Anti-Müllerian hormone and polycystic ovary syndrome: a mountain too high?

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Abstract

Anti-Müllerian hormone (AMH) was initially thought to be produced solely by the foetal male during sexual differentiation to promote regression of the Müllerian ducts. Over the last decade, however, a new and interesting role has emerged for AMH in the ovary. In human ovaries, AMH is produced by granulosa cells from 36 weeks of gestation until menopause, with the highest expression being in small antral follicles. AMH production gradually declines as follicles grow; once follicles reach a size at which they are dominant, it has largely disappeared. Its removal from these larger follicles appears to be an important requirement for dominant follicle selection and progression to ovulation as AMH has an inhibitory role in the ovary, reducing both primordial follicle initiation and follicle sensitivity to FSH by inhibition of aromatase. It is for this reason that AMH is a focus of interest in polycystic ovary syndrome (PCOS). Serum levels are doubled, and granulosa cell production is greatly increased. Interestingly, there appear to be two groups of women with PCOS who can be distinguished by their AMH level: one group consists of those who have high levels which do not reduce with treatment and who respond less well to induction of ovulation, and a second group consists of those in whom the level is less elevated and reduces on treatment and who seem to respond rather better. Understanding the reason for the raised AMH in PCOS may give clues as to the mechanism of anovulation. To conclude, AMH appears to have a major inhibitory role during folliculogenesis, which may contribute to anovulation in PCOS.

Introduction

Anti-Müllerian hormone (AMH), also known as Müllerian-inhibiting substance, is a member of the transforming growth factor-β (TGFβ) superfamily, which includes more than 35 structurally related peptides including activins, inhibins, bone morphogenetic proteins (BMPs) and growth differentiation factors. Many of these are involved in the reproductive function of both sexes (Itman et al. 2006, Knight & Glister 2006). The AMH gene is located on chromosome 19 (Cohen-Haguenauer et al. 1987, Cate et al. 1990) and encodes a 140 kDa dimeric glycoprotein. AMH is synthesised as a pro-hormone, which undergoes cleavage at the site of action to generate a biologically active C-terminal fragment (Pepinsky et al. 1988, Wilson et al. 1993, Rey et al. 2003).

Members of the TGFβ superfamily exert their effects through serine/threonine kinase receptors. AMH acts on its own specific type II receptor, AMHR2 (Imbeaud et al. 1995, Visser et al. 1995), to signal through a BMP-like pathway, recruiting one of the type I receptors; ALK 2, 3 or 6. Downstream signalling of the AMH receptor involves cytoplasmic effectors known as receptor-related SMAD proteins (R-Smads 1, 5 and 8) and a common SMAD4 protein (Visser 2003). Once AMH binds to AMHR2, the type I receptor becomes recruited forming a receptor complex. This results in activation of the type I receptor, which causes phosphorylation of R-Smads. These proteins bind to the common SMAD4 protein, resulting in translocation of the complex into the nucleus and binding directly to the DNA to regulate gene expression or interacting with other DNA-binding proteins (Massague & Wotton 2000).

AMHR2 is essential for signalling was demonstrated by the fact that its disruption in mice caused persistence of the Müllerian ducts (Mishina et al. 1996, di Clemente et al. 2003). In humans, mutations of either the AMH or the AMHR2 gene are the cause of persistent Müllerian duct syndrome (Josso et al. 2005).

Ovarian AMH

Bioactive AMH was first detected in granulosa cells in the 1980s (Vigier et al. 1984). It was later reported that AMH was produced from 36 weeks of gestation in human GCs (Rajperts-de Meyts et al. 1999) and was expressed until menopause. Many studies followed demonstrating AMH expression in rats (Ueno et al. 1989, Baarends et al. 1995), mice (Munsterberg & Lovell-Badge 1991), sheep (Sweeney et al. 1997) and...
human (Weenen et al. 2004, Stubbs et al. 2005, Modi et al. 2006) ovaries using in situ hybridisation or immunostaining. It is still unclear precisely when during folliculogenesis AMH expression begins with the studies on primordial follicles producing equivocal results (Stubbs et al. 2005, Modi et al. 2006), but it is clear that the highest expression of AMH is found in preantral and small antral follicles. The latter being those involved in FSH-dependent cyclic recruitment (Ueno et al. 1989, Baarends et al. 1995, Weenen et al. 2004). After selection, the level of expression gradually declines in the mural GCs with the AMH-positive staining becoming localised to the cumulus GCs (Munsterberg & Lovell-Badge 1991). Direct measurements of AMH protein production by human GCs and follicular fluid in 2007 confirmed that the highest concentrations were in small antral follicles and became very low or undetectable in follicles ≥10 mm (Pellatt et al. 2007a), as shown in Fig. 1. The cessation of production of AMH from these follicles suggests that this is an important requirement for selection of the dominant follicle.

