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석사학위 청구논문

Roles of Müllerian Inhibiting Substances in Stromal Basal Function during Implantation

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성신여자대학교 대학원

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이 논문을 석사학위논문으로 제출함

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Submitted in partial fulfillment of the

requirements for the degree of master.

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2015년 5월

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논 문 개 요

자궁내막은 가장 복잡한 조직 중 하나로 이는 임신과 착상과정에 대한 반응으로 역동적인 변화를 겪는다. 탈락막화는 자궁내막 기질세포가 형태적 기능적으로 탈락막 세포로 증식하고 분화하는 것을 말한다. 자궁이 탈락막화를 겪는 동안 세포의 증식, 분화, 세포자살을 포함하는 기능적이고 조직적인 변화를 겪는다. 착상과정과 이때 관찰되는 자궁내막의 변화의 내분비학적 조절과 세포수준에서의 연구가 많은 진척을 이루어 왔다. 이러한 복잡한 상태는 스테로이드 호르몬, 사이토카인들, 그리고 성장인자들에 의하여 조절받는다. Transforming growth factor β (TGF-β) 구성원들은 TGF-β, inhibins, activins, born morphogenetic proteins (BMP), 그리고 Müllerian inhibiting substance (MIS)를 포함한다. 이들은 세포의 증식, 분화, 면역반응, 혈관형성과 세포의 외기질의 리모델링 조절한다. 또한 근래들어 발달한 유전자 발현과 관련된 대량정보 획득 기술과 분석 방법을 이용하여 탈락막 형성과 관련된 분자 수준에서 많은 사실들이 밝혀졌다. 다른 한편 임신동안 자궁의 조직학적 변화는 기능층에서 주로 관찰되며 다른 세포층은 그 원래적 성격을 유지하고 있다. 그러나 이러한 조직적 유지의 조절 기작에 대한 이해는 매우 미미한 상태이다. 이를 위해 유전자 네트워크를 통한 조절적 접근이 시도되고 있으나 아직미미하다. 한편 임신 기간동안 자궁의 기능 유지와 변화는 착상한 배아와의 상호 관계성에 의해 조절되는 것으로 추측되고 있어 국부적 수준에서의 조절에 대한 이해가 필요하다. 암컷 생식 수관계에서 MIS 의 발현은 잘 알려져있다. 성분화 이후 성체의 난포나 자궁에서 발현되고 난포의 숫자적 변화를 예측하는 예측지표로 사용되고 있다. 또한 배아발생 시기에 성분화 조절 이외에 난자나 과립세포의 세포 분열을 억제하는 기능이 있음이 알려져 왔다. MIS 가 자궁에서 발현되는 것이 알려져 왔으나 다른 조직에서와는 달리 자궁에서의 역할에 대한 이해는 미미하다.

따라서 이 연구에서는 탈락막화 과정에서 발현되는 MIS 가 그 수용체를 통하여 자궁내막 기질세포의 분화를 조절하는데 관여함으로써 자궁의 기능조절에 관여함을 알아보고자 하였다. 이를 위해 본 연구에서는 생쥐의 자궁내막기질세포를 분리하여 탈락막화 유도와 기질세포에서 MIS 수용체 II 를 adenoviral system 을 이용하여 과발현을 시켜 실험에 사용하였다. 그리고 사람 재조합 MIS 단백질(rhMIS)을 처리하거나 처리하지 않음으로 MIS 의 생리학적 역할을 mRNA 수준, 단백질수준에서 알아보고 탈락막 마커를 사용하여 탈락막 분화 여부를 확인하였다. 이전 실험에서 MIS 는 탈락막화된 세포에서 발현되는 것이 밝혀졌고 또한 혈청에도 일정량 있음으로 MIS 적중생쥐를 이용하였다. 탈락막화 유도 배양액에서 탈락막세포로 분화 유도한 후 유세포분석기로 세포증식 정도를 알아보았다. MIS knock out 생쥐의 기질세포증식은 대조군에 비하여 유의하게 증가하였다. 한편 MISRⅡ 과발현과 rhMIS 를 처리해준 세포에서 탈락막 분화 마커들이 유의적으로 감소하는 것으로 확인되었다. 또한 탈락막화된 세포의 특징인 세포내 글리코겐 축적과 지질 축적을 Periodic-acid-Schiff (PAS)와 Oil-Red-O 염색과 그 정도를 분석하였다. 예측한 것 처럼 MIS 와 MISRⅡ를 처리해준 세포에서 유의적으로 글리코겐과 지질이 감소하였다. 이러한 결과는 탈락막 반응이 진행되는 시기에 MIS 는 MISRⅡ를 통하여 자가분비 또는 측분비 방식으로 기질세포의 증식과 탈락막 분화를 조절하여 자궁의 기능 유지에 관여함을 예측할 수 있었다.

