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Anti-Mullerian Hormone Serum Concentrations in Prenatal and Postnatal Period in Murine

Dae Young Kim

Division of Life Science, College of BioNano Technology, Gachon University, Incheon 406-799, Korea

ABSTRACT

Mullerian inhibiting substance (MIS) is a member of the TGF-β (transforming growth factor-β) family whose members play key roles in development, suppression of tumour growth, and feedback control of the pituitary-gonadal hormone axis. MIS is expressed in a highly tissue-specific manner in which it is restricted to male Sertoli cells and female granulose cells. The serum levels of MIS in prenatal and postnatal ICR mice were measured using the enzyme-linked immuno-solvent assay (ELISA) using the MIS/AMH antibody. Mice were grouped by age: the significant periods were at the onset of development. During sex organ differentiation, no remarkable difference between female and male foetus MIS serum levels (both<0.1 ng/ml) was observed. However, MIS serum levels in pregnant mice markedly changed (4.5～12.2 ng/ml). After birth, postnatal female and male mice serum MIS levels changed considerably (male: <0.1～138.5 ng/ml, female: 5.3～103.4 ng/ml), and the changing phase were diametrically opposed (male: decreasing, female: fluctuating). These findings suggest that MIS may have strong associations with not only develop- ment but also puberty. For further studies, establishing the standard MIS serum levels is of importance. Our study provides the basic information for the study of MIS interactions with reproductive organ disability, cancer, and the effect of other hormone or menopause. We hypothesise that if MIS is regularly injected into middle-age women, meno- pause will be delayed. We detected that serum MIS concentration curves change with age. The changing phase is different between males and females, and this difference is significant after birth. Moreover, MIS mRNA is expressed during the developmental period (prenatal) and also in the postnatal period. This finding indicates that MIS may play a significant role in the developmental stage and in growth after birth.

(Key words : anti-mullerian hormone (AMH), ELISA, developmental stage, mice)

INTRODUCTION

Mullerian Inhibiting Substance (MIS), also known as Anti-Mullerian Hormone (AMH), is a member of the TGF-β (transforming growth factor β) family and is related to the growth and male sex differentiation factor (Visser and Themmen, 2005). During fetal development, MIS is synthesised by testicular Sertoli cells and induces the degeneration of the Mullerian duct, which form the anlagen of female sex organs (Munsterberg and Lovell-Badge, 1991; Chris et al., 2002; Bedecarrats et al., 2003; Salhi et al., 2004; Visser and Themmen, 2005).

MIS starts to be expressed at 12.5 days post coitus (dpc) at the onset of sex differentiation. Although its volume reduces after birth, ovarian granulose cells of the growing follicle synthesise MIS (Munsterberg and Lovell-Badge, 1991; Visser and Themmen, 2005). In a previous study conducted by Visser and colleagues, MIS expression initiated in the columnar granulose cell of the primary follicles in the mouse (Rajpert-De Meyts et al., 1999; Visser and Themmen, 2005; Visser et al., 2006), which indicates that MIS functions during fetal differentiation and also in the postnatal period (Durlinger et al., 1999; Fujisawa et al., 2002). Moreover, the MIS expression region of the postnatal period is different from the expression region during the differentiation period. This difference suggests that MIS has a special role in female growth (Donahoe et al., 2003; Bedecarrats et al., 2003). In postnatal females, MIS expression is the highest in the granulose cell of the preantral and the small antral follicle, and its decrease in expression correlates with follicle developmental stages during which MIS interacts with other female hormones (Visser and Themmen, 2005). Due to the MIS-mediated inhibitory effect on FSH, MIS is not expressed during the final step of FSH-dependent follicle growth; therefore, fast primary follicle reinforcement is accom-

* Correspondence : E-mail : davekim@gachon.ac.kr

plished without MIS (Durlinger et al., 1999; Bedecarrats et al., 2003).

During the estrous cycle, MIS interactions and serum MIS concentrations in mice genetically null for MIS have been studied (Durlinger et al., 2002a; Durlinger et al., 2002b). Moreover, previous studies have clarified that the low level of MIS expression initiates follicular development and ovulation during the mouse estrous cycle (Racine et al., 1998; Lukas-Croisier et al., 2003; Schmidt et al., 2005; Themmen, 2005). In human reproduction, serum MIS levels have been reported as a marker for ovarian dysfunction (van Rooij et al., 2002; Visser et al., 2006).

