and Vale, 2003). In this manner inhibin could antagonize the paracrine actions of BMPs, a subgroup of growth factors and cytokines from the TGFβ superfamily.

A BMP essential for follicle development to the secondary stages is BMP15 (also known as Growth Differentiation Factor 9B). The earliest role of BMP15 is the initiation of granulosa cell proliferation in primary follicles (Moore *et al*., 2003). Upon further follicular development and with rising FSH levels, the granulosa cells acquire FSHR which stimulates cumulus expansion and the formation of antral cavity (Dong *et al*., 1996). BMP15 also enhances estrogen and progesterone synthesis with the stimulation of aromatase expression and inhibits the premature luteinisation of large follicles (Otsuka *et al*., 2001). The abundance of BMP15 in large antral and Graafian follicles causes the down-regulation of FSHR expression, as a consequence, maintaining a control on progesterone production until ovulation (Otsuka *et al*., 2000). BMP15 also stimulates granulosa cell secretion of Kit Ligand which bind to its receptor c-kit in the oocyte. Kit Ligand actions cause negative feedback on BMP15-dependent granulosa cell proliferation (Otsuka and Shimasaki 2002).

The importance of BMP15 as a fertility regulator is evident in Inverdale and Hanna ewes (Galloway *et al*., 2000). Genetic mutations in the proregions (Y235C, R68W, A180T and 262insLeu have been shown to be associated with POF in an Italian population study (Di Pasquale *et al*., 2004). Additional genetic variations have been shown to be significantly associated with POF in the Indian population (Dixit *et al*., 2006), however no variations were identified in the New Zealand population cohort (Chand *et al*., 2006). A recent German study reported the A180T mutation in the proregion as a polymorphism and did not identify any other BMP15 genetic variations (Ledig *et al*., 2008).

Another TGFβ ligand that signals via BMPRII is GDF9. It is known paracrine roles within the ovarian follicle include (i) granulosa cell proliferation and differentiation (Elvin *et al*., 1999, Eppig, 2001, Gilchrist *et al*., 2006, Mottershead *et al*., 2008) and cumulus expansion, with the induction of hyaluronan synthase 2 (HAS2), cyclooxygenase 2 (COX2), and steroidogenic acute regulator protein (StAR) (Dong *et al*., 2000). Recently missense mutations have been identified in the GDF9 proregion (P103S) (Kovanci *et al*., 2007), mature protein (T238A) (Zhao *et al*., 2007) and S186Y (Laissue *et al*., 2006) in some populations but not others (Chand *et al*., 2006) indicating rare mutations in this gene are linked to POF. The oocyte derived factor, GDF9 is expressed in follicles in the primary stage and onwards, and the deletion of GDF9 renders mice infertile as folliculogenesis is blocked from the primary follicle stage onwards (Elvin *et al*., 2000, McPherron and Lee 1993). The phenotype of a double knockout transgenic mouse, in which the genes encoding inhibin α subunit and GDF9 have been deleted ($Inha^{-/-}GDF9^{-/-}$) (Wu *et al*., 2004) clearly identifies a role for inhibin in the determination of ovulation rates.

The $Inha^{-/-}GDF9^{-/-}$ mouse exhibits an interesting phenotype, where the block in folliculogenesis typically observed in GDF9 knockout mice, is removed. Histological examination of the $Inha^{-/-}GDF9^{-/-}$ mouse ovaries, show the presence of healthy follicles at all stages of folliculogenesis, until the late pre-antral stage, after which there is formation of granulosa cell tumours. This transgenic model demonstrates a vital role of inhibin as a potent modulator of ovarian granulosa cell proliferation. In the absence of inhibin, transgenic mice are observed to have uncontrolled granulosa cell proliferation resulting in ovarian tumours, as observed in $Inha^{-/-}GDF9^{-/-}$ and $Inha^{-/-}$ transgenic mice. Correlating these observations to altered mechanisms in the POF ovary, it is suggested that in carriers of the *INHA* G769A mutation, a reduction in bioactivity of inhibins could allow uncontrolled granulosa cell proliferation leading to abnormal folliculogenesis (Fig. 3).