Neither AMH staining nor AMH mRNA expression was observed in oocytes, corpus luteum, atretic follicles or theca cells in mice, rats or human ovaries (Ueno et al. 1989, Baarends et al. 1995, Weenen et al. 2004, Modi et al. 2006), confirming that the GCs are the only source of AMH in the ovary.

AMHR2 was shown to have a similar pattern to AMH mRNA expression in rodent follicles (Baarends et al. 1995), in which expression of both was colocalised in the GCs of preantral and small antral follicles. Interestingly, the mRNA for the AMH receptor was also found in the theca from a range of follicle sizes as well as in GCs and granulosa-luteal cells (GLCs) from human ovaries (Ingraham et al. 2000, Hanna et al. 2006). The effects of AMH on theca function are yet to be determined, but this poses the possibility of a new signalling pathway between granulosa and theca in the developing follicle.

A reliable marker of ovarian function?

In females, AMH has been heralded as a marker of ovarian ageing and reserve in humans (Van Rooij et al. 2002, de Vet et al. 2002). In mice, the higher production of AMH by small antral follicles reflected the remaining follicle pool (Kevenaar et al. 2006). Women of <25 years of age had higher serum AMH concentrations than those aged 35 years and above (Pilotonen et al. 2005), and when women were followed longitudinally for a period of between 1 and 7 years, there was a decrease in serum AMH levels, with levels becoming undetectable when menopause was reached (Pilotonen et al. 2005).

It has also been suggested that AMH may be a better predictor for successful IVF treatment and oocyte maturation than traditional markers (Muttukrishna et al. 2005, Silberstein et al. 2006, La Marca et al. 2007). A lower serum AMH concentration preceding or during assisted reproductive techniques was strongly associated with reduced oocyte yield and low oocyte quality (Silberstein et al. 2006, La Marca et al. 2007). This might be expected if serum AMH concentrations produced by the small follicles indirectly reflect the remaining follicle pool. Ironically, the opposite appears to be true for successful treatment of infertility in women with polycystic ovary syndrome (PCOS), in that those who had the highest concentrations of AMH seemed to respond less well. This is discussed in detail below. In terms of predicting outcome, a recent systematic review of possible predictors of outcome of IVF has found that any tests of ovarian reserve, including AMH, had only ‘modest-to-poor’ predictive properties and were unsuitable as a basis for clinical decision making (Broekmans et al. 2006). Understanding the importance of AMH in normal ovarian function may assist in improving treatment regimens and in improving success rates in the future; however, it appears that at least at the moment, these tests are insufficiently robust as a basis for clinical decision making.

Function of AMH in the normal ovary

Some of the most informative studies examining AMH action in the ovary have been performed by knocking out AMH or its receptor. AMH knockout (AMHKO) mice (Durlinger et al. 1999, 2001, 2002) are fertile, but have an increase in the number of growing follicles resulting in depletion of the primordial pool and early cessation of ovulation (Durlinger et al. 1999); an effect which was reversed by culture of ovaries from 2-day-old mice with AMH (Durlinger et al. 2002). These results were confirmed by culture of mouse AMHR2-null or

Figure 1 AMH concentration in GC and follicular fluid from normal ovaries: left panel: AMH production was measured in the medium conditioned by GCs from 14 follicles (range 4–19 mm) collected from 12 individual patients. Levels of AMH declined as the follicle size decreased with very low or undetectable levels of AMH in follicles ≥10 mm (Pellatt et al. 2007a), as shown in Fig. 1. The cessation of production of AMH from these follicles suggests that this is an important requirement for selection of the dominant follicle.