Several studies have examined whether hormone and sex-organs are connected with diseases such as cancer, and studies have reported that MIS partially influences cancer development (Dutertre et al., 2001; Donahoe et al., 2003; Hoshiya et al., 2003; Stubbs et al., 2005).

During development, MIS is important for reproductive organ differentiation; therefore, the studies investigating the interaction between MIS and sex-organ deformity significantly impact multiple conditions, including hermaphroditism (Dawes et al., 1999; Bedecarrats et al., 2003; Donahoe et al., 2003; Salhi et al., 2004; Visser and Themmen, 2005). Specifically, determining the normal expression range of MIS during sex-organ differentiation may lead to the early diagnosis of reproductive organ abnormalities at developmental stages; however, the standard MIS serum levels have not yet been determined.

The objective of this study was to measure the normal levels of MIS from fetal developmental to the adult (after puberty) in the mouse. The results of the present study will contribute to the understanding of MIS function during the entire lifespan of the animal and its expression differences between the two sexes.

MATERIAL AND METHODS

1. Animals

ICR mice were used in all experiments as subjects. The animals were bred at 18～26℃ with 30～70% humidity, ASPEN POROUS® bedding, and Rodent NIH-31 Modified Auto feed (Samtako Bio Korea). The mice were divided into 18 groups by development stage and age.

At embryonic 14 day (E14) mouse organogenesis is complete, and the overt sexual dimorphism of the gonad is obvious by E12.5 (Josso et al., 2005). The mice were divided into 4 categories: prenatal, postnatal, puberty and adults. Each category was divided into several groups with a gap of 2～5 days (Prenatal: 2 days, Postnatal: 5 days, Puberty: 2 days, and Adult: 5 days).

The mating day was indicated as day 0. The birth group (embryonic 19～21 days) was examined after the pups sucked the first colostrums. Postnatal mice were grouped by a 5-day interval from 5 to 20 days. The MIS levels in the pubertal group were measured on days 22, 24, 26, 28, and 30 (Before the onset of the vaginal opening, Leptin Accelerates the Onset of Puberty in Normal Female Mice, Rexford S. Ahima), and in the postnatal group, the MIS levels were measured on days 35, 40, and 50. All procedures in this study were in compliance with the Animal Welfare Act Regulations and with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996).

2. Serum Collection

Before the sampling of blood, adult and pregnant mother mice were anesthetised with Avertin. Avertin was used at a 1.2% working solution (Avertin working solution: 0.5 ml stock solution+39.5 ml PBS) with a final concentration of 0.02 ml/g body weight and was administered intraperitoneally. Blood was collected from the heart by syringe with an 18 G needle in adult mice and with a pipette in foetus mice. The blood samples were centrifuged by micro-centrifuge at 3,000 rpm, 4℃ for 20 min. After separating the serum from the blood, we stored the samples in a －150℃ deep freezer.

3. PCR

1) DNA Extraction

To determine the sex of the prenatal mice, a PCR-based method was used. Genomic DNA was extracted from peritoneum lysate through proteinase K digestion at 55℃ and denaturation at 72℃ using the DNeasy tissue kit (Qiagen Hilden, Germany).

2) Oligonucleotide Primers

Sry was chosen as a Y-specific target sequence, and the Nds3 locus, a polymorphic microsatellite locus located on mouse X chromosome, was chosen as a control (McClive and Sinclair, 2001). The sequences of the PCR primers were as follows: SRY2 forward: 5'- TCTTAAACTCTGAAGAA GAG-AC-3' SRY4 reverse 5'-GTCTTGCCTGTATGTGATGG-3' and

NDS3 forward: 5'- GA GTGCCTCATCTATACTTACAG-3' NDS4 reverse: 5'-TCTAGTTCATTGTTGATTAGTTGC -3'(2) (Kunieda et al., 1992).

3. PCR Amplification and Gel Electrophoresis

Fifty microlitres of the reaction mixture containing 1 μl of 50 pM/μ1 sequence-specific primer (Bioneer Inc., Daejon, South Korea), 0.25 μ1 of 5 units of HotStar Taq™ Polymerase (Qiagen, Hilden, Germany), 2 μ1 of 10 xPCR buffer (2 mM Tris-HCl, 50 mM LCl, 1.5 mM $MgCl_2$, pH 8.4; Qia- gen), 2.5 μ1 of each Advantage UltraPure dNTP mix® (Clontech) were added into the PCR tube. PCR amplification was carried out for 30 cycles with denaturing for 20 sec at 94℃, annealing for 20 sec at 55℃, extension for 20 sec at 72℃, and elongation step for 5 min at 72℃ using PCR system (TC-512, TECHNE, UK).

