Molecular Human Reproduction Vol.10, No.2 pp. 77–83, 2004 DOI: 10.1093/molehr/gah015

Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment

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Anti-Müllerian hormone (AMH) is a member of the transforming growth factor-β superfamily, which plays an important role in both ovarian primordial follicle recruitment and dominant follicle selection in mice. However, the role of AMH in folliculogenesis in humans has not been investigated in detail. In the present study, AMH expression was assessed using immunohistochemistry in ovarian sections, obtained from healthy regularly cycling women. To this end, a novel monoclonal antibody to human AMH was developed. AMH expression was not observed in primordial follicles, whereas 74% of the primary follicles showed at least a weak signal in the granulosa cells. The highest level of AMH expression was present in the granulosa cells of secondary, preantral and small antral follicles ≤4 mm in diameter. In larger (4–8 mm) antral follicles, AMH expression gradually disappeared. In conclusion, in the human AMH expression follows a similar pattern as compared to the mouse and rat, suggesting an important role of AMH in folliculogenesis.

Key words: Anti-Müllerian hormone/folliculogenesis/primordial follicle recruitment

Introduction

Anti-Müllerian hormone (AMH), also referred to as Müllerian inhibiting substance (MIS), is a transforming growth factor-β family member which is involved in regulation of folliculogenesis (Grootegoed *et al*., 1994; Baarends *et al*., 1995). During male fetal sex differentiation, AMH is synthesized by testicular Sertoli cells and induces degeneration of the Müllerian derivatives that form the anlagen of the oviducts, the uterus and the upper part of the vagina (Josso *et al*., 1977, 1998). During mouse and rat female fetal development, no ovarian AMH activity can be detected, but AMH mRNA expression is present in ovarian granulosa cells as early as day 4 after birth, coinciding with the initiation of primary follicle growth (Ueno *et al*., 1989a,b; Durlinger *et al*., 2002a). Immunohistochemistry and mRNA *in situ* hybridization (ISH) studies in rodents and sheep revealed specific expression of AMH in granulosa cells of early growing, preantral and small antral follicles, whereas the signal was lost in non-atretic large antral follicles and all atretic follicles (Bezard *et al*., 1987; Baarends *et al*., 1995). In human fetal and neonatal ovarian tissue, AMH expression is not detected before 36 weeks of gestation (Rajpert-De Meyts *et al*., 1999). Data on postnatal expression are lacking.

Follicle growth and differentiation is a complex process. The initiation of growth and early differentiation appears to be regulated independently of stimulation by gonadotrophins as indicated by the presence of preantral follicles in FSH knockout (FSHKO) mice. At later stages, growth and differentiation and the selection of the cohort are largely dependent on FSH activity (Fauser and van Heusden, 1997). Two important regulation steps can be identified (McGee and Hsueh, 2000); the initiation of growth of follicles from the primordial pool (initial recruitment) and rescue of the growing follicles from atresia (cyclic recruitment). The gonadotrophin FSH is an essential factor in cyclic recruitment as indicated by the absence of antral follicles in ovaries of FSHKO mice (Kumar *et al*., 1997; Durlinger *et al*., 2001) and absence of large antral follicles in hypophysectomized women (Schoot *et al*., 1992). Some of the factors involved in the regulation of the initiation of growth of primordial follicles (initial recruitment) have been identified and include factors such as kit-ligand (SCF) (Parrott and Skinner, 1999) and nerve growth factor (NGF) (Dissen *et al*., 2001).

Studies in AMH knockout (AMHKO) mice indicate that AMH exhibits an inhibitory effect on initial follicle recruitment. Immediately after birth, a normal-sized primordial follicle stock has been formed in AMHKO animals, but depletion of the primordial follicle pool is accelerated, resulting in premature cessation of the estrus cycle (Durlinger *et al*., 1999). Moreover, ovarian follicles of AMHKO mice treated *in vivo* with exogenous FSH appear to be more sensitive to FSH compared with follicles from wild type mice, resulting in an increased number of follicles reaching the ovulatory stage compared with wild type mice (Durlinger *et al*., 1999, 2001). These findings suggest an involvement of AMH in mouse primordial follicle selection and growing follicle cyclic recruitment.

Since it is unknown whether AMH plays a similar role in human folliculogenesis, we have investigated the expression pattern of AMH using immunohistochemistry, in human ovaries.