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INTRODUCTION

The Uterus is composed of heterogeneous cell-types that undergo dynamic changes by the physiological status (Tan et al., 1999). The endometrium is one of the most complex tissues; it undergoes dynamic change because it has to remodel in response to implantation and pregnancy processes (Joswig et al., 2003). Implantation of blastocyst into the uterine endometrium is accomplished by the existence of histologically and functionally preparing uterine endometrium. These changes are primarily dependent on coordinate interactions mediated by ovarian steroid hormone estrogen and progesterone (Tan et al., 1999). The steroid hormones progesterone (P) and estrogen (E) act to control uterine competency for implantation. A primary role of P is to induce differentiation of the endometrial stromal cells into morphologically distinct decidual cells (Cheon et al., 2002).

Decidualization describes the differentiation of endometrial stromal cells into morphologically and functionally distinct decidual cells (Dimitriadis et al., 2012). In rodents decidualization begins only following implantation of a blastocyst into the endometrium of a hormonally primed uterus (Farrar et al., 1992; Shuya et al., 2011). The main feature of this event is the transformation of endometrial fibroblasts into a new type of cells, the decidual cells, which exhibit epithelial characteristics (Teodoro et al., 2003). Cell proliferation, differentiation and apoptosis are observed during undergoes decidualization.

High-throughput methodology improved the information about the involved genes and a few genes including Heparin-binding epidermal growth factor (HGEGF), Leukemia inhibitory factor (LIF), Bone morphogenetic protein (BMP4), Cytotoxic T lymphocyte-associated protein 2 beta (CTLA2- β), transcription factor CCAAT enhancer-binding protein β (CEBP- β), HomeoboxA10 (HOX10) known

as a important regulator in implantation and decidualization (Rodolfo et al., 2014).

On the other hand, histological characters of decidualized uterus are critical, although the mechanisms how those characters are maintained and changed are not clear. The undifferentiated stroma have to be remained in deep stroma layer and the decidualizing cells are stay in embryo surrounding stroma layers. However, the mechanism of tissue stability during decidualization is not clear.

Müllerian inhibiting substance (MIS), also known as anti-müllerian hormone (AMH) is member of the TGF- β superfamily of growth factor. MIS, 140-kDa dimeric glycoprotein is composed of two identical disulfide-linked subunits of 535 amino acids. During embryo development MIS is secreted by Sertoli cells in the embryonic testis and induces the regression of Müllerian duct. In female embryos, the absence of testicular tissue, and therefore of MIS, permits the development and differentiation of the Müllerian duct into its adult derivatives (Jeff et al., 2009). It has fundamental role in the regression of the Müllerian ducts in the male fetus, the initial step of organogenesis of the male genital tract. In the absence of MIS, Müllerian ducts develop into uterus, fallopian tube and the upper part of the vagina.

MIS exerts its effect by binding to a heterodimeric transmembrane serine/ threonine kinase cell surface receptor complex consisting of the MIS type Ⅰ and type Ⅱ receptors (MISR Ⅰ, MISR Ⅱ, respectively). The ligand and the MISR Ⅰ – MISRII heterodimer is the signaling receptor (Maclaughlin et al., 2010). Current evidence suggests that these MISR Ⅰ is part of the activin-like kinase(ALK) family and may in fact be ALK2, ALK3, and ALK6 (Visser., 2003; Belville et al., 2005). The activated MISR Ⅰ phosphorylates receptor-specific Smads. The heterodimeric MISR Ⅰ - MISR Ⅱ complex then triggers a downstream signaling cascade of phoshporylation activating Smad 1, 5, 8 (Tran et al., 2006). The Smad

signaling complex then enters the nucleus and interacts with transcription factors to induce gene expression, apoptosis, and, in male embryos, regression of the Müllerian duct (Catlin et al.,1997).