The products were electrophoresed in 1% agarose (Intron, Korea) gels with Tris/borate/EDTA (TBE) buffer. After electrophoresis at 100 V for 30 min, the amplified fragments were visualised directly by ethidium bromide staining and ultraviolet illumination.

4. ELISA

The Mullerian Inhibiting Substance (MIS) serum concentration was measured by ELISA with the Diagnostic Systems Laboratory, Inc. DSL-10-14400 ACTIVE® MIS/AMHELISA. The assay had a 0.025～15 ng/ml standard range and 0.017 ng/ml sensitivity. The serum samples that had a higher concentration than the highest standard were diluted prior to the assay. Standards, controls and serum samples were added to each well and incubated on an orbital micro plate shaker for 1 hr at room temperature at 500 to 700 rpm. After incubation and washing, the wells were treated with another anti-MIS/AMH detection antibody-biotin conjugate. After a second incubation and washing step, the wells were incubated with streptavidin-horseradish peroxidase (HRP). After a third incubation and washing step, the wells were incubated with the substrate tetramethylbenzidine (TMB). An acidic stopping solution was then added, and the degree of enzymatic turnover of the substrate was determined by a dual wavelength absorbance measurement at 450 nm. The absorbance measured was directly proportional to the concentration of MIS/AMH by the ELISA reader system (Versamax, Molecular Devices Inc., USA).

RESULTS

1. PCR – Sexing of the Mouse Embryo

The mice in the prenatal groups were sexed using PCR. The SRY primer was used to amplify specific DNA in the SRY region, which denoted a male foetus. The SRY primer amplification band locus was 385 bp, and the NDS locus was 241 bp (Fig. 1). As shown in Fig. 1, we detected the Sry gene using our specific primer sets. B denotes the PCR of a female foetus using the SRY primers, C denotes the PCR of a male foetus model using the SRY primers. E and F denote the PCR of the control foetus models using the NDS primers. We utilised this protocol from embryonic day 8 to postnatal day 10 (no distinguishable sex organs).

2. ELISA – Serum MIS level

1) Prenatal (Developmental Stage); E12 to Birth

The detected serum MIS concentrations are shown in Fig. 2. In male and female mice, the serum MIS concentrations had similar increasing patterns until birth. Although the comprehensive pattern was similar, several detailed data values were different. No sexual difference at the contiguous period (E12 and E14) to sex determination was observed. Moreover, the serum MIS level in males was lower than the level in females at E14 (male-15.0 μl/ml and female-18.6 μl/ml). After E18,

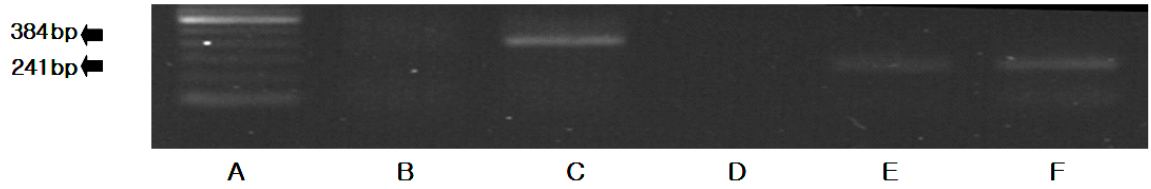

Fig. 1. PCR sexing of mouse embryo-agarose gel electrophoresis. A; 100 bp DNA ladder, B; female embryo. PCR with SRY primer, C; male embryo. PCR with SRY primer, D; BLANK, E; female embryo. PCR with NDS primer, F; male embryo. PCR with NDS primer.

male and female data were distinguishable (male-91.2 μl/ml and female-13.3 μl/ml.

2) Postnatal (before puberty); Birth to P20

The decreasing female MIS value after P5 confirmed that the MIS expression was reduced after primary follicle formation (Fig. 3). Contrary to the slow decreasing pattern observed in the female, the MIS values in males rapidly decreased. This pattern continued until puberty when the levels of testosterone, the primary factor of male puberty, increased, which inhibited is MIS expression. However, MIS expression was stimulated by FSH, a G protein coupled receptor, and cAMP, a NFkB signaling pathway component.