Materials and methods

Subjects

This study was approved by the Ethics Review Committee of Erasmus MC and informed consent was obtained from all subjects. All subjects had undergone surgery in the period 1998–2002. Uni- or bilateral oophorectomy was performed because subjects were carrying a gene mutation (BRCA1 or 2), because family history indicated a severe increase of the incidence of ovarian cancer (Blanchard and Hartmann, 2000) or because of other gynaecological conditions. Only women with a regular menstrual cycle (interval 21–35 days) and aged <46 years were included. Women with polycystic ovaries were excluded. Similarly, if ovarian cancer was present in one or both ovaries, patients were excluded.

Formalin-fixed paraffin-embedded ovarian tissue blocks were collected either from the Pathology Department of the Erasmus MC (Rotterdam, The Netherlands) or from the Pathology Department of MC Rotterdam-Zuid (Rotterdam, The Netherlands).

Ovarian morphology

After oophorectomy, tissue segments were fixed for 24 h in formalin and subsequently embedded in paraffin. Histological examination was carried out to exclude ovarian pathology. For the present study, the haematoxilin and eosin-stained (HE) sections of each tissue segment were re-examined. Samples that did not contain any follicles >2 mm in size or had poor morphology were excluded. Of each evaluated tissue sample, 150 fresh 4 μm serial sections were mounted on Starfrost microscopic glass slides (Menzel-Glaser, Germany). Every fifth slide was stained with HE and evaluated for follicle stages to use for the experiment and stored at 4°C. Follicular size was calculated by measuring two perpendicular diameters as described before (van Cappellen *et al*., 1989). Adjacent sections were used for immunostaining.

Follicles were grouped according to the classification proposed by Gougeon (1986): primordial follicles (oocyte with one layer of flat pre-granulosa cells), primary follicles (oocyte with one layer of cuboidal granulosa cells), small secondary follicles (two to six layers of granulosa cells, no theca cells), preantral follicles (class 1), and antral follicle stages (antrum formation is present) with diameters <1 mm (classes 2 and 3), 1–2 mm (class 4), 2–4 mm (class 5), 4–6 mm, 6–8 mm (class 6) and >8 mm (class 7). The criteria for an atretic follicle are nuclear pyknosis and disappearance of granulosa cells.

Immunohistochemistry of AMH

Since the specificity and sensitivity of the detection of immunohistochemical staining of AMH is largely dependent on the quality of the first antibody, we decided to use two different, completely independently developed antibodies. The first, MIS C-20 (Santa Cruz Biotechnology, USA), was a commercially available goat polyclonal anti-AMH antibody. The second, a mouse monoclonal antibody, was specifically developed using the C-terminal 32 amino acids of human AMH. The use of these independently obtained antibodies ensures specific detection of the AMH expression in the ovaries.

For the development of the mouse monoclonal antibody, a synthetic peptide was synthesized corresponding to a peptide sequence close to the C-terminus of human AMH, VPTAYAGKLLISLSEERISAHHVPNMVATECG, and coupled to a purified protein derivative of tuberculin (Groome and Lawrence, 1991). Outbred Tyler's Original (T/O) mice (Southend on Sea, Essex, UK) underwent an immunization regime over a 4 month period. The animals were killed and their spleens removed for fusion to SP2/0 murine myeloma cells, following a standard protocol (Goding, 1986). The hybridoma supernatants were initially screened by enzyme-linked immunosorbent assay using standard protocols (Harlow and Lane, 1988), against recombinant human AMH coated to Nunc immunoplates using 0.2 mol/l bicarbonate buffer pH 9.4 as diluent for the AMH. Reactive clones were expanded and recloned by limiting dilution. Supernatants were then titrated against recombinant AMH and the best reacting clones were selected, expanded and isotyped. As each clone was found to be IgG1, all were purified on a protein A column using a high salt protocol (Harlow and Lane, 1988) before assessment. Clone 5/6A was selected after screening on rat and mouse ovarian sections using immunohistochemistry.