Thus, the effects of inhibins are numerous and current understanding is that these effects are largely mediated through its antagonist action on activin and BMPs. However new evidence showing differences in bioactivities and betaglycan receptor affinity of inhibin A and B implies the existence of other, as yet unknown, mechanisms of inhibin function (Makanji *et al*., 2008). As inhibin has a potent influence on ovarian function via mechanisms described above, a decrease in inhibin biological activity, due to the *INHA G769A* mutation, could lead to an accelerated loss in follicle numbers, leading to POF (Fig. 4). The decrease in inhibin bioactivity could lead to more potent activin, BMP15 and GDF9 effects resulting in increased number of primordial follicles being recruited for folliculogenesis, enhanced follicular development to antral stages with increased granulosa cell proliferation, cumulus cell differentiation and expansion, and

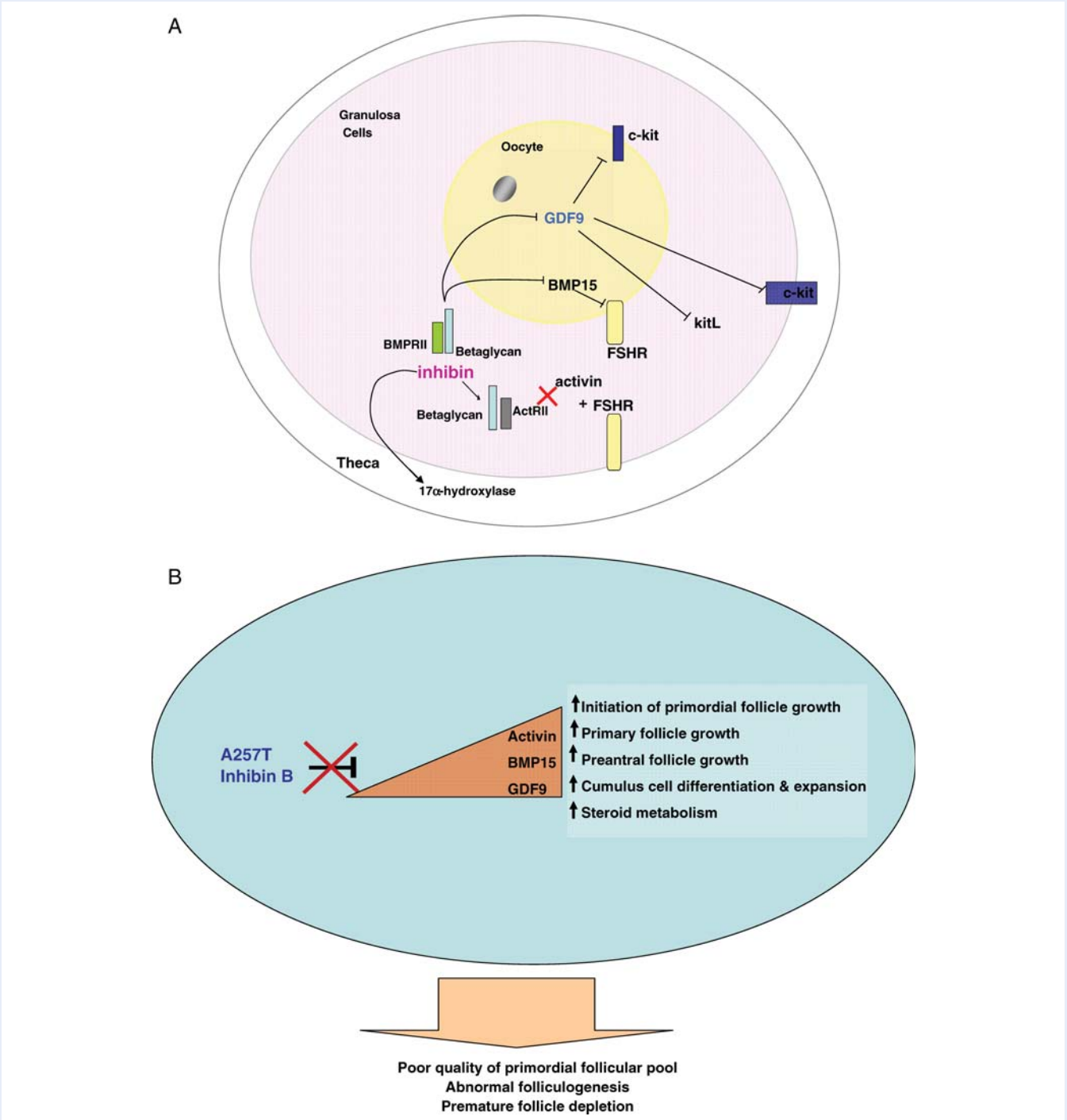

Figure 4 Paracrine roles of inhibin within the ovarian follicle and proposed mechanisms of POF in *INHA* G769A mutation carriers.

(**A**) The effects of GDF9, KitL, BMP15, activin and inhibins and their interactive roles are summarized in this illustration. (**B**) Reduced inhibin bioactivity, due to the *INHA G769A* mutation, could lead to more potent activin, BMP15 and GDF9 effects resulting in increased number of primordial follicles being recruited for folliculogenesis, enhanced follicular development to antral stages with increased granulosa cell proliferation, cumulus cell differentiation and expansion, and a dysregulation in steroid hormone production at the theca and granulosa cell level.

a dysregulation in steroid hormone production at the theca and granulosa cell level. Clearly further study is required to complete our understanding on how the paracrine actions of inhibin are facilitated within the granulosa cell and to recognize the impact of the *INHA* G769A mutation within this cell type (Fig. 4).