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wild-type ovaries beneath the chorioallantoic membrane of chick embryos (‘in ovo’). In this position, the pieces of tissue become vascularised thereby preventing the normal loss of follicles which occurs in culture. There was an increase in follicle growth compared to wild-type in those pieces lacking AMH receptor (Gigli et al. 2005).

In human tissue, the picture is rather less straightforward. In cultured human ovarian cortical biopsies, AMH treatment (100 ng/ml) reduced primordial follicle growth compared to untreated tissue in one study (Carlsson et al. 2006), whereas in another study, AMH actually increased the numbers of primordial follicles growing (Schmidt et al. 2005). The latter study used a higher dose of AMH, and the tissue was previously cryopreserved which may account for the difference.

In an effort to clarify the stage of follicle at which AMH may be able to act, we looked for the presence of AMHR2 in individual preantral human follicles and found that it was rarely expressed (Rice et al. 2007). This indicates that AMH may not have a role at this stage of follicle development in humans. The expression of AMHR2 in rodent preantral follicles (Baarends et al. 2005) and the effect by AMH observed by McGee et al. (2001) on preantral growth indicate that AMH acts on its receptor in these small follicles in the rodent. This suggests that there is a species difference in the expression of AMHR2.

In antral follicles, the overall effect of AMH is to reduce follicle sensitivity to FSH. A number of in vitro studies have demonstrated similar findings, in that FSH-stimulated follicle growth was inhibited by the addition of AMH. In rat GCs, FSH- and cAMP-stimulated aromatase activity was significantly reduced after AMH treatment (di Clemente et al. 1994). In the same report, AMH reduced aromatase mRNA expression in cAMP-stimulated cells and also reduced LH receptor mRNA expression in porcine GCs stimulated with FSH (di Clemente et al. 1994). Even in the low FSH environment of the AMHKO mouse, there was an increase in the number of growing follicles compared to wild-type littermates (Durlinger et al. 2001). An inhibitory effect of AMH on FSH-stimulated aromatase mRNA expression and oestradiol (OE₂) production has also been shown in human GLCs (Grossman et al. 2007). Our preliminary data in humans support these reports, in that AMH, in the presence of FSH or LH, significantly reduced aromatase mRNA expression and activity in GCs from small (4–9 mm) and large (≥10 mm) follicles respectively (Pellatt et al. 2007b). These studies collectively demonstrate an inhibitory role of AMH in antral follicle growth, and it can be envisioned that high concentrations of AMH in small antral follicles would hold back FSH responsiveness and steroidogenesis and acquisition of LH receptors until the time of follicle selection. By the time the intercycle rise in FSH has occurred, AMH production ceases, concentrations fall and the follicle is ‘released’ to produce OE₂.

Intriguingly, the factor causing inhibition of AMH production in these selected follicles remains unknown and, in our hands at least, it is not the obvious candidate, FSH itself (Pellatt et al. 2007a). Discovery of this inhibitor is important as it may provide a clue as to why AMH production is high in PCOS.

There is one other important question which arises regarding the role of AMH in these small follicles. It is well described that AMH causes regression of the Müllerian duct by inducing cellular apoptosis (Roberts et al. 1999). It is therefore interesting to speculate as to why the high concentrations in small follicles are not similarly damaging. In the Müllerian duct, atresia occurs in a pattern from cranial to caudal following the AMHR2 gradient. We have previously shown the presence of the receptor in both theca and granulosa of small healthy follicles, so why do these follicles similarly not undergo atresia due to cellular apoptosis? Is it possible that in ovarian cells the requisite intermediary pathways are absent? The only study to date to our knowledge investigating the effect of AMH on ovarian cell growth did show that it was in fact decreased (Kim et al. 1992), and this is hard to reconcile with the known increase in granulosa and theca cell division which occurs in small follicles. Figure 2 illustrates how AMH may modulate ovarian follicle development. Research into the role of ovarian AMH and the expression pattern of the receptor clearly needs to address this issue, particularly in light of the high concentrations found in PCOs.