Immunohistochemical staining was performed on the stored 4 μm thick formalin-fixed paraffin-embedded tissue sections according to standard procedures (Durlinger *et al*., 2002a). After deparaffinization, the sections were quenched for 20 min in 3% H_2O_2/methanol solution to block endogenous peroxidase activity. Subsequently the sections were pretreated by 15 min heating in 0.01 mol/l citric acid buffer (pH 6) in a microwave oven at 700 W. After cooling for 30–45 min at room temperature, the sections set up for staining with MIS C-20 were rinsed in phosphate-buffered saline (PBS) and incubated for 15 min at room temperature with normal rabbit serum in 5% (w/v) bovine serum albumin (BSA) in PBS (Dako, Denmark). The preincubation step was followed by incubation at 4°C overnight with primary polyclonal antibody MIS C-20 diluted 1:1000 in 5% BSA in PBS. After incubation, the sections were rinsed in PBS and subsequently treated for 30 min at room temperature with biotinylated rabbit anti-goat antibody (dilution 1:400; Dako). This was followed by incubation for 30 min with streptavidin–biotin–peroxidase complex (diluted 1:200 in PBS; Dako) and colour development for 4 min with 0.075% 3,3′-diaminobenzidine tetrahydrochloride (Sigma–Aldrich, USA) at room temperature.

After pretreatment, the sections scheduled for staining with 5/6A were incubated with normal goat serum in 5% BSA for 30 min at room temperature. This was followed by incubation at 4°C overnight with primary monoclonal antibody 5/6A, diluted 1:500. After incubation, the sections were rinsed in PBS and subsequently treated for 15 min at room temperature with biotinylated goat anti-mouse antibody (dilution 1:400; Dako). The same colour development procedure was used. After immunostaining, sections were counterstained with haematoxylin for 3 min.

For each section, the adjacent section was incubated with 5% BSA/PBS in the absence of the primary antibody (negative control). To validate the specificity of the 5/6A antibody, a preabsorption experiment was performed. The 5/6A antibody (0.67 g/l) was combined with a 5-fold excess (by weight) of the peptide, which was used for the development of the 5/6A antibody (see above). The 5/6A antibody with the blocking peptide (final dilution 1:500) and the 5/6A alone (dilution 1:500) were incubated at 4°C overnight. Adjacent sections were incubated with 5/6A, 5/6A with blocking peptide or 5% BSA/PBS (negative control), using the procedure described above.

Mouse ovarian tissue was used as a positive control since the expression pattern of AMH in these animals has been well described (Durlinger *et al*., 2002a). Sections were analysed and scored using a Zeiss Axioplan 2 microscope (Germany). Granulosa cells of each follicle were scored negative (–) if absolutely no staining was present as compared to adjacent control tissue sections. If staining was present in only some granulosa cells, the follicle was scored 'weakly positive' (+/–). Follicles were scored positive (+) if specific AMH staining was present in almost all or all granulosa cells. When granulosa cells of a single follicle stained much stronger than granulosa cells in other follicles in the same slide, this follicle was scored strongly positive (++). The absolute intensity of staining varied between experiments, but the relative intensity of signals in the different follicle classes remained similar. Pictures were taken using a Coolsnap Pro Color camera and ImagePro® Plus software (Media Cybernetics, Inc., USA). Using a multivariate analysis of variance the number of follicles within each class that stained for AMH with MIS C-20 was compared with the number of follicles within each class that stained for AMH with 5/6A.

Human AMH production and western blot analysis

Full length human AMH cDNA was isolated from human testis by RT–PCR using the primers 5′-CTCGAGCTGCCAGGGACAGAAAGGGCT-3′ and 5′-CTCGAGTTGCTGGTCTTTATTGGGGCG-3′. The hAMH cDNA was fully sequenced, compared with the published sequence (Cate *et al*., 1986), and subcloned into the pcDNA3.1 expression vector (Invitrogen, The Netherlands). To allow efficient hormone processing in HEK293 cells, an optimized cleavage site (RARR) was created by quick change site-directed mutagenesis according to the manufacturer (Stratagene Europe, The Netherlands) as described previously (Nachtigal and Ingraham, 1996). A 6HIS epitope tag was introduced into hAMH at position Pro-30 by site-directed mutagenesis. Human embryonic kidney 293 (HEK293) cells were stably transfected with the cDNA encoding the modified hAMH. Recombinant bioactive AMH ligand was obtained from conditioned media collected from stably transfected HEK293 cells expressing

Table I. Clinical data of all subjects

Subject no.	Age (years)	Diagnosis	Cycle length (days)	Body mass index (kg/m^2)
1	34.2	BRCA-1	28	30.5
2	39.9	BRCA-2	28	20.4
3	35.5	BRCA-1	28	21.8
4	35.2	HBOC	34	21.3
5	35.8	BRCA-1	28	22.1
6	38.8	BRCA-1	28	24.7
7	44.0	HBOC	28	32.7
8	33.8	Endometriosis	25	32.7
9	18.9	Benign cyst	28	ND
10	39.9	Hysterectomy	28	18.0
11	38.8	Hysterectomy	28	24.4
12	33.3	Endometriosis	28	ND
Median	35.7		28	23.3
Range	18.9–44.0		25–34	18.0–32.7