Recently it is revealed that MIS is also expressed in the female. MIS has been demonstrated to play an important role in the ovarian function. MIS is expressed by the granulosa cells of ovarian follicles (Vigier et al., 1984) and appears to regulate early follicle development (Durlinger et al., 2002). MISRII is detected in the endometrial tissue. MIS is a suppressor of cell division, and it blocks meiosis II in the ovary and inhibits granulosa cell division (Kim et al., 1992). However, its roles during decidualization in uterus are not unmasked. Therefore, In present study used adenovirus carring MISR II. This MISR II infected in *in vitro* cultured uterine stroma cell. So, the present study aimed to investigate the physiological roles of MIS in differentiation during implantation.

MATERIALS AND METHODS

Experimental animals

All experiment involving animals were conducted according to the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health. CD-1 and MIS knockout mice (MIS-KO) were maintained under standard condition at Sungshin Women's University diurnal rhythm kept under the 14L : 10D schedule with light-on at 06:00 hr and clean room system. Animals were fed a standard rodent diet and water *ad libitum* from weaning at 21 days after birth.

MIS-KO mice were purchased from The Jackson Laboratory and maintained. Genotyping was performed according to the direction of the Jackson Laboratory.

Total RNA extraction

Total RNAs of uterine stroma cells were extracted using TRIzol reagent (Invitrogen, San Diego, CA, USA) according to manufacturer's instruction with modification. Briefly, the samples were homogenized with Trizol reagent (0.8ml/100mg) and stored for 5 min at room temperature (RT). The chloroform of 0.16 ml/1 ml TRIzol reagent were added to the homogenates and shake vigorously for 15 sec. After then, the mixture kept for 15 min at RT and centrifuged 12000 RPM for 15 min at 4℃. After RNA in the aqueous phase was transferred into new tube, the RNA was precipitated by mixing 0.5 ml isopropyl alcohol, mixed softly, kept for 10 min at RT. And centrifuged 12,000 RPM for 8 min at 4℃. The supernatant was removed, The RNA pellet was

washed briefly in 0.8ml 75% ethanol, mixed by inverting, and centrifuged 7,500 RPM for 5 min at 4℃. The supernatant was removed, dried to remove ethanol and added 15 ul DEPC treated water. Total RNA quality and quantity were assessed by Agilent bioanalyer™ 2100 analysis.

First strand cDNA synthesis

In order to perform reverse-transcription, 5 μg total RNAs were used. First strand cDNAs were synthesized using Accuscript first strand cDNA synthesis kit (Stratagene, CA, USA) according to the manufacturer's instruction. Briefly, reaction reagents were 5 μg total RNA, 5.0 μl Accuscript buffer (10X), 1.0 μl oligo dT primer (0.5 μg/μl), 1.0 μl random primers (0.1 μg/μl), 2 μl dNTP mix (100 mM), RNase- free water. Reaction mixture was incubated at 65℃ for 5min, placed the tube at RT to allow the primers to anneal to RNA for 10min, after then added 4.0 μl DTT(100 mM), 2.0μl RNase block ribonuclease inhibitor (40 U/ml), 1.0μl Accuscript multiple temperature RT. The mixture was incubated at 42℃ for 1 hr and 70℃ for 15min to terminate cDNA synthesis.

Quantitative RT- PCR

For quantification of expression level, transcripts of target genes were amplified using RT-PCR (Table 1) and the specific primers (Table 2). The primer parameters were 50% GC contents, avoiding repeat base pair and lengthening 20-24 mer. Quantitative RT-PCR was performed using SYBR Premix Ex Taq™ (TaKaRa, Japan) and Thermal Cycler Dicd Real Time System TP800 (TaKaRa, Japan). Each reaction was run in triplicate and consisted of

1 μl cDNA. Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. The fold change in gene with the appropriate melting temperature. The fold change in gene expression was calculated using the △△Ct method with the housekeeping gene, ribosomal protein, Rplp0, as the internal control.

Preparation of an Adenovirus Encoding MISR II

The mouse MISR II coding region (1,7 kbp) was ligated into the pENTR™/D-TOPO (Invitrogen, cat #: K2400-20) vector; the vector had been amplified using Oneshot® Chemically competent E.coli cells. Clones were then purified and DNA was extracted using plasmid miniprep (GENEALL). The DNA clones screening confirmed sending to company (Macrogen). The product was then transferred into a pAd/CMV/V5-DEST™ Gateway Vector using LR recombination reaction. (attL- containing entry clone and the attR- containing pAd/CMV/V5-DEST™). The vector was amplified One Shot® ccdB Survival™2T1 Chemially Competent E.coli cells (Invitrogen, Life Technologies) and purified using QIA prep spin miniprep kit (Qiagen). The DNA clones screening confirmed sending to company (Macrogen). Subsequently, the pAd/CMV/V5-DEST™ vector encoding MISR II was linearized enzymatically with Pac I and transfected into 293A cells using DNA-Lipofectamine® 2000 complexes. The cells then produced an adenovirus encoding MISR II. The primary adenoviral stock was collected in media to prepare crude viral lysates. Different dilutions of adenovirus were obtained using titer method and used to infect 293A cells. This resulted in a titer of 1×10^{8}pfu/ml. The secondary cytopathic adenoviral stocks were then stored at - 80℃ after the same freeze-thaw cycles.