3) Puberty; P20 to P30

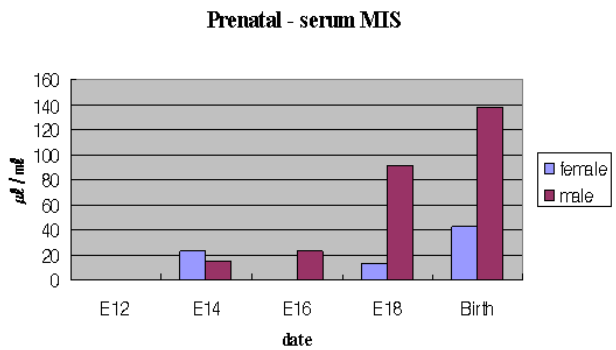

Fig. 2. Prenatal group (E12 to Birth) ELISA results: category data Ⅰ. E12 data are not shown, male: 0.1 μl/ml, female: 0.1 μl/ml. E16 female data: 0.1 μl/ml. Male and female serum MIS levels increased gradually, and the level in males (138.5 μl/ml) is the highest at birth.

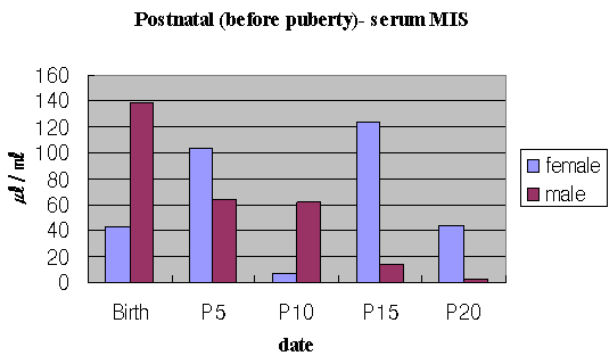

Fig. 3. Postnatal group (Birth to P20) ELISA results: category data Ⅱ. Male serum MIS levels gradually decreased from 138.5 μl/ml to 2.3 μl/ml, but female serum MIS levels had a marked change before puberty.

At puberty, the serum MIS level was the highest in females (Fig. 4). At P20 (20 days after birth), the male and female groups entered puberty, and the serum MIS levels decreased, and the decrease gap was greater in females compared to males. MIS competes with other hormones that promote sex organ growth during generation and degeneration, and we have no results that oppose this hypothesis; however, we suggest that serum MIS levels decline at puberty.

4) After Puberty; P30 to P50

After puberty, the P30 to P50 mice groups, who are regarded as adults had their serum sampled at 5 or 10 days interval. During this period, the serum MIS levels gradually decreased

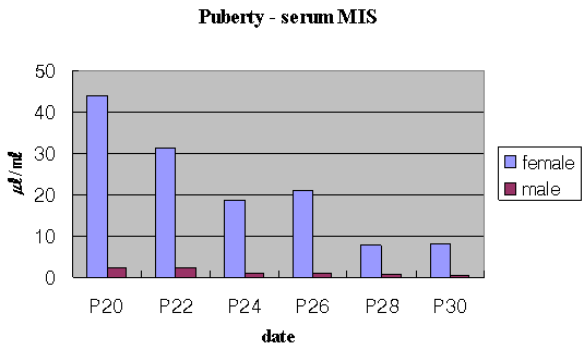

Fig. 4. Puberty group (P20 to P30) ELISA results: category data Ⅲ. MIS levels decreased in both females and males, but in females, the decreases was more rapid. The range in females was 43.9 μl/ml to 8.01 μl/ml and in males was 2.3 μl/ml to 0.25 μl/ml.

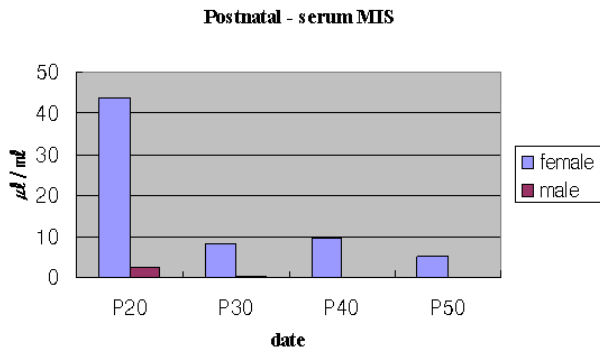

Fig. 5. Postnatal (after Puberty) group (P30 to P30, P20 contained Puberty) ELISA results: category data Ⅳ. Female and male MIS serum levels did not markedly change; however, the level became undetectable in males after puberty; however, the MIS level was preserved after puberty in females. These data do not show the MIS level after P50.

in males and females as shown in Fig. 5. No unusual differences were observed between males and females, but the large range in the females was caused by the previous level.

