

Developmental expression and cellular distribution of Müllerian inhibiting substance in the primate ovary

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Abstract

Ovarian follicle formation during development and follicle maturation in adulthood are crucial determinants of female fertility and disruptions in these processes may result in subfertility or infertility. Among the several factors that are involved in ovarian physiology, Müllerian inhibiting substance (MIS) also known as anti-Müllerian hormone has emerged as an important marker to predict the follicle reserve. However, the roles of MIS in human ovarian physiology are unknown. To gain an insight into the potential roles of MIS in human ovarian differentiation during development and its regulation in adulthood, the expression profiles of MIS mRNA in the developing and adult human and monkey ovaries was examined by *in situ* hybridization. The results revealed that in the fetal human ovaries, MIS is specifically expressed at low levels in the granulosa cells of the developing primordial follicles; a small subset (~2–3%) of oocytes express high amounts of MIS. In the adult human and monkey ovary, MIS mRNA is expressed at low levels in the primordial follicles, maximally in the primary and secondary follicles, and the expression is downregulated in the antral and atretic follicles. MIS expression is extinguished in the granulosa cells only after ovulation. These observations strongly favor the regulatory roles of MIS in folliculogenesis. MIS in the primate ovary may exert its effect during the primordial follicle formation to the terminal granulosa cell differentiation. The presence of MIS in a small subset of oocytes in the fetal ovary further points towards its additional role during fetal oocyte development.

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Introduction

The process of ovarian follicle growth and differentiation is a dynamically controlled event involving initial recruitment of the follicles from the primordial pool followed by a phase of growth. The growing follicles are next selected for differentiation to ovulate or undergo atresia (McGee & Hsueh 2000). This cyclic process of follicle selection unto ovulation or atresia is regulated and is under strict endocrine, autocrine, and paracrine control.

Among the several local factors involved in follicular differentiation, Müllerian inhibiting substance (MIS) also known as anti-Müllerian hormone (AMH) has been demonstrated to play a crucial role in the regulation of folliculogenesis in rodents. Essentially a male hormone, produced by the Sertoli cells of the fetal testis (Lee & Donahoe 1993, Lee *et al.* 1994), MIS mRNA and protein has been detected in the granulosa cells of the adult rodent ovary. The expression is specifically detected in the early preantral and antral follicles; the expression is lost in the ovulatory or the atretic follicles (Uneo *et al.*

1989a, Hirobe *et al.* 1992, Durlinger *et al.* 2002a,b). Interestingly, targeted disruption of MIS in female mice leads to rapid depletion of the primordial follicles (Durlinger *et al.* 1999). The ovaries of these mice have more preantral and small antral follicles as compared with wild-type littermates. In addition, the follicles of MIS knockout mice demonstrate altered sensitivity to FSH (Durlinger *et al.* 2001). Based on these studies, it has been suggested that MIS may play a role in follicle selection in the rodent ovary (Durlinger *et al.* 2002a, Visser *et al.* 2006).

In human females, circulatory levels of MIS have been found to correlate with the ovarian reserve, the levels are altered in cases of abnormal folliculogenesis (Fallat *et al.* 1997, Cook *et al.* 2002, Penarrubia *et al.* 2005, Piltonen *et al.* 2005, van Rooij *et al.* 2005, reviewed in Visser *et al.* 2006) suggesting a role of MIS in human ovarian functions. However, the roles of MIS in human ovary have not been well defined. Limited and controversial information exist on the expression profiles of MIS in the human ovary. During development, MIS protein has

been demonstrated to express in the human fetal ovary only in the primary follicles late in gestation; however, MIS mRNA has been reportedly detected even at 13 weeks of development (Voutilainen & Miller 1987, Rajpert-De Meyets *et al.* 1999). In the adult ovary, expression of immunoreactive MIS has been restricted to the granulosa cells of primary, secondary, preantral, and antral follicles; the expression is downregulated in the atretic follicles. The granulosa cells of the primordial follicles, the oocytes, or the ovarian stroma do not stain positive for MIS (Weenen *et al.* 2004). Contrasting these observations, in a recent study, immunoreactive MIS has been detected in the oocytes, the ovarian stroma and the thecal cells along with the granulosa cells of the growing follicles (Stubbs *et al.* 2005). These observations differ from those reported in rodents where MIS mRNA or protein has not been detected in the fetal ovary, primordial follicles, oocytes, or the interstitial cells of the adult rat ovary (Uneo *et al.* 1989a, Hirobe *et al.* 1992, Baarends *et al.* 1995, Durlinger *et al.* 2002a). In murine system, the expression of MIS is identical in all the follicles of the same class (Uneo *et al.* 1989a, Hirobe *et al.* 1992). However, in the human ovary, heterogeneity has been reported in the expression MIS protein in the granulosa cells of the developing follicles, particularly the antral follicles (Weenen *et al.* 2004, Stubbs *et al.* 2005). The differences in the pattern of MIS expression between species and the contrasting observations reported in human studies makes it difficult to speculate the precise roles of MIS in ovarian physiology.

To gain better insight into the roles of MIS in the process of follicle formation, growth, and differentiation, particularly in the primates, the detailed ontogeny of MIS mRNA expression in the developing and the adult human ovary was examined. Additionally, the pattern of MIS mRNA expression in the ovary of adult cyclic bonnet monkeys is also compared, to determine species-specific changes, if any, that may occur. We chose to study the mRNA expression of MIS rather than protein, as it would permit us to exactly determine the site of protein synthesis and decide, if MIS expression during folliculogenesis is regulated transcriptionally. The aim of the study is to examine the detailed cellular localization and expression pattern of MIS mRNA in the process of follicular development prenatally and during adult folliculogenesis in primates.

Material and Methods

The present study was approved by the local ethics committee. All tissues were collected after informed consent. All the samples were anonymous retaining only the essential clinical information.

Human fetal ovaries were collected from second-trimester, legal abortions done at the Obstetric and Gynecology Department of King Edward Memorial Hospital (KEMH). The details of collection of these

gonads and anthropometric analysis have been reported earlier (Modi *et al.* 2003).