Protein extraction and Western blotting analysis

Before protein extraction, cells were washed using cold Y-PBS (0.7mM PMSF, 1 mM Benzamidine-HCl, 4 μg/ml Leupeptin, 2 μg/ml Aprotinin, 2 mM EDTA). Uterine stroma cell were homogenized in cold homogenization buffer (50 mM Tris-Cl, 150 mM NaCl, 10 mM β -mercaptoethanol, 2 mM $CaCl_2$, 0.1 mM PMSF, 1μM Leupeptin, 1 μM Pepstatin, 0.5 mM EDTA, 15% Glycerol, 0.1% NP-40). The homogenates were centrifuged to remove insoluble materials. The protein concentration was determined using protein dye reagent (Bio-Rad Laboratories, Inc., Richmond, CA) by Bradford assay. 30 μg /ml of protein were boiled in SDS/β-mercaptoethanol sample buffer, and loaded onto each lane of 10% SDS-PAGE. The proteins were separated by electrophoresis and then electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc., Richmond, CA) in transfer buffer (25 mM Tris base, 192 mM Glycine, 0.1% SDS, 20% Methanol, pH 8.3). The membranes were blocked in 5% skimmed dry milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 1 hr at RT, and washed three times with TBST. The membranes were incubated for 1 hr with rabbit polyclonal MISRII antibody (dilution 1:500); rabbit monoclonal beta actin antibody (dilution 1:500); rabbit ploYclonal progesterone receptor antibody (1:500). After incubation, membranes were washed three times and incubated for 30 min with horseradish peroxidase conjugated goat anti-rabbit IgG (dilution 1:1000);. The bands were detected using ECL solution (GE Healthcare, Little Chalfont, UK) by Kodac Image station.

Primary endometrial cells culture and *in vitro* decidualization induction and treatment of recombinant human MIS treatment

For *in vitro* decidualization of mouse endometrial stromal cells (mESCs), mESC were isolated of CD-1 female mice in PMSG primed. Uteri were cut into $1mm^3$ pieces and incubated in DMEM:F12 (Sigma-Aldrich Corp., St. Louis, MO) supplemented with 2 mg/ml Collagenase Type I (Gibco, Rockville, MD, USA) and 100 μg/ml penicillin/streptomycin (Sigma-Aldrich Corp., St. Louis, MO) for 2 hr at 37℃ in shaking incubator with 150 RPM. After incubation, the solution was passed through 100 μm and 40 μm sieve (BD Falcon, BD Biosciences, San Jose, CA) and centrifuged at 1000 rpm for 5 min. The mESCs were then resuspended in DMEM:F12 supplemented with 10% charcoal dextran-stripped FBS (cFBS; Sigma -Aldrich Corp., St. Louis, MO). mESCs were plated in 4-well cell culture plates onto glass cover slip at 1×10^5 cells per well, and after 30 min, medium was aspirated, and changed fresh media containing 15 nM β-estradiol (E_2) and 10% cFBS. After 24 hr, medium was changed to DMEM:F12 with 10% cFBS without steroid hormone. After two day, decidualization was induced by DMEM:F12 media containing 10% cFBS, 1 nM β-estradiol (E_2) and 1 μM progesterone (P_4) (Sigma-Aldrich Corp., St. Louis, MO). Control samples received empty vector supplementation. MISRII overexpression and MISRII overexpression + 50 ng/ml rhMIS were treated with 1 nM E_2 and 1 μM P_4. The cells were harvested at -48 hr, 0 hr, 24 hr, 48 hr, 72 hr, 96 hr, and 120 hr respectively.

Cell viability assay

The stroma cells were transfected with MISR II and rhMIS and cultured in decidual induction medium. After 120hr, the cells were collected by trypsinization, and stained with Trypan blue to cell viable test, which was carried out in hemocytometer. Cells were diluted 1:1 in 0.4% Trypan blue (Gibco Laboratories) and counted.