3. MIS mRNA Detection

As shown in Fig. 6, MIS 3r was more sensitively detected in the ovary compared to the uterus and the cervix.

DISCUSSION

The aim of this study was to measure the normal serum MIS levels in the mouse from the foetal stage to the adult stage. In this study, we observed that serum MIS levels in mice were different depending on age (prenatal, postnatal, puberty and adults) and sex. In the prenatal group and prepuberty groups, the males had high levels of serum MIS, but after puberty, the MIS level was higher in the female mice.

The highest serum MIS levels in male and female mice existed at birth and puberty, suggesting that its expression is correlated with other growth factors such as IGF (Donahoe et al., 2003). Interestingly, the male growth rate is usually slower than the growth rate of the female when they are young (Ahima et al., 1997; Bronson, 2001; Keene et al., 2002). Sex characteristics form during puberty; therefore, MIS and other sex hormones have very important functions during this time. Previous studies have revealed that MIS-deficient mice (AMHKO) have an increased number of growing follicles despite a lower serum FSH level compared to wild-type mice (Durlinger et al., 2001; Durlinger et al., 2002b; Bedecarrats et al., 2003).

At birth (E20), males had a peak MIS value promoted by the maternal FSH surge. MIS expression is stimulated by FSH through a G-protein coupled receptor, cAMP and the NFkB signalling pathway. In contrast, females had a peak MIS value after birth (P5). MIS expression is increased during folliculogenesis. In rodents, mice, rats, and other animal, folliculogenes is initiated after birth. At folliculogenesis, transition follicles produce MIS to inhibit primordial follicle growth to granulose cells and regulate the population of growing follicle pools. MIS is not the only regulator of initial recruitment and SCF, GDF, bFGF, and NGF have stimulatory effects on the initiation of follicle growth (Durlinge et al., 2002a).

In female mice, the MIS serum levels are higher than the levels in male mice after birth except at day 10. The important