Fetal samples: Twenty-eight second-trimester fetal ovaries (13–23 weeks of gestation) were analyzed in the present study. Somatic tissues from two male and two female fetuses aged 20 weeks were also collected.

Neonatal samples: Ovaries were obtained from two female neonates that had expired soon after birth and were undergoing autopsy to determine the cause of death.

Adult samples: Adult human ovaries were collected from three women (33–39 years) undergoing panhysterectomy for reasons other than ovarian disorders.

To examine the expression profile of MIS mRNA in the granulosa cells post-ovulation, two cumulus oocyte complexes (COCs) were obtained from a woman undergoing superovulation and oocyte retrieval for *in vitro* fertilization owing to male factor infertility. These were kindly donated for research purposes and collected from the Malpani Infertility Clinic (Mumbai, India). The protocol of superovulation and oocyte retrieval has been detailed previously (Malpani *et al.* 2002).

Monkey ovaries: Ovaries from three adult regularly cycling bonnet monkeys (*Macaca radiata*) were collected in the late follicular phase. The cyclicity and the hormonal profiles of the animals have been reported previously (Patil *et al.* 2005).

Tissue processing

The tissue was immediately fixed in 10% buffered formaldehyde for 48–96 h and processed for routine paraffin embedding and sectioning. Sections of 5 μ m thickness were mounted on aminosailine-coated glass slides air-dried and stored in dust-free boxes at room temperature until use. Care was taken at all stages to avoid RNase contamination. The COCs were directly fixed on glass slides, air dried, and immediately fixed in 4% buffered formaldehyde for 20 min, dehydrated in alcohol, and stored until use.

In situ hybridization

Expression of MIS transcripts was studied in the ovaries using non-radioactive *in situ* hybridization (ISH). All the reagents were purchased from Roche and Sigma. All glass and plastic wares used were autoclaved and baked; all solutions and water were treated with diethylpyrrocarbonate (DEPC) to inactivate RNase prior to use. The general protocol for ISH has been reported previously (Modi *et al.* 2005, Shah *et al.* 2005).

Probe preparation

The probe used for *in situ* hybridization was an oligonucleotide (5'-CGGTAGGCGCCTTGAGCTCCT-CAGCGG-3') commercially synthesized (Gibco) and labeled with Digoxigenin (Dig) using the 3' tailing kit

according to the manufacturer's instructions (Roche). The specificity of the probe sequence was established by examining its homology with other sequences in the database. All database searches were carried out using the BLAST search engine at www.ncbi.nlm.nih.gov at low stringency.

Hybridization

For ISH, the sections were deparaffinized in xylene, hydrated, and refixed in 2% paraformaldehyde for 10 min. The COCs were directly refixed in paraformaldehyde for 5 min and processed further. After washing in 0.1 M PBS (pH 7.0), the slides were incubated in $2 \times \text{SSC}$ ($1 \times \text{SSC} = 0.15 \text{ M}$ sodium chloride and 0.015 M sodium citrate, pH 7) for 15 min at room temperature. Pre-hybridization was carried out at room temperature for 1 h in a pre-hybridization cocktail containing 50% formamide, $4 \times \text{SSC}$, $5 \times \text{Denharts}$ solution, 0.25% yeast tRNA, 0.5% sheared Salmon sperm DNA, and 10% dextran sulphate. After pre-hybridization, the sections were hybridized overnight at 56°C with the labeled probe diluted in the same cocktail at a concentration of 2–5 pmol/ μl . The sections were stringently washed in varying concentrations of SSC containing 0.1% Tween 20 ($4 \times \text{SSC}$, 20 min twice; $2 \times \text{SSC}$, 20 min twice; $1 \times \text{SSC}$, 20 min twice; $0.5 \times \text{SSC}$ 15 min twice; and $0.1 \times \text{SSC}$ 15 min once) followed by blocking for 2 h at room temperature in blocking solution containing 2% normal sheep serum, 0.1% Triton X-100 in 0.1 M Tris-HCl buffer (pH 7.5). The washing stringency described above was derived after extensive experimentation using human fetal testicular tissue. This stringency-permitted detection of the signals only in the Sertoli cells, increasing the wash stringency above this (by either increasing the temperature or reducing the salt concentration) lead to loss of signals from the Sertoli cells.

After blocking, the sections were incubated overnight at 4°C in alkaline phosphatase-conjugated anti-Dig antibody diluted (1:500) in the above blocking solution. The slides were then extensively washed in 0.1 M Tris-HCl (pH 7.5) and equilibrated in 0.1 M Tris-HCl (pH 9.5) for 10 min. Detection was carried out at pH 9.5 for 35 min at room temperature in a solution of nitro blue tetrazolium and 5-bromo-4-chloro-2-indoyl phosphate containing 0.2% levamisole as described previously (Modi *et al.* 2003) and mounted in aquamount. The sections were viewed light microscopically and representative areas were photographed using Olympus BX-60 microscope (Japan). The sections incubated using a sense probe served as negative controls and fetal testicular sections were used as positive control.

Semiquantative analysis

The ovarian sections were scored semiquantatively for extent of MIS expression by two independent observers.

An arbitrary score was given: 0, no expression; 1, negligible, but detectable expression; 2, mild expression; 3, moderate expression; and 4, strong expression. The final score was calculated as $\Sigma \text{grade} \times \text{number of cells counted} / \text{total number of cells}$. Values are expressed as mean \pm s.e.m. and statistical analysis was performed using Student's *t*-test.

Follicular nomenclature

The follicles were grouped according to the standard classification. Briefly, follicles with a single layer of flat granulosa cells were termed primordial follicles and those with a single layer of cuboidal granulosa cells as primary follicles. The follicles with more than one layer of granulosa cells and a well-defined theca layer were termed secondary or preantral follicles. Follicles containing an antral cavity were antral follicles and those follicles with a large antral cavity were termed ovulatory follicles. Follicles containing the granulosa cells scattered in the antral cavity and having degenerative changes were termed atretic follicles.