HBOC = Hereditary Breast and Ovarian Cancer; BRCA-1 = Breast Cancer gene 1; BRCA-2 = Breast Cancer gene 2; ND = not determined.

modified hAMH as described (Durlinger *et al*., 2002a). AMH was purified from the medium using the NiNTA superflow Ni-column (Qiagen, The Netherlands). Subsequently, AMH was eluted from the column using Hanks' balanced salt solution (HBSS) (Gibco BRL, Invitrogen, The Netherlands) containing 250 nmol/l imidazole (Sigma-Aldrich Chemie BV, The Netherlands) and stored in siliconized tubes (Biozym, The Netherlands) in the presence of 0.1% BSA. Finally, AMH was desalted over a PD10 column (Amersham Pharmacia Biotech, The Netherlands) to remove the imidazole. Ni-column elution buffer run through a PD10 column constituted the control medium. Samples were stored at –20°C.

Western blot analysis was performed using the mouse monoclonal antibody 5/6A. Proteins from conditioned medium were separated using 12% polyacrylamide gel electrophoresis under reducing conditions. Proteins were transferred to nitrocellulose membrane and incubated with the 5/6A antibody at a 1:500 dilution, followed by a secondary peroxidase-conjugated goat anti-mouse antibody (Sigma-Aldrich Chemie BV, The Netherlands) at a 1:10 000 dilution. Proteins were visualized by ECL plus western blotting detection system (Amersham Biosciences, The Netherlands).

Results

Subjects

Tissue blocks from 12 different subjects were included. These ovaries were collected from regularly cycling women with ages ranging from 19 to 44 years, median 36 (Table I). All ovaries were of normal size. Seven patients underwent a prophylactic bilateral oophorectomy. Five were carriers of gene mutations (four BRCA1 and one BRCA2). Two subjects had a positive family history of breast and ovarian cancer without a BRCA1/BRCA2 gene mutation. Two patients underwent a unilateral oophorectomy because of endometriosis, where the ovaries were not affected, two because of a hysterectomy and one because of a benign cyst. Data regarding patients' last menstrual period preceding surgery are lacking since most patients did not recall this date precisely.

Ovarian morphology

The mean number of follicles in each sample differed considerably (mean MIS C-20 34.5 and mean 5/6A 25.9). Due to the age of the subjects (median age 36 years), the number of follicles with a diameter >6 mm was low (n = 8). No follicles with a diameter >10 mm were found. Specific stain deposition only occurred in the cytoplasm of granulosa cells. Oocytes of follicles in both control and antibody-incubated sections did show a weak, non-specific, brown staining.

Immunohistochemistry of AMH

Both antibodies revealed identical staining patterns, both in terms of intensity and specificity, when tested on mouse ovary sections (data not shown). Subsequently, we applied the antibody on the human material in this study. Addition of the peptide, used for development of the 5/6A antibody, showed elimination of the immunohistochemical staining (Figure 1K) and therefore validates the specificity of this novel antibody.

The results of the immunohistochemical detection of AMH in the human ovaries are shown in two ways. In Figure 1, examples are given of the staining patterns and intensity in individual follicles of different classes. In Tables II and III and in Figure 2, the results of the quantitative determinations of numbers of follicles are given.

In all primordial follicles examined, AMH expression was absent (Figure 1B and C; Tables II and III). AMH immunostaining could first be observed in granulosa cells of follicles in the primary stage. Approximately 75% of secondary follicles with two to six granulosa cell layers were positive or strongly positive for AMH immunostaining (Figure 1B and C). The strongest staining was observed in preantral (more than six GC layers) and small antral follicles ⩽4 mm (Figure 1E and F). In these follicle classes, all granulosa cells were positive (Table II and III). This was observed with both the polyclonal and monoclonal anti-AMH antibodies.

AMH staining disappears rapidly with increasing follicle size (Figure 1H and I), with AMH staining being almost lost in follicles with a diameter >8 mm. These follicles show very weak staining of the granulosa cells, which was almost exclusively restricted to the cumulus granulosa cells. All atretic follicles showed no immunohistochemical staining. No corpora lutea were present in the sections. There was no statistically significant difference between the number of follicles that stained for AMH between the two different antibodies (P = 0.8).