Oil-red-o and PAS stain of endometrial stromal cells

After induction, the cells were washed with phosphate buffered saline (PBS). The cells were fixed with 0.16% picric acid in 0.4% paraformaldehyde in room temperature for 30min, and then briefly washed with PBS. The cells were stained with freshly prepared Oil Red O solution for 1hr. The morphology was analyzed with microscope.

After induction, the cells were washed with phosphate buffered saline (PBS). The cells were fixed with 0.16% picric acid in 0.4% paraformaldehyde in room temperature for 30 min, and then briefly washed with PBS. The cells were stained with 0.5% periodic acid solution for 20 min, and then briefly washed with H_2O, and then stained Schiff reagent for 20 min. Washed with warm water and counter stained with Mayer's hematoxylin for 1min. Washed with tap water for 10 min. The morphology was analyzed with microscope. The images were analyzed using NIH Image J software for cell stain intensity.

Alkaline phospatase(ALP) staining

After induction, the cells were washed with phosphate buffered saline (PBS). The cells were fixed with 0.16% picric acid in 0.4% paraformaldehyde in room temperature for 2 min, and then briefly washed with PBST. It was incubated in 200 μl staining solution at room temperature for 10min to identify the blue ALP positive cells. Cells were washed with H_2O to stop the staining reaction. The morphology was analyzed with microscope. The images were analyzed using NIH Image J software for cell stain intensity.

BRDU/7-AAD cell cycle analysis

Harvested cell at time points as indicated were labeled with 5-bromo-2'-deoxyuridine (BrdU) (BD-Bioscience) and cultured at 37℃ in 5% CO_2 for 24 hr. The cells were trypsinized, fixed, and stained with anti-BrdU and 7-aminoactinomycin-D(7-AAD) following the protocol provided by the manufacturer. The cell cycle data was collected via FACSCalibur (BD Biosciences).

Statistics

All experiments were conducted at least in triplicate. The Student's t-test was performed to evaluate the statistical significance between control and experiment group. Results were presented as mean ± SEM. Values of $P < 0.05$ were considered to be significantly different.

Table 1. Thermal cycler schedule

step		Temperature (℃)	Time
Hold	Hold	94	30 sec
3 step PCR (45 cycle)	Denaturation	95	1 min
	Annealing	59	30 sec
	Extension	72	1 min
Dissociation	Denaturation	95	15 sec
	Annealing	60	30 sec
	Extension	95	15 sec
Hold		4	Indefinitely

Table 2. Sequence- specific of primers

Gene	symbol	NCBI gene reference	Premer sequence (5'-3')	
MISR II	MISR II	NM_144547.2	S	CACCAGGATGCTGGGGACACT
			AS	CTACTCATTTACATACACCTGAACAGTGTCGCCT
Decidual relate prolactin protein	dPRP	NM_010088.2	S	CTGCTGGTGGTTTCAAACTTGC
			AS	GGTGGGTTTGTGACATTAGAGTGG
Cytotoxic T-lymphocyte antigen 2β	CTLA2 β	NM_009883	S	ACAGAAGACTCATGTGGGAGGAGA
			AS	TTCTCAGCTTTCTGTGGGCA
Progesteron receptor	PR	NM008829.2	S	CATGACTGAGCTGCAGGCAAA
			AS	AAGCTCTGGCCCAAAGAGACA
Ribosomal protein, large, P0	Rplp0	NM_007475	S	CGACCTGGAAGTCCAACTACTTCCT
			AS	ATGCTGTTGGCCCAAATAAGGTGC

Table 3. Antibody information

name		company
Progesterone receptor	-Rabbit polyclonal	Santa cruz
MISR II	-Rabbit polyclonal	Santa cruz
β-Actin	-Rabbit polyclonal	Abcam
Goat Anti-Rabbit IgG-HRP conjugate		Bio-Rad

RESULTS

Overexpression MISR II gene in mouse uterine stroma cell during decidualization

To evaluate the cell specific expression and time-dependent expression, western blotting was performed with MISRII specific antibody. The normalization was performed with β-actin protein.

Empty vector treat group (control group) was showed endogenous MISRII expression as expected (Fig. 1A). Previously, it was explored that MISRII is expressed in decidualized stromal cells. After 48 hr of transfection, its level was about two times higher than control (Fig. 1C).

From these results, it is confirmed that the expression profiles of MISRII construct group were overexpressed about 2 fold than empty vector group. So we are using these construct by next experiment.