Results

The probe used in this study was highly specific to MIS, since the sequence did not share significant homology ($> 50\%$) with any of the other sequences in the database. MIS mRNA was visualized as brown to purple staining in the nucleus and cytoplasm of all positive samples. MIS transcripts were abundantly localized to the nucleus and cytoplasm of the Sertoli cells, but not in germ cells or other somatic cells of the human fetal testis (Fig. 1G). The specificity of staining is further evident by the absence of any signals in fetal somatic tissues like kidneys, heart, membranes, liver, lungs, placenta, and umbilical cord (data not shown).

MIS expression in the developing human ovary

MIS transcripts were consistently detected in all the ovarian samples examined from 13 to 23 weeks of development (Fig. 1A–F). However, the expression was at lower levels than that observed in the fetal testis (Fig. 1G). In the early developing ovary at 13–15 weeks of gestation, MIS transcripts were seen only in the cytoplasm and the nucleus of pregranulosa/somatic cells, the germ cells were MIS negative (Fig. 1B). As the development progressed (16–18 weeks), MIS-positive granulosa cells enveloped the MIS-negative oocytes marking the initiation of folliculogenesis (Fig. 1C and D). At 20 weeks and above, a large number of oocytes surrounded by MIS-positive granulosa cells (developing primordial follicles) were observed all over the ovarian parenchyma; naked oocytes not surrounded by groups of MIS-positive somatic cells were also detectable (Fig. 1E and F). As the development progressed, most oocytes formed

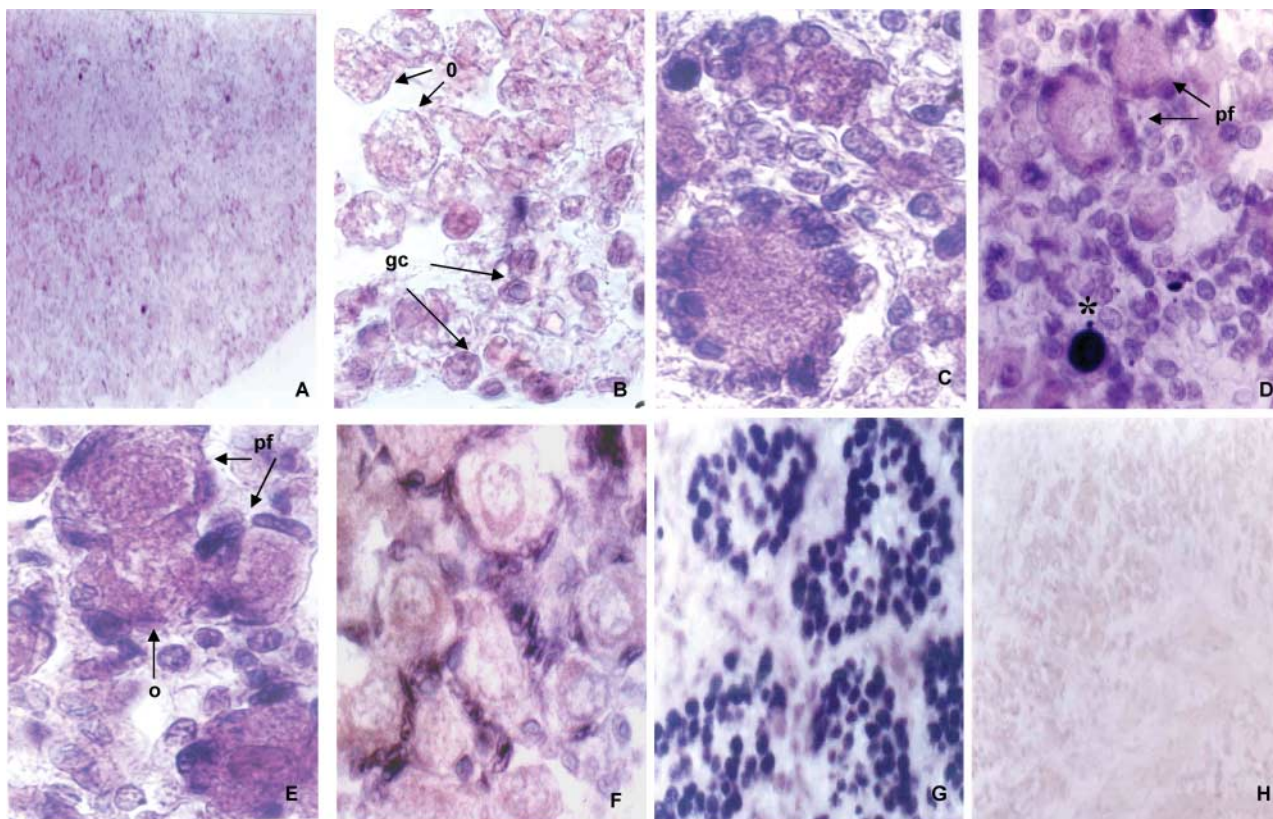


Figure 1 Expression of Müllerian inhibiting substance (MIS) mRNA in the developing human ovary. A is *in situ* hybridized 18-week-old fetal ovary, B is fetal ovary at 13 weeks showing week expression in the somatic (presumably pregranulosa) cells (gc); the oocytes (o) are MIS negative. C is ovary of a fetus at 16 weeks showing a developing follicle. D and E are ovaries at 18 and 20 weeks of development respectively showing developing primordial follicles (pf) and naked oocytes (o). Oocyte showing strong MIS expression is marked (*) in D. F is fetal ovary at 23 weeks of development showing a group of well-defined primordial follicles. G is fetal testis at 16 weeks of gestation as positive control. L is 16-week-old fetal ovary hybridized with a sense probe. Magnifications A 10 \times , B–F 100 \times and digitized, G and H 20 \times .

primordial follicles and MIS was detectable in the granulosa cells. At 23 weeks of development, along with the primordial follicles a single primary follicle was observed, MIS expression was higher in the granulosa cells of the primary follicle as compared with that in the primordial follicles (not shown).

Although most of the oocytes/oogonia were MIS negative, strong MIS mRNA expression was observed occasionally in some of the germ cells in the developing ovary (Fig. 1D). These cells had MIS expression nearly similar to that observed in the Sertoli cells of the fetal testis. The cells occurred at a frequency ranging from 1 to 3% with no appreciable difference between 13 and 23 weeks of development. Interestingly, the MIS-positive oocytes were generally not surrounded by the layer of somatic cells. The negative control did not show any staining (Fig. 1H).