Human AMH production and western blot analysis

For further validation of the novel antibody 5/6A, a western blot analysis was performed. No AMH protein was detected in the control medium and in concentrated medium of COV343 cells, a human granulosa tumour cell line (Figure 3) which does not express AMH mRNA (J.Visser, unpublished observations). Purified recombinant rat AMH, purified recombinant human AMH as well as non-purified hAMH in concentrated medium of hAMH-producing HEK293 cells is detected by this novel antibody.

Discussion

In the present study, the AMH protein expression pattern in granulosa cells of follicles in human adult ovaries was investigated. Immunoreactive AMH protein was observed in primary follicles and continued to be expressed in follicles in the antral stage. The highest level of AMH expression was present in granulosa cells of secondary, preantral and small antral follicles ⩽4 mm in diameter. In larger (4–8 mm) antral follicles, the AMH expression gradually disappeared. The almost identical patterns obtained with two different antibody tools, a goat polyclonal and a newly developed mouse monoclonal antibody, indicate the value of these observations.

This pattern of AMH immunostaining is comparable to that previously found in human fetal and postnatal ovarian tissue (Rajpert-De Meyts *et al*., 1999). In that study, however, the emphasis was not on AMH expression in different stages of normal folliculogenesis, but rather on ovaries in different stages of development and under different disease conditions (Rajpert-De Meyts *et al*., 1999).