Phenotypic heterogeneity among *INHA* G769A mutation carriers

As in many other genetic diseases, the relationship between *INHA* G769A and POF is not simple, and the genetic variant may be

acting more like a susceptibility allele than a disease-associated mutation with 100% penetrance. Studies have shown the occurrence of asymptomatic carriers suggesting incomplete penetrance and/or a multi-genetic cause of POF.

Examples of such cases of genetic heterogeneity are observed in a number of other similar genetic conditions such as retinitis pigmentosa (Kajiwara *et al*., 1994; Waseem *et al*., 2007) where analysis indicates up to 30 separate genes involved, with different modes of transmission. Genetic studies are difficult with infertility disorders due to the inability to ascertain large families over multiple generations, as by definition, infertility within a family will lead to small families. However, using a candidate gene approach many genes have now been identified as causes of idiopathic POF, such as FSHR, FOXL2, NOBOX and FMR1 (Fassnacht *et al*., 2006; Qin *et al*., 2007; Beysen *et al*., 2008; Laissue *et al*., 2008; Simpson, 2008).

In the New Zealand population, the clinical details of the asymptomatic carrier identified were not available as the DNA sample was provided anonymously, so the possibility that this DNA sample was from a female who experienced menopause early cannot be discounted. The second asymptomatic carrier was identified during the genetic assessment of the family of a carrier. In this family, the mother was shown to be an asymptomatic carrier, although her 16-year-old daughter who also carried the mutation was diagnosed with POF. Similarly, in the Marozzi study, the asymptomatic carrier was the sister of the POF subject with the *INHA* G769A change. In this particular family, the other sibling (male) also carried the inhibin mutation. From the two family histories available, the prevalence of unaffected carriers could be a relatively common occurrence in families with a history of POF.

Asymptomatic carriers of the *INHA* G769A mutation occur in a higher frequency in the Italian population (Corre *et al*., 2009) also explaining similar incidence observed in a study of control and pre-eclampsia patients (Ciarmela *et al*., 2005). Additionally asymptomatic mutation carriers are present in an Argentinean study (Sundblad *et al*., 2006).

The presence of *INHA* G769A mutation in the control cohort, in the New Zealand and Argentinean studies, may be due to inappropriate selection of samples. In the New Zealand study, controls included males and females whereas Sundblad *et al*. report six of the eight mutation carriers found in the control population were under the age of 40 years. Similarly the two asymptomatic controls in the Indian study were under the age of 40 years (Prakash *et al*., 2009). Population-control replaced with phenotype-specific controls where women known to have experienced menopause at the normal age of onset of $\geq$50 years of age may be a more appropriate control for these studies.

From all the population data available it appears that the Indian population is most profoundly affected by the *INHA* G769A mutation. Can this observation complement existing understanding that genetic variability is confounded by ethnic-dependent factors?

The age of onset of POF in carriers of the mutation varies amongst different population groups. This suggests that mutation carriers within some ethnicities may be at greater or lower risk of developing POF, due to other genetic or environmental influences. Furthermore the age of onset of menopause in the New Zealand and Indian population is considerably lower (13.6 years earlier) compared with that of Italian women who carried the *INHA* G769A mutation. The incidence of menopause before the age 25 years in the New Zealand and Indian carrier population was 100 and 90%, respectively, although in the Italian POF population, none of the carriers experienced menopause before the age of 30 years. In POF patients who were carriers of the *INHA* G769A mutation, approximately 30% experienced primary amenorrhoea.

Can phenotypic heterogeneity among *INHA* G769A mutation carriers be related to ethnic-dependent factors?

Is the Indian population most profoundly affected by the *INHA* G769A mutation due to ethnic-dependent factors? A recent analysis of pregnancy outcomes in polycystic ovarian syndrome (PCOS) patients using IVF techniques supports variability between South Asian (Indian) and Caucasian populations. In terms of clinical pregnancy outcomes, Caucasian PCOS patients had a 2.5 times higher chance of ongoing pregnancy compared with South Asian PCOS patients (Palep-Singh *et al*., 2007). This discrepancy was attributed to increased sensitivity among South Asians to gonadotrophin stimulation which resulted in a greater change in FSH levels. According to this study increase in basal FSH concentration by one unit reduced the odds of pregnancy by 18.6%. Lower rates of pregnancy outcome are also observed in Asian and African American women compared with Caucasian women who undergo IVF treatment (Feinberg *et al*., 2006; Purcell *et al*., 2007).