**AMH and PCOS**

PCOs are characterised by an increase in follicle number, and this increase has been shown to occur at the earliest stages. The previously demonstrated ability of AMH to alter early follicle growth therefore made it a candidate for causing this change. In order to investigate this possibility, the expression of AMH in fixed stained sections of PCOs from anovulatory or ovulatory women was determined by immunostaining (Stubbs et al. 2005). The percentage of positively stained primordial and transitional follicles from anovulatory ovaries was significantly lower than from normal and ovulatory PCOs (ovPCOs); however, the intensity of staining in the preantral and antral follicles was similar among all three groups (Stubbs et al. 2005). This suggests that the absence of AMH in anovulatory PCOs (anovPCOs) at the early stage of follicle development may allow for an increased number of follicles to initiate growth. It is perhaps the later appearance of theca that may increase the production of AMH in these follicles, but until the factors controlling AMH production are determined, this will remain speculation.

AMH has been shown to be two- to three-fold higher in serum from women with PCOS than in women with normal ovaries (Fallat et al. 1997). This was initially thought to be due to the increase in the number of small...
AMH as a regulator of normal follicle growth and development: AMH production in preantral follicles is variable, but has been detected from the primary stage onwards. It is unclear whether preantral follicles express AMHR2. AMH production by surrounding larger follicles is thought to inhibit primordial follicle initiation by a paracrine action. AMH production by granulosa cells (pink layer) increases to the small antral stage by an unknown mechanism. AMH may ‘fine tune’ follicle development by inhibiting early maturation of these follicles. It may reduce follicle sensitivity to FSH, thereby inhibiting aromatase mRNA expression and activity. The effects on cell proliferation are uncertain, but it does not clearly have the apoptotic effect seen in the Müllerian duct during differentiation. AMHR2 has been detected in theca (blue layer) from a range of follicle sizes, but its actions are unknown. As the follicles develop and grow, AMH levels decline, but the causative factor/s remains to be discovered. The decrease in AMH then releases the inhibitory effect, allowing these larger follicles to become responsive to FSH, and stimulating aromatase and oestradiol production.

The cause of the increased AMH production in PCOS is unknown; however, in 2007, we demonstrated that AMH production was on average 75 times higher per granulosa cell from anovPCOs than from cells from normal ovaries (Fig. 3). Similarly, concentrations of AMH were found to be five times higher in follicular fluid from unstimulated follicles from women with anovulatory PCOS compared to women who were ovulatory (Das et al. 2008). In a further study, the serum concentration of AMH correlated with the severity of symptoms, with again the ovulatory group having lower concentrations than those who were equally hyperandrogenic but anovulatory (Piouka et al. 2009). Interestingly, follicle number only added 5.3% to the variance in the concentration of AMH. The fact that raised AMH production was an intrinsic property of granulosa cells in PCOS was later confirmed by the finding of raised levels of AMH mRNA in these GCs, even after stimulation for IVF (Catteau-Jonard et al. 2008). Together, these studies demonstrate that the increase in AMH concentration is largely due to the increase in production of AMH by each follicle and not just a consequence of an increase in follicle number.

Figure 2 AMH as a regulator of normal follicle growth and development: AMH production in preantral follicles is variable, but has been detected from the primary stage onwards. It is unclear whether preantral follicles express AMHR2. AMH production by surrounding larger follicles is thought to inhibit primordial follicle initiation by a paracrine action. AMH production by granulosa cells (pink layer) increases to the small antral stage by an unknown mechanism. AMH may ‘fine tune’ follicle development by inhibiting early maturation of these follicles. It may reduce follicle sensitivity to FSH, thereby inhibiting aromatase mRNA expression and activity. The effects on cell proliferation are uncertain, but it does not clearly have the apoptotic effect seen in the Müllerian duct during differentiation. AMHR2 has been detected in theca (blue layer) from a range of follicle sizes, but its actions are unknown. As the follicles develop and grow, AMH levels decline, but the causative factor/s remains to be discovered. The decrease in AMH then releases the inhibitory effect, allowing these larger follicles to become responsive to FSH, and stimulating aromatase and oestradiol production.

Figure 3 Comparison of AMH concentration from normal ovaries, ovPCOs and anovPCOs: AMH production was measured in the medium conditioned by GCs isolated from follicles ranging from 2 to 10 mm in size from normal ovaries (n=7), ovPCOs (n=9) and anovPCOs (n=5). AMH production was significantly different between normal, ovPCOs and anovPCOs. Mean concentration of AMH in GCs from anovPCOs was 75 times higher than the mean for normal ovaries. Note the log scale. (Graph reproduced, with permission, from Pellatt L, Hanna L, Brincat M, Galea R, Brain H, Whitehead S & Mason H 2007 Granulosa cell production of anti-Müllerian hormone is increased in polycystic ovaries. Journal of Clinical Endocrinology and Metabolism 92 240–245. Copyright 2007, The Endocrine Society).
testis, androgens actually inhibit AMH production (Rey et al. 1993), so a different control mechanism would have to be present in the ovary for androgens to cause the rise seen in PCO.

Another candidate for the cause of the increase in AMH in PCOS is insulin. Hyperinsulinaemia is known to affect anovulatory women more than ovulatory women (Conway & Jacobs 1993), and falling insulin concentrations do correlate with the return of ovulatory cycles (Dunaif et al. 1988). La Marca et al. (2004a) found no correlation between serum AMH and androgen levels, but did observe a direct correlation between AMH and insulin insensitivity. Insulin has been shown to enhance gonadotrophin-stimulated steroid production in GCs and theca (Willis et al. 1996); therefore, the raised AMH concentrations may be secondary to an effect of insulin on androgen levels. Although this is a possible cause, other studies have failed to find a direct correlation between insulin and AMH concentrations (Pigny et al. 2003, Eldar-Geva et al. 2005), and even when insulin levels have reduced with treatment, a fall in serum AMH has not followed directly (Bayrak et al. 2007, Carlsen et al. 2009). It is possible that there is an intrinsic over-expression of the AMH gene causing the raised production of the protein in the PCO or that the currently unknown factor driving the androgen production also increases AMH. Discovering the answers to these questions may have important implications for the treatment of this condition.

**AMH and response to treatment in PCOS**

One interesting and important finding from our study was that women with PCOs could be clearly divided into ovulatory or anovulatory by identifying whether or not their GCs were ‘low’ or ‘high’ AMH producers. In women with PCO but regular cycles, AMH production was still significantly higher than normal; however, GCs from anovPCOs produced on average 18 times more AMH than GCs from ovPCOs (Fig. 3). The results were tightly grouped with no overlap (Pellatt et al. 2007a). Is it possible therefore that in order to begin ovulating the GC production of AMH in follicles in anovPCOs has to be greatly reduced? A partial answer to this question has come from the results of some recent publications regarding response to treatment in women with PCOS. One study showed that pre-treatment of AMH was a reliable predictor of reproductive response to weight loss (Moran et al. 2007), in that although the degree of weight loss was similar, it was only those women with lower AMH who responded with an increase in the number of ovulatory cycles. This was followed by an investigation of the reverse phenomenon, i.e. whether improvement in reproductive function was accompanied by reduced AMH (Thomson et al. 2009). Weight loss did improve reproductive function, but again only in those patients who already had significantly lower serum AMH at the start and in neither group did the weight loss result in a reduction in AMH. We are therefore hypothesising that in this group of women with PCOS and chronic anovulation, the high GC production of AMH does not reduce and is preventing a response to weight loss treatment. It is only in those with less elevated AMH that the ovary can be coaxed into overcoming its inhibitory effects (Fig. 4).

Is this hypothesis supported by the results of other treatments for induction of ovulation? The data are rather scarce; however, in one study, the response to clomiphene citrate in obese patients with PCOS was again dependent on initial AMH concentration (El-Halawaty et al. 2007). Unfortunately, AMH response during treatment was not measured in this study. With regards to metformin treatment, there are a number of studies that have correlated reduced AMH with responsiveness; however, the AMH measurement has been made during or after treatment as opposed to correlating pre-treatment levels with subsequent response.

This hypothesis may appear to be contradicted by the number of studies indicating that high AMH is a positive predictor of outcome of IVF; however, the majority of these studies have not focussed on women with PCOS and involve women with different or non-specified ovarian morphology. When the concentrations of AMH were measured in follicular fluid collected at the time of oocyte retrieval for IVF only from women with PCOs, the results were very interesting. Although again AMH was higher compared to ovulatory women, the concentrations in both small and large follicles were found to be lower in those women who began a pregnancy (Desforges-Bullet et al. 2010). This indicates that even following a stimulation protocol, it is those women with PCOS producing the relatively lower levels of AMH who have the best outcome.

Although metformin is now one of the most common treatments for PCOS, to our knowledge, there are no studies in which the initial concentration of AMH has been correlated with response to treatment, but it is clear that AMH concentrations do fall during treatment, even if this does take some time. Certainly over the course of a single week, metformin did not reduce the serum AMH level despite there being a reduction in antral follicle number (Bayrak et al. 2007). This is not surprising given that in other studies it was not until after 4, 6 or 8 months of metformin treatment that AMH levels fell (Fleming et al. 2005). It was assumed that the time taken reflected the growth of a new cohort of follicles from the earliest stages, and that these follicles had developed in a partially normalised endocrine environment. One problem with interpreting AMH levels in serum is that it is only a reflection of the total ovarian output, and this will depend on both the follicle number and size distribution given that production is highest in the small antral follicles.
One of the most reliable methods of induction of ovulation in PCOS is FSH treatment. Women treated with recombinant human FSH to induce ovulation had lower serum concentrations of AMH after treatment (LaMarca et al. 2004b), again indicating that reducing AMH is an essential part of the response. Indeed, it has generally been anticipated that FSH would be the factor responsible for the reduction in AMH production seen following follicle selection in natural cycles. Indeed, we observed a significant reduction in concentrations of AMH protein in the conditioned medium from GCs from PCOS which had been treated with FSH. Surprisingly, however, this was not seen in cells from normal ovaries (Pellatt et al. 2007a). Curiously, acute stimulation with FSH (24 h) in women with PCOS had no effect on serum AMH concentrations (Wachs et al. 2007). An earlier in vitro study in mice demonstrated a reduction in AMH in response to FSH and OE2 (Baarends et al. 1995). It may be possible that it is not FSH per se that is affecting AMH levels, but it is the FSH-stimulated OE2 production, and this effect may take a little longer to become evident. In support of this, a number of groups have shown a negative correlation between OE2 and serum AMH concentrations in women with PCOS (Fallat et al. 1997, LaMarca et al. 2004a); however, OE2 production by the follicle is the end point of many processes and factors, and again these results are difficult to interpret. It seems that there is a fine balance between AMH production and follicle sensitivity to FSH, which will need to be examined further. If this was the case, then it could be seen that those women with very high intra-follicular concentrations of AMH may be prevented from inducing the aromatase and subsequently the OE2 which is the very important thing required to reduce the AMH. One further piece of information which adds to the puzzle is that AMH itself was able to stimulate FSH b-subunit mRNA expression in a pituitary cell line (Bedecarrats et al. 2003). It is difficult to reconcile this with anovulation in PCOS because, if this is the case, why do women with high serum AMH concentrations not have higher FSH levels also? There are clearly a number of research questions which remain to be answered.

**Conclusion**

AMH has an inhibitory role in the ovary, and the increased production by GCs from anovPCOS may therefore contribute to cessation of follicle development. It appears that a decrease in AMH is an essential part of reproductive response to treatment and that those women with the highest concentrations have the worst outcome. The evidence has led us to hypothesise that there is a subgroup of women with PCOS who have elevated levels of AMH and who are the most resistant to treatment. AMH is unlikely to be the sole cause of anovulation, but its effects on aromatase expression and
Oestradiol (E2) production suggest that it is involved in follicle growth and selection, and that very high concentrations actually prevent the normal process of removal of the AMH ‘break’ from the follicle. The function and regulation of the production of AMH in the normal ovary warrant further investigation if we are to unravel the complexities of its action in PCOS.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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