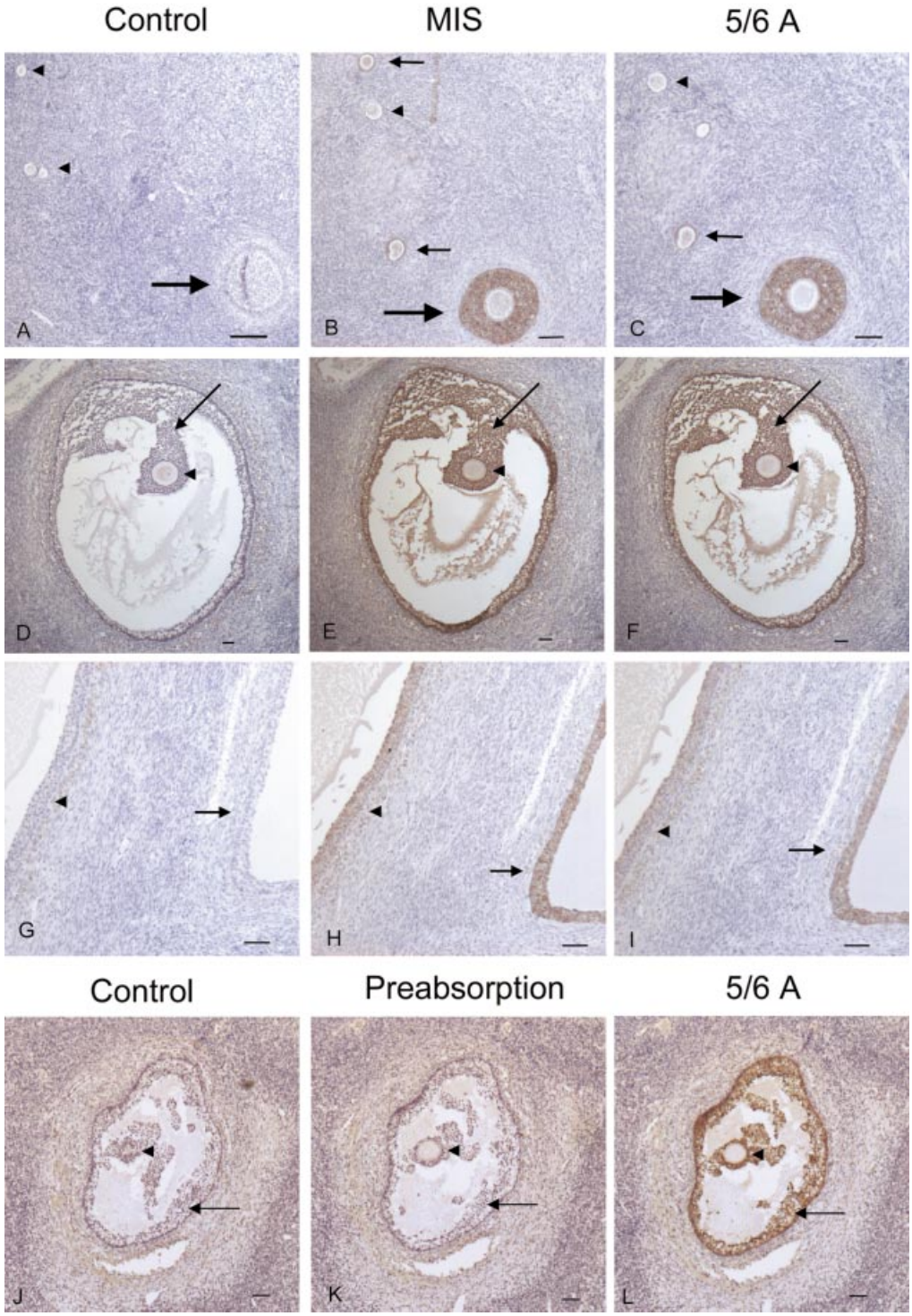

Figure 1. Micrographs of anti-Müllerian hormone (AMH) immunohistochemical stained human ovarian tissue sections. Specific (brown) AMH stain deposition is present in the cytoplasm of granulosa cells. Scale bar = 100 μm. Sections **A**, **D** and **G** are controls; sections **B**, **E** and **H** were stained using the MIS C-20 antibody; sections **C**, **F** and **I** using the 5/6A antibody. (**A**–**C**) Adjacent sections at ×100 magnification with a primordial follicle (arrowhead), a primary follicle (small arrow) and a secondary follicle (large arrow). Primordial follicles show no immunostaining of the cytoplasma of the granulosa cells, whereas the primary follicles show normal staining (+) with both the MIS C-20 and 5/6A antibodies. Secondary follicle shows strong staining (++) with both antibodies. (**D**–**F**) Adjacent sections at ×40 magnification with a small antral follicle <1 mm. The oocyte shows weak, non-specific brown staining (arrowhead), whereas the granulosa cells, especially of the cumulus (arrow), show strong staining (++) with both antibodies. (**G**–**I**) Adjacent sections at ×100 magnification showing two larger antral follicles. The follicle on the left side with a diameter of 6.1 mm shows weak staining (+/–) of the granulosa cells (arrowhead), whereas the smaller follicle on the right side (arrow) with a diameter of 2.5 mm shows normal staining (+) with both antibodies. (**J**–**L**) Adjacent sections at ×40 magnification with a small antral follicle <1 mm. The oocyte shows weak, non-specific brown staining (arrowhead). The granulosa cells show strong staining (++) with the 5/6A antibody. When the peptide is added to the antibody, no immunohistochemical staining occurs (**K**).

Table II. Number of follicles scored for all subjects after immunostaining with MIS C-20

	MIS C-20				Total
	–	+/–	+	++	
Primordial	269	–	–	–	269
Primary	12	43	14	3	72
Secondary 2–6 GC layers	–	5	12	3	20
Preantral >6 GC layers	–	–	5	1	6
Antral					
<1 mm	–	–	10	5	15
1–2 mm	–	–	3	3	6
2–4 mm	–	1	6	2	9
4–6 mm	–	–	8	1	9
6–8 mm	–	3	2	–	5
>8 mm	–	3	–	–	3
Total non-atretic	281	55	60	18	414
Atretic	12	–	–	–	–

– = no staining; +/− = weak staining; + = normal staining; ++ = strong staining; GC = granulosa cell.

Table III. Number of follicles scored for all subjects after immunostaining with 5/6A

	5/6A				Total
	–	+/–	+	++	
Primordial	190	–	–	–	190
Primary	27	11	13	1	52
Secondary 2–6 GC layers	–	2	7	5	14
Preantral >6 GC layers	–	1	2	2	5
Antral					
<1 mm	1	1	10	3	15
1–2 mm	1	–	7	1	9
2–4 mm	–	2	5	2	9
4–6 mm	–	3	6	–	9
6–8 mm	–	2	3	–	5
>8 mm	–	3	–	–	3
Total non–atretic	219	25	53	14	311
Atretic	12	–	–	–	–

– = no staining; +/– = weak staining; + = normal staining; ++ = strong staining.