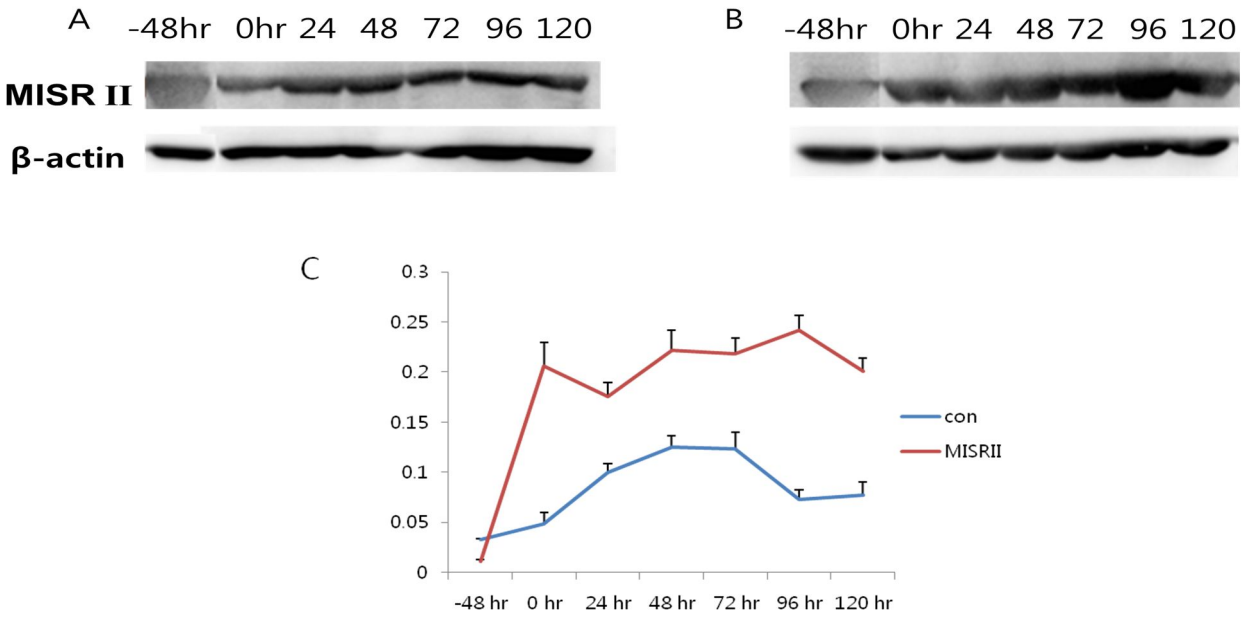


Fig 1. Overexpression MISR II gene in mouse uterine stroma cell
(A) Control group; empty vector treatment and 1 nM β-estradiol (E_2) and 1 μM progesterone (P_4) treatment.
(B) MISRII overexpression in the presence of E_2 and P_4.
(C) Normalization of the MISRII protein.

Viability of MISR II overexpressing cells

Whether the transfection is caused of cell death, cell viable assay was performed mentioned in Materials and Methods. In all group, there was no difference in viability (Fig. 2).

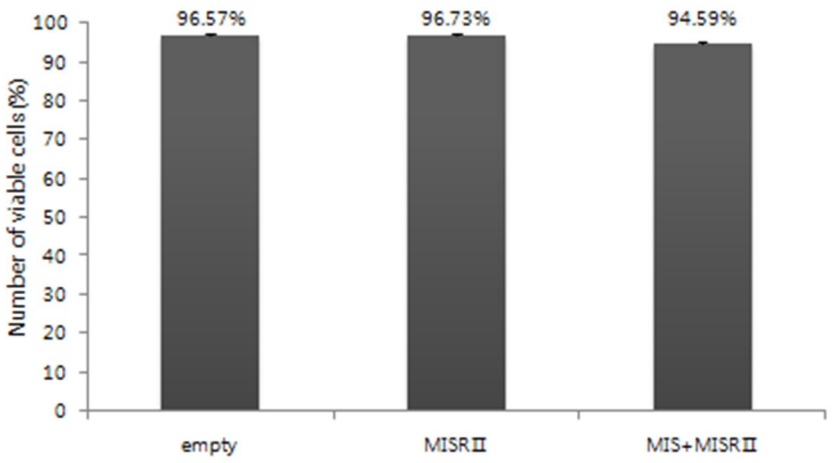