A similar mechanism could explain variable disease phenotypes in *INHA* G769A mutation carriers among the South Asian POF population. A decline in inhibin bioactivity would cause increased secretion of FSH; and in a susceptible population, a small variability in FSH levels but a heightened sensitivity to gonadotrophin stimulation would explain the earlier onset of POF observed in this population group. A subtle change in FSH levels in a hypersensitive system could account for increased follicular loss, likely through aberrant folliculogenesis and increased atresia.

The degree of variability in ovarian FSH responsiveness is observed in normal and anovulatory infertile women undergoing gonadotrophin induction of ovulation (Imani *et al*., 2002). Factors such as body mass index, serum leptin levels, menstrual cycle history (oligomenorrhea versus amenorrhea), ovarian response to clomiphene citrate medication, initial serum FSH levels, free IGF-I and IGFBP-1 levels are significantly correlated with individual FSH response dose. It has also been shown that follicular growth is dependent on the duration of FSH elevation above a critical threshold rather than the degree of elevation (Schipper *et al*., 1998). Further evidence is provided from a clinical case report where a small increase in FSH levels caused ovarian hyperstimulation, in a 37-year-old woman with an autonomous FSH-secreting pituitary adenoma. She presented with irregular cycles, and multiple ovarian cysts (Djerassi *et al*., 1995). Observations during IVF procedures in older women demonstrate that more follicles are recruited in the form of multiple small follicular cysts (Romeu *et al*., 1987) and an increased tendency for dizygotic twinning occurs in older mothers (Gilfillan *et al*., 1996). It can be postulated that the amount of FSH required varies greatly because of individual variation in ovarian FSH sensitivity or the FSH threshold. This accounts for variability in response during gonadotrophin stimulation in IVF procedures in relation to ethnicity or ageing. These studies illustrate the importance

of FSH as a critical initiator of the progression of small responsive follicles to the antral stage and subsequent maturation towards the preovulatory stages. During this time ovarian factors work in concert to establish the follicular microenvironment necessary for successful ovulation. It is suggested that in certain populations, for example, in the East Asian population who appear to respond differently to FSH than Caucasians, the presence of the inhibin mutation resulting in impaired inhibin activity may have more pronounced effects on ovarian function.

Genetic variation in INHA as a susceptibility gene for POF

During the screening of the inhibin α promoter region, a C>T polymorphism was identified, and we showed that the promoter variant c.-16C>T was significantly under-represented in POF patients, and has thus been suggested to protect women from developing POF (Harris *et al*., 2005). A further *INHA* promoter variant c.-124A>G was found to be co-ordinately inherited with a highly variable TG repeat element at −300 bp, and several haplotypes were identified that differ in repeat element length (between 76 and 94 bp) and sequence. The *INHA* promoter haplotype C has the shortest repeat length and was found to be in linkage disequilibrium with the -16T allele (Harris *et al*., 2005). In further studies, we were able to conclude that *INHA* promoter variants, specifically haplotype C, are associated with the development of POF (Woad *et al*., 2008). The C haplotype is significantly underrepresented in patients (15/118; 12.7%) compared with controls (50/238; 21%) ($P = 0.037$, Fisher's exact test). It is unclear whether these polymorphisms in the *INHA* promoter might result in reduced inhibin expression, but this indicates that promoter polymorphisms may be another mechanism for the transcriptional regulation of the inhibin α subunit (Woad *et al*., 2008). Therefore, we suggest that variants within the inhibin gene cause it to act as a susceptibility allele, whereby women who carry this particular variant more likely to develop POF.

Conclusions

POF is a common condition and is of growing concern to the general population due to the increasing trend of delaying pregnancy. The use of hormonal contraceptives often conceals this condition until the woman decides to stop the contraception regime, in preparation to have a child. Diagnosis is often delayed due to an extensive variability in symptoms and no predicting factors for idiopathic POF, the largest subset of aetiology. The issues regarding personal health and quality of life of the affected women with the occurrence of menopause at a young age is also of concern. Hence there is a clear need for improved diagnostic screening before the onset of disease.

The identification of an autosomal mutation in the inhibin α subunit gene (*INHA* G769A) that is significantly linked to POF is of benefit in terms of improving our understanding of the role of inhibin in the regulation of ovarian biology and fertility. Although the reduction of inhibin B bioactivity by the *INHA* G769A mutation is clearly not the only cause for the condition, evidence suggests that this change may serve as a susceptibility factor, increasing the likelihood of POF. Other predisposing factors including other genes, ethnicity and lifestyle factors will likely play a role, but at the moment, the roles of these factors remain largely unknown. Furthermore these studies contribute to the overall understanding of oligogenetic diseases such as POF.

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