From our previous mouse and rat studies, it became apparent that AMH plays an important role during both initial and cyclic recruitment of ovarian follicles. AMH, produced by the pool of growing follicles, acts as a feedback signal by inhibiting the initial recruitment of primordial follicles (Durlinger *et al*., 2002b). In the rat, AMH expression negatively correlates with future atresia of follicles that undergo selection, suggesting that AMH may be involved in the process of cyclic recruitment (A.L.Durlinger, unpublished thesis, 2000). In addition, we found that FSH-dependent growth of mouse follicles *in vitro* is attenuated by the addition of AMH to the culture, indicating that AMH is one of the factors determining the sensitivity of ovarian follicles to FSH (Durlinger *et al*., 2001). This role of AMH is underlined by its characteristic pattern of expression in the mouse and rat ovary. As soon as primordial follicles are recruited for growth, AMH is expressed in the first differentiating granulosa cells indicated by a change from flat to cuboidal. AMH expression is lost in rat and mouse follicles during the stage at which they undergo cyclic recruitment, i.e. large preantral and small antral follicles.

The role of AMH in initial and cyclic recruitment might be similar in the human ovary, since the pattern of expression of AMH in human follicles is similar to that of rodents and sheep. From the primary stage onwards, all follicles express AMH, whereas expression is lost from follicles at sizes >8 mm. Thus, also in the human, the pool of growing follicles produces AMH, which could act on the remaining primordial follicles, inhibiting their recruitment. In addition, AMH expression in the human disappears in large-sized antral follicles (6–8 mm), which are the follicles that undergo cyclic recruitment (Fauser and van Heusden, 1997). Interestingly, AMH expression in these follicles was the strongest in the granulosa cells of the cumulus, similar to the pattern found in rat and mouse (Baarends *et al*., 1995; Durlinger *et al*., 2002b). In the human ovary, a cohort of small healthy antral follicles that reaches a diameter of 3–5 mm, and that has just become dependent on FSH for further growth, will grow into larger antral follicles (6–8 mm) once a certain threshold level of serum FSH is reached (Pache *et al*., 1990). Only a single follicle from this cohort is selected to gain dominance and ovulate every cycle (van Santbrink *et al*., 1995; Fauser and van Heusden, 1997). Since we could not study follicles >10 mm, the question whether AMH expression is lost in the follicles that are selected for dominance remains unanswered. However, the increasingly lower expression that is found in the larger follicle classes suggests that this may be the case.

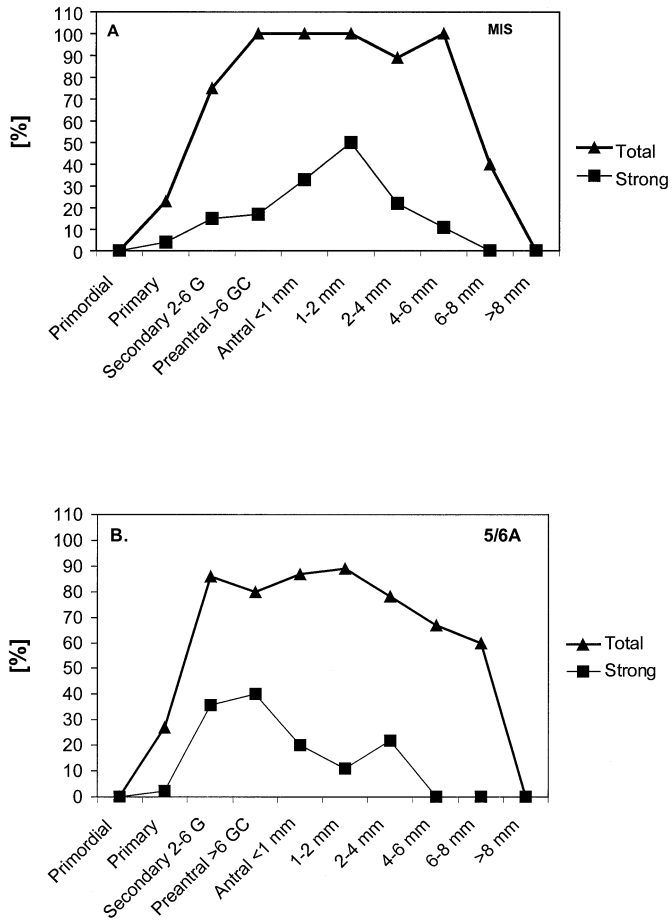

Figure 2. Graphical summary of the immunohistochemical data in Tables II and III. Only the percentages of follicles with strong (++) and total staining (+ and ++) are shown. The total staining in the graphical depictions represents an addition of the percentage of follicles within a certain class with normal (+) and strong (++) staining (total staining; triangles). For comparison the percentage of follicles of a certain class that show strong staining is depicted separately (strong staining; squares). Staining increases rapidly with the stage of the follicles and decreases when follicles are >4–6 mm. In the upper graph (**A**), staining with the MIS antibody is shown. In the lower graph (**B**), staining with the novel 5/6A antibody is shown.