The semiquantitative analysis of MIS mRNA expression in the granulosa cells was performed to evaluate if MIS mRNA expression in the ovary is regulated developmentally in the second trimester. As evident from Fig. 2A, no significant difference is noted in the expression of MIS in the fetal ovaries during the second trimester.

MIS mRNA expression in the neonatal ovary

In the newborn ovary, MIS expression was observed only in the granulosa cells of the primordial follicles at levels similar to those observed in the fetal ovary (Fig. 3A). None of the oocytes were MIS positive. Occasionally, MIS mRNA was detected in the stromal cells of the neonatal ovary.

In one of the neonatal ovary, some primary, secondary, and small antral follicles were detected along with the primordial follicles (Fig. 3B). MIS transcripts were observed in the granulosa cells of all these follicles, and the levels of MIS expression varied with the stages of folliculogenesis (Fig. 3B and C). As compared with the primordial follicles, MIS mRNA increased significantly in the granulosa cells of the primary and the secondary follicles. The small antral follicles had heterogeneous pattern of MIS mRNA expression with some of the granulosa cells being strongly positive for MIS mRNA whereas the others were MIS negative. In all, 90 follicles in different stages were analyzed semiquantitatively for the MIS expression levels in the granulosa cells (Fig. 2B). MIS expression increased significantly in the primary and the secondary follicles as compared with primordial

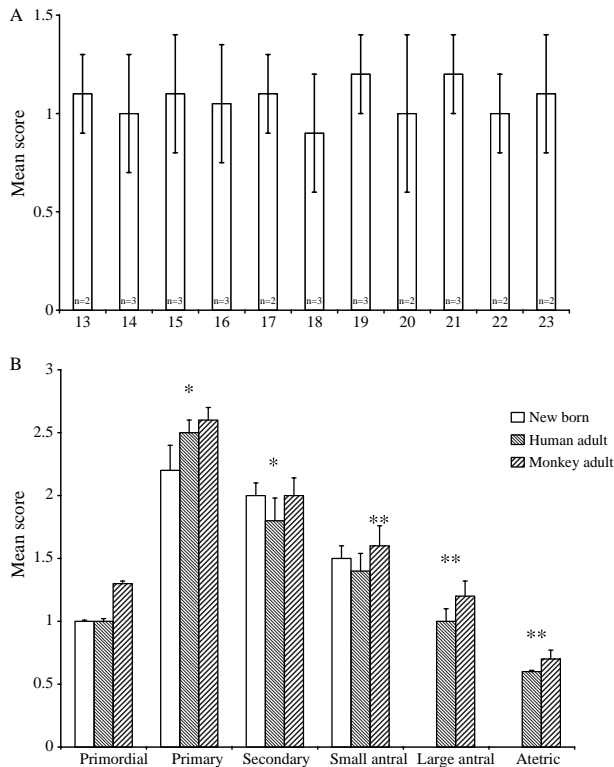


Figure 2 Semiquantitative assessment of Müllerian inhibiting substance (MIS) mRNA expression in human fetal neonatal and adult ovary. (A) Analysis of 28 fetal ovaries (n) between 13 and 23 weeks of gestation. (B) Analysis of MIS expression in the granulosa cells of the developing follicles from ovaries of a neonate, adult humans, and monkeys. Data is expressed as mean score \pm S.E.M. for the intensity of MIS expression. *Significantly different ($P < 0.05$) as compared to primordial follicles. **Significantly different ($P < 0.05$) as compared to primary follicles.

follicles (Fig. 2B). MIS expression was lower in the small antral follicles as compared with the secondary or preantral follicles (Fig. 2B). No staining was visible in the negative control (Fig. 3D).

MIS mRNA expression in the adult human ovary

The results of MIS mRNA staining in the human ovary and the results of semiquantitative analysis are represented in Figs 4 and 2B respectively. In all, 108 follicles from three adult ovaries were analyzed. MIS was specifically expressed in the granulosa cells of the follicles; the oocyte, theca cells, and the corpus luteum were generally negative for MIS mRNA. Occasionally, in some areas, the ovarian stroma showed presence of MIS transcripts.

The granulosa cells of the primordial follicles had low levels of MIS mRNA; the intensity of staining was highest in the primary and the preantral follicles (Fig. 4A–C). However, as the follicle size increased with the formation of the antrum, MIS mRNA expression reduced. The granulosa cells of the

small antral follicles had reduced MIS mRNA expression as compared with the preantral follicles, the intensity of staining and the number of cells expressing MIS reduced further in the large antral follicles (Fig. 4D). By semiquantitative analysis (Fig. 2B), MIS mRNA expression was found to be significantly lower in the small antral follicles as compared with secondary follicles, the expression of MIS reduced as the antral cavity enlarged. The mean staining intensity of MIS mRNA in large antral follicles was similar to the primordial follicles. MIS transcripts were minimal in the atretic follicles and in corpora lutea (Fig. 4G and H).

In the large antral follicles, differences in the levels of MIS transcripts were observed in the cumulus cells as compared with the mural ones. In these follicles, the cumulus cells had MIS expression higher than the mural cells (Fig. 4E and F). However, the differences were not statistically significant.

To determine if MIS expression still persisted or extinguished in the cumulus cells, post-ovulation, *in situ* hybridization was performed on COCs obtained from a woman undergoing IVF for male factor infertility. MIS transcripts were detected in the granulosa cells of the COCs the oocyte was MIS negative (Fig. 4I–K). Interestingly, closer examination revealed that the transcripts were exclusively localized to the cytoplasm and the nuclear staining was absent in most of the cells (Fig. 4J and K). The negative control is shown in Fig. 4L.

MIS expression in the monkey ovary

The results of *in situ* hybridization for MIS in the monkey ovary are represented in Fig. 5 and the semiquantitative analysis of gene expression is shown in Fig. 2B. The profiles of MIS expression in the ovaries of bonnet monkeys were identical to that observed in human ovary. MIS mRNA was detected in the granulosa cells of the growing follicles; the oocyte, theca cells, and the corpus luteum were negative for MIS mRNA. Occasionally, the ovarian stroma showed presence of MIS transcripts.

As seen in the human ovary, MIS expression increased in the primary and secondary follicles as compared with the primordial ones (Fig. 5A). As the antral cavity formed, the expression of MIS was downregulated in some granulosa cells (Fig. 5B). In large antral follicles, the number of granulosa cells with MIS expression reduced further (Fig. 5C). MIS expression was minimal in the ovulatory follicles (Fig. 5D). Differences were observed in the levels of MIS expression in the mural versus the cumulus granulosa cells. While the mural granulosa cells had low levels of MIS transcripts, the cumulus cells had relatively higher expression (Fig. 5G and H). MIS transcripts were negligible in the atretic follicles

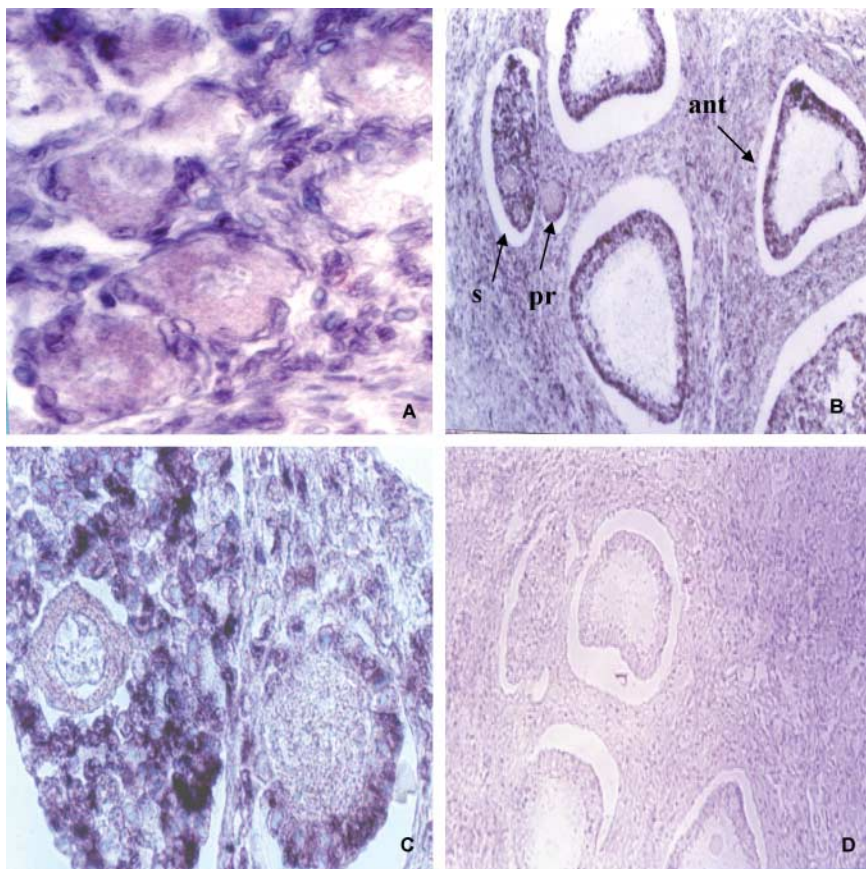


Figure 3 Müllerian inhibiting substance (MIS) mRNA in the neonatal ovary. (A) Newborn human ovary containing primordial follicles showing MIS expression in the granulosa cells of primordial follicles. (B) Expression of MIS in the growing follicles in a newborn ovary. Note the increase in expression in the primary (pr) and secondary (s) follicles, and a marginal drop in the antral (ant) follicles. (C) Enlarged view of the primary and large secondary follicles. (D) Negative control. Magnifications A 100 \times , B and D 20 \times , C 40 \times and digitized.

(Fig. 5E and F). The negative control did not show any appreciable staining. (Fig. 5I).

In all, 228 follicles from ovaries of three different animals were analyzed. In the monkey ovary, the granulosa cells of the primordial follicles had very low levels of MIS mRNA; the expression increased and was maximal in the primary follicles (Fig. 4A). Nearly all the granulosa cells of the primary follicles stained positive for MIS mRNA, the expression increased by almost twofold over the primordial follicles.

As the follicular development proceeded, the expression of MIS was maximal in all the granulosa cells of the preantral follicles. The strongest staining for MIS was apparent in the preantral follicles that had 2–4 layers of granulosa cells (Fig. 4A). Semiquantitative analysis revealed MIS expression to be marginally lower in the preantral follicles as compared with the primary follicles, although the differences were not significant.

Among the small antral follicles, in the follicles that had small antral cavity, the expression was similar to the secondary follicles (Fig. 4B), the intensity of staining and the number of cells expressing MIS reduced in the follicles with larger antrum (Fig. 4C). By semiquantitative analysis, MIS mRNA expression was found to be identical in the secondary follicles and the small antral follicles.

The expression of MIS reduced as the antral cavity enlarged further. In large antral follicles, the expression of MIS mRNA dropped as compared with the small antral follicles; the numbers of cells expressing MIS were also reduced (Fig. 4D). MIS expression was high in some of the granulosa cells, but was conspicuously low or even absent in others. The mean staining intensity of MIS mRNA in large antral follicles was similar to the primordial follicles.

As seen in the human ovary, in the large antral follicles, although the MIS expression was low and heterogeneous, the cumulus cells stained more strongly for MIS mRNA as compared with the mural cells. Semiquantitative analysis confirmed the observation where the expression of MIS in the cumulus cells was higher than that in the mural cells (not shown); however, the difference failed to reach statistical significance.

MIS expression was observed at very low levels or almost undetectable in the atretic follicles (Fig. 4E). Semiquantitatively, lowest expression of MIS was observed in these follicles.

No staining was visible in any of the sections hybridized with the sense probe demonstrating the specificity of staining (Fig. 4F).

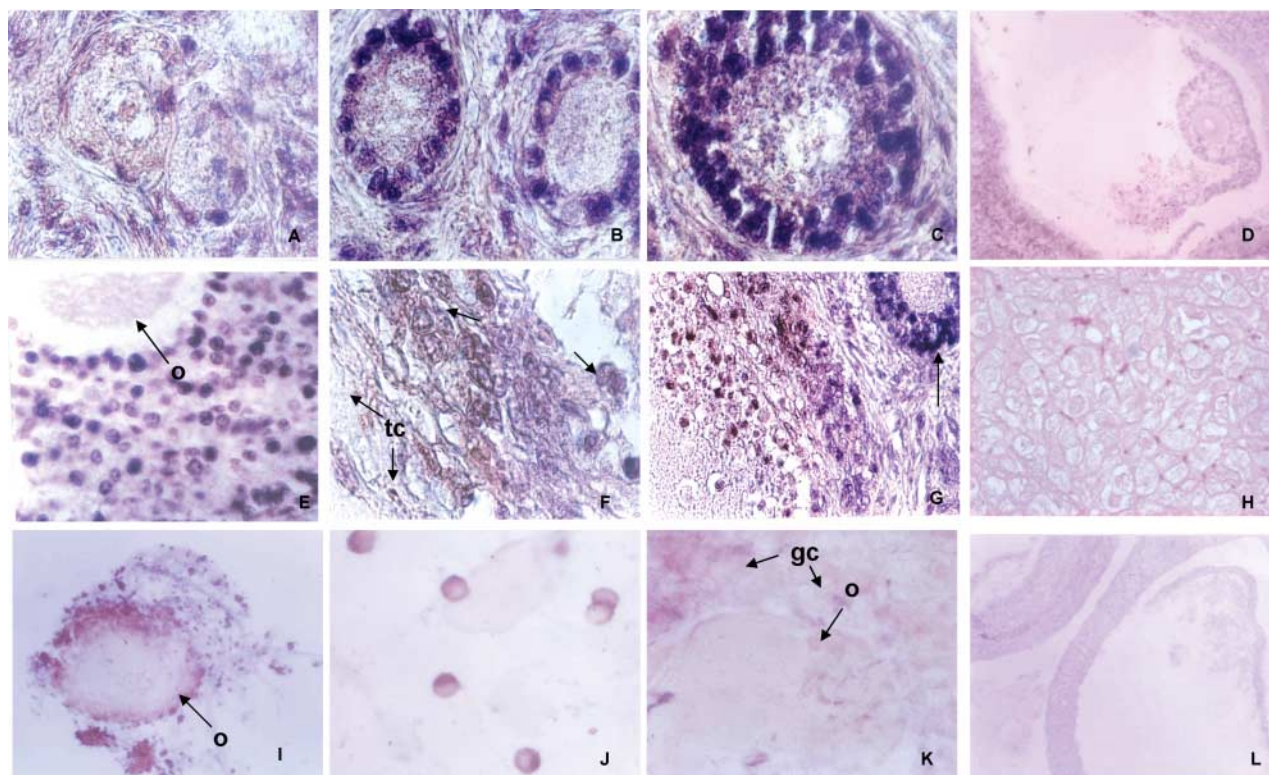


Figure 4 Cellular localization of Müllerian inhibiting substance (MIS) transcripts during folliculogenesis in the human ovary. (A) Primordial follicles. (B) Primary follicles. (C) Secondary follicle. (D) Large antral follicle. (E) Cumulus granulosa cells and the oocyte (o) of a large antral follicle. (F) The mural cells (arrow) and the theca (tc) layer of the same. Note the difference in the intensity of the staining of MIS mRNA in these cells. (G) An atretic follicle and a secondary follicle (arrow). (H) Corpus luteum that is negative for MIS mRNA. (I–K) Photographs of human cumulus oocyte complex (COC) stained for MIS mRNA: I is low magnification of the COC showing staining in the granulosa cells while the oocyte (o) has no staining; J is higher magnification of the granulosa cells; note the staining only in the periphery (cytoplasm) no nuclear signals are evident; K shows the granulosa cells (gc) closest to the oocyte (o) do not show MIS expression. Negative control is shown in L. Magnifications A, B, C, E, F, J and K 100 \times and digitized, D and L 20 \times , G, H and I 40 \times .

Discussion

The results of the present study demonstrate that MIS mRNA is expressed in the granulosa cells of the fetal and the neonatal ovary, in adulthood, the pattern of expression varies according to the stages of folliculogenesis. During fetal life, MIS transcripts are expressed constitutively at low levels in the developing granulosa cells, a small number of oocytes express high levels of MIS transcripts. In the adult human and monkey ovaries, the granulosa cells express MIS mRNA at low levels in the primordial follicles, which increases in the primary and the preantral follicles, the expression drops with the formation of the antral cavity. MIS, at low levels, is persistently expressed in the granulosa cells of the large antral and the ovulatory follicles; the expression is further downregulated upon ovulation or at follicular atresia. The pattern of MIS expression during fetal life and in adulthood suggests its roles in follicular formation and the autocrine/paracrine regulation of adult folliculogenesis.

In the human fetal ovary, *in situ* hybridization revealed MIS expression in the granulosa cells at 13 weeks; the

expression continues during follicular formation where MIS-positive granulosa cells envelope the oocytes to form the primordial follicles. To the best of our knowledge, this is the first report demonstrating the *in situ* localization of MIS in the fetal ovary and its association during folliculogenesis. Our findings corroborate the earlier results where MIS mRNA has been detected in the mid-trimester human fetal ovary; the levels have been estimated to be about 1.2% of the age-matched testicular tissue (Voutilainen & Miller 1987). Contrasting these observations, immunoreactive MIS is not detectable in the human fetal ovaries in the second trimester, MIS-positive granulosa cells are found in the primary follicles only after 30 weeks of development (Rajpert-De Meyets *et al.* 1999). It is possible that, during early gestation, the amount of MIS protein may be extremely low and/or may be rapidly utilized, the levels may rise during late gestation or as the follicle matures to the primary stage. Indeed, we observed an increase in MIS mRNA expression in the granulosa cells of the primary follicles as compared with the primordial follicles in the fetal and the neonatal ovary.

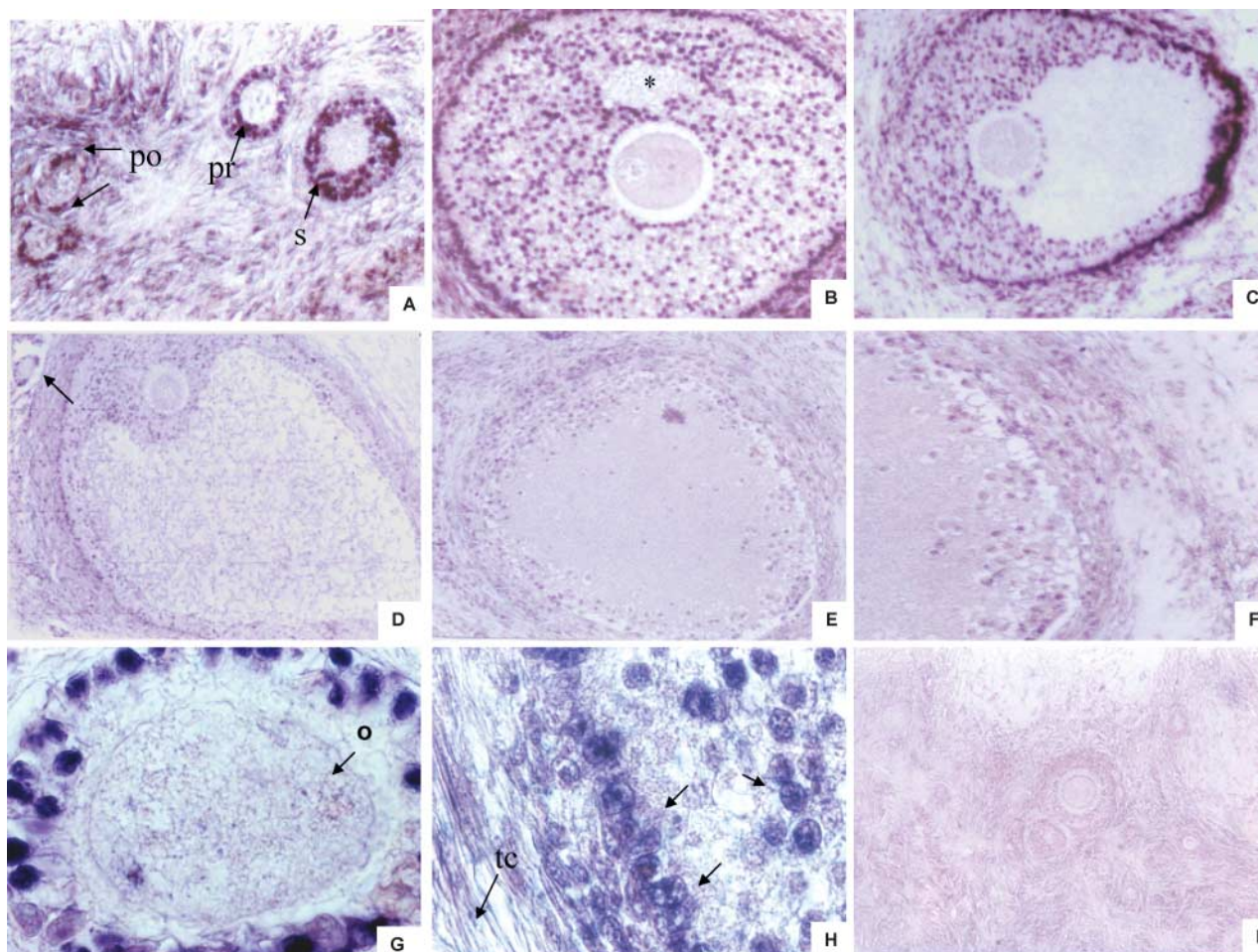


Figure 5 *In situ* hybridization for Müllerian inhibiting substance (MIS) transcripts in the bonnet monkey ovary. (A) Bonnet monkey ovary showing staining in the granulosa cells of primordial (po), primary (pr) and secondary (s) follicles. (B) Large preantral follicle (*antral cavity is just developed) showing strong MIS expression in the granulosa cells. (C) Antral follicle with reduced MIS expression. (D) Low levels of MIS mRNA in a large preovulatory follicle, compared with the primordial follicle (arrow). (E and F) Atretic follicle showing minimal MIS expression. (G) Cumulus granulosa cells and the oocyte (o) of a large antral follicle and H shows the mural cells (arrow) and theca (tc) layer of the same. Note the difference in the intensity of the staining of MIS mRNA in granulosa cells between G and H. (I) Negative control.

At present, the precise functions of MIS in the fetal ovary are difficult to speculate in the absence of any supportive experimental data. MIS is absent in the fetal mouse ovary (Uneo *et al.* 1989b); the ovarian phenotype of the MIS knockout is essentially normal (Mishina *et al.* 1996). However, it is noteworthy that the fetal rodent ovary is responsive to MIS actions since female mice overexpressing MIS have streak ovaries owing to the loss of follicles during development (Behringer *et al.* 1990, Lyet *et al.* 1995, Josso *et al.* 1998).

A unique observation made in this study was the expression of MIS in a small subset of oocytes. MIS mRNA was detected at high levels in a small population of oocytes/oogonia of fetal human ovary; the frequency was estimated to be ~1–3%. These findings are novel and surprising since MIS has not been reported in the germ line of other mammalian species (Bezard *et al.* 1987, Uneo *et al.* 1989a,b, Hirobe *et al.* 1992, Durlinger

et al. 2002a, Juengel *et al.* 2002). A striking feature of the MIS-positive oocytes was the absence of its association with the granulosa cells. Since oocytes not enveloped by the granulosa cells during development die by apoptosis (DePol *et al.* 1999, Modi *et al.* 2003), it is tempting to suggest that the MIS-expressing oocytes may be undergoing apoptosis. Indeed, *in vitro* incubation of fetal rat ovary with MIS leads to oocyte attrition (Vigier *et al.* 1987); gonadal dysgenesis is observed in prenatal ovaries overexposed to MIS (Behringer *et al.* 1990, Lyet *et al.* 1995). Based on the observations made in this study and the experimental evidences, it appears that supraphysiological levels of MIS are detrimental to ovarian development; whether low levels of MIS are conducive to ovarian formation needs to be investigated.

The expression profiles of MIS in the adult human and monkey ovary are identical and comparable to that observed previously in other species (Bezard *et al.* 1987,

Uneo *et al.* 1989a, Baarends *et al.* 1995, Juengel *et al.* 2002, Visser *et al.* 2006). Table 1 compares the results of different studies on cellular localization of MIS transcripts and protein in the adult cycling ovary. As evident, the results reported herein and those demonstrated by Stubbs *et al.* (2005) are comparable, the results differ with other studies. While in this study and that reported by Stubbs *et al.* (2005), MIS is detected in the granulosa cells of primordial follicles until ovulatory follicles; weak, but specific MIS transcripts and protein are detectable in the stromal cells. Contrasting these observations, previous studies have reported the absence of MIS in the primordial follicles and stroma (Bezard *et al.* 1987, Uneo *et al.* 1989a, Baarends *et al.* 1995, Juengel *et al.* 2002). To our knowledge, granulosa cells of the primordial follicles and the ovarian stroma as sites of MIS synthesis have not been reported earlier. There could be several explanations for such contrasting observations. Differences in sensitivity of the methods used, different fixatives, and staining protocols are some of the likely contributing factors. One striking observation reported by Stubbs *et al.* (2005) is the presence of MIS protein in the oocyte and theca cell cytoplasm, the mRNA for the same is not detectable in the same cell types (present study). The presence of MIS protein but not the mRNA in the theca cells may not be surprising, as the MIS receptor is expressed by these cells (Baarends *et al.* 1995); however, the expression of MIS in the oocyte is intriguing. MIS or its receptors have not been detected in the oocytes of the adult ovary of other species; additional studies are needed to define the origin and the role of MIS in the oocytes in the human ovary.

In the granulosa cells, MIS expression is dynamically regulated during folliculogenesis. Increase in MIS expression was evident in the primary and the secondary follicles as compared to the primordial follicles; little heterogeneity was evident in follicles within the same stage. MIS mRNA expression was downregulated as the antral cavity formed; this was more obvious in the granulosa cells of the large antral follicles. MIS transcripts were minimal in the atretic follicles. These results complement the recent observations where MIS protein has been immunolocalized predominantly in the

granulosa cells of secondary and small antral follicles (Weenen *et al.* 2004, Stubbs *et al.* 2005) and indicate that the differential pattern of MIS protein expression during folliculogenesis is associated with the changes in MIS transcription and not due to differential utilization of the protein.

The changes in expression of MIS during various stages of early folliculogenesis may be an inherent property of the process and may not be under strict endocrine control. This is evident from the observation that MIS expression in the neonatal ovary, having spontaneously growing follicles until the early antral stage, was similar to that observed in adult human and monkey ovary. Interestingly, induction of MIS expression has also been observed in the primary follicles of day 4 neonatal mouse ovary (S Dharma, T Nandedkar, and D Modi, unpublished data). These observations tempt us to hypothesize that the dynamic regulation of MIS during early folliculogenesis may be under the control of some local autocrine and paracrine factors, the downregulation of MIS in the antral follicles may be in response to an endocrine signal.

The pattern of MIS transcription in the granulosa cells of human and monkey ovary reinforces the concept of stage-specific requirement of MIS in the process of follicular growth and maturation. In the primate ovary, increase in MIS transcription is observed in the granulosa cells of the growing (primary and secondary) follicles, the expression is downregulated upon their differentiation to antral follicles. This indicates that MIS may be required for follicle growth in the early phase; its downregulation is associated with differentiation. In support of these observations, it has been demonstrated that MIS is reported to promote the growth of human primordial follicles (Schmidt *et al.* 2005) and inhibition of differentiation of the preantral follicles (McGee *et al.* 2001) *in vitro*. In this context, it is also of interest to note that MIS transcription is extinguished in the granulosa cells only post-ovulation (as no nuclear MIS transcripts were evident in the granulosa cells obtained in the COC from an IVF cycle). Thus, it is likely that MIS may regulate granulosa cell proliferation while loss of MIS expression may lead to its differentiation. Thus, MIS may be

Table 1 Comparison of Müllerian inhibiting substance expression in the adult ovary.

	Uneo <i>et al.</i> (1989a), Baarends <i>et al.</i> (1995)	Durlinger <i>et al.</i> (2002a,b)	Weenen <i>et al.</i> (2004)	Stubbs <i>et al.</i> (2005)	Present study
Species	Murine	Mouse	Human	Human	Human
End product	mRNA	Protein	Protein	Protein	mRNA
Primordial	–	–	–	+	+
Primary	+/-	+/-	+	++	++
Secondary/preantral	++	++	++	+++	+++
Small antral	+++	+++	+++	+++	+++
Large antral/ovulatory	+/-	+	+	+	+/-
Oocyte	–	–	–	+	–
Theca	–	–	–	+	–
Stroma	–	–	–	+	+

involved in the regulation of follicular growth and granulosa cell differentiation in the primate ovary.

An interesting observation made in this study is the difference in MIS expression in the cumulus versus mural granulosa cells of the large antral follicles. MIS transcripts were higher in the cumulus cells as compared to the mural ones. Although the differences were not statistically significant, the observations point towards disparate granulosa cell functions within the follicular compartment. Whether this is due to differential functional requirements of the cumulus and the mural cells of the preovulatory follicle, or of the differences in the responsiveness to an inhibitory signal, needs investigation.

In summary, the results of the present study in both human and monkeys strongly favor the regulatory roles of MIS in the folliculogenesis particularly in the process of follicular growth and differentiation. Based on the expression profiles and the results of some *in vitro* studies, it seems likely that the roles of MIS in ovarian functions in the rodents and primates may differ. MIS in the primate ovary may exert its effects in a larger temporal window initiating from the primordial follicle growth to terminal granulosa cell differentiation. The presence of MIS in the granulosa cells and a small subset of oocytes in the fetal ovary, point towards its additional role during fetal ovarian development that needs to be explored.

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