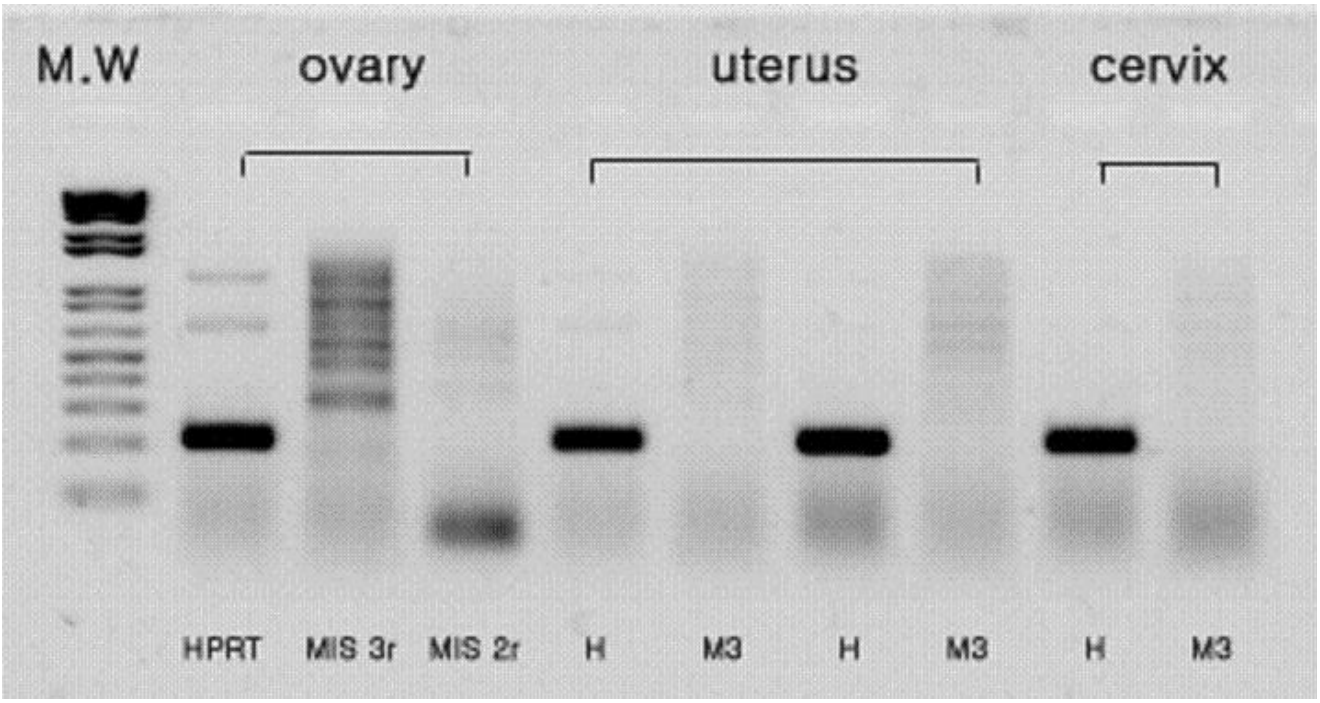

Fig. 6. Primer and organ selection This gel represents which primer was appropriate for the experiments and MIS mRNA detection. Among the two primers, MIS 3r detected MIS mRNA more sensitively and MIS mRNA was observed in ovaries but not in the uterus or the cervix.

functions of MIS in foetal developmental stages have been reported in previous studies (Munsterberg and Lovell-Badge, 1991; Morrish and Sinclair, 2002; Visser and Themmen, 2005). However, the role of MIS has not been identified in postnatal and puberty. The timing of puberty may be thought of as the period when reproduction is first possible, is crucial to the regulation of population growth and density and involves a complex interaction between hormones (Ahima et al., 1997). However, serum MIS ELISA data suggest that MIS may have important functions in female mice after birth, especially during puberty and follicle growth (Durlinger et al., 1999; Durlinger et al., 2001; Schmidt et al., 2005; Visser and Themmen, 2005).

MIS was detected in mouse serum until the postnatal date 50 (P50); however, these data do not definitively prove a connection between MIS and menopause; therefore, the interaction between MIS and menopause are only corroborative. In a former study of humans, early follicular phase hormone measurements at 3-year intervals revealed that serum MIS levels decreased significantly; however, serum FSH and inhibin B levels did not change during this interval. Analysis revealed that both serum MIS levels and follicle numbers declined with age Visser et al., 2006). Moreover, premature menopause and reproductive disability may shed light on these findings.

These are the first studies that showed a time course of serum MIS levels from the embryo to the adult in mice in both sexes. With the recognition that postnatal MIS expression may have physiological significance, the study of MIS has entered an exciting new era Bedecarrats et al., 2003). Our data can be a standard of serum MIS levels at different ages and in both sexes for further functional studies. Our study of MIS in mice also suggests that MIS may be used as therapeutics. For example, in the postpubertal individuals with Klinefelter's syndrome, MIS expression is weak with clear differences in intensity (Rajpert-De Meyts et al., 1999), and MIS is a sensitive marker of granulosa cell tumour progression (Dutertre et al., 2001).

We detected serum MIS concentration in ICR mice. The changing phase of the expression of MIS is different with age and sex. In the prenatal group and the pre-puberty groups, males had higher levels of serum MIS compared to females. After birth, male MIS levels started to reduce. During puberty, female MIS level increased and reached its peak point, and after-puberty, the MIS level in females remained higher than the male MIS levels. Serum MIS levels showed significant change during the essential period (sex differentiation, birth and pubertal period). During the developmental period, MIS regulates the degeneration of the Mullerian duct of males, and in the pubertal period, MIS regulates female folliculogenesis. These aspects are the result of the interaction between MIS and other hormones (IGF, FSH, SCF, GDF, bFGF, and NGF). We also found that MIS mRNA expression in female and male tissue is maintained during this entire period (prenatal and postnatal). All of these results indicate that MIS is engaged in the developmental stages and growth after birth, suggesting that MIS may provide therapeutic effects for prostate, breast, uterus and cervix cancers and be a marker of granulosa cell tumours and delayed menopause. The serum MIS levels from our study may be used as a MIS standard in ICR mice for future studies.

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