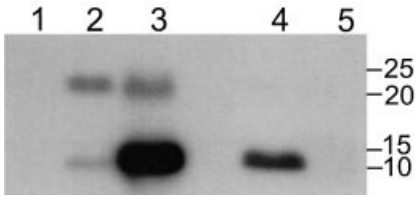

Figure 3. Detection of anti-Müllerian hormone (AMH) with the mouse monoclonal antibody 5/6A. No AMH protein is detected in the control medium (lane 1) and in concentrated medium of COV434 cells, a human granulosa tumour cell line (lane 5). The 5/6A antibody detects purified recombinant rAMH (lane 2), purified recombinant hAMH (lane 3), and non-purified AMH in concentrated medium of hAMH-producing HEK293 cells (lane 4). Upon purification, a stable AMH dimer is formed. Relative molecular mass (kDa) of the standards is indicated on the right.

Since AMH may be involved in initial and cyclic recruitment, it is of great interest to investigate the role of AMH in diseases associated with ovarian dysfunction. Indeed, in women with polycystic ovary syndrome (PCOS), a chronic anovulatory disorder (World Health Organization classification 2), serum AMH levels appear to be strongly increased (Cook *et al*., 2002; Laven *et al*., 2003). Normogonadotrophic anovulatory infertility appears to be caused by disturbed dominant follicle selection. The size of the population of antral follicles is increased, while the number of primary and secondary follicles in the polycystic ovary are about twice those observed in the normal ovary. Thus, it would be of great interest to investigate the AMH expression pattern in ovarian tissue collected from women with this anovulatory disorder.

AMH has also been shown to be an excellent marker for ovarian ageing (de Vet *et al*., 2002; van Rooij *et al*., 2002). The number of follicles in the ovary of a woman reaching the end of her reproductive period is the major determinant of the timing of both the period prior to menopause and menopause itself. Serum levels of AMH in regularly cycling women decrease over time and there is a strong correlation between AMH and the number of antral follicles (de Vet *et al*., 2002; van Rooij *et al*., 2002). It appears that the size of the recruited cohort of follicles is closely linked to the remaining primordial follicle pool. Post-menopausal women are infertile due to the exhaustion of the primordial follicle pool, whereas women in the years before menopause, whose ovaries still contain follicles, have a reduced fertility of which the cause is not completely known (Richardson and Nelson, 1990; Te Velde and Pearson, 2002). Through its inhibitory effect on primordial follicle recruitment, AMH may regulate the efficiency of the use of the primordial follicle pool and therefore may be involved in the determination of the age at which menopause arises. The pattern of AMH immunostaining as observed in the present study is supportive of the suggestion of an inhibitory role of AMH in follicle recruitment. Another argument in favour of this inhibitory role of AMH is the observation of an accelerated depletion of resting follicles in women in the 35–45 year old age group, when the levels of AMH decrease rapidly as a result of a strongly diminished number of growing follicles.

In conclusion, the pattern of AMH immunostaining in the human ovary suggests a role of AMH in both the processes of initial and cyclic recruitment. These results confirm for the first time previous observations in the rat and mouse and may be of great importance for the role of dysregulation of AMH function as a cause of female infertility, and the possible treatment.

Acknowledgements

The authors gratefully acknowledge Dr Patricia Ewing (Department of Pathology, Erasmus MC (Rotterdam, The Netherlands) for her advice regarding the morphological examination of all tissue samples, Dr Richard Cate, Biogen, Cambridge, MA, USA for his kind gift of human recombinant AMH, Bas Karels for assistance with the histology and Annemarie de Vet for her preliminary work. This work was financially supported by a research grant from Organon NV (EMF fonds 2764005), the Stichting Voortplantingsgeneeskunde Rotterdam (SVG) and by the European Commission through the 'OVAGE' grant (QLK6-2000-00338).

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Submitted on October 6, 2003; accepted on October 20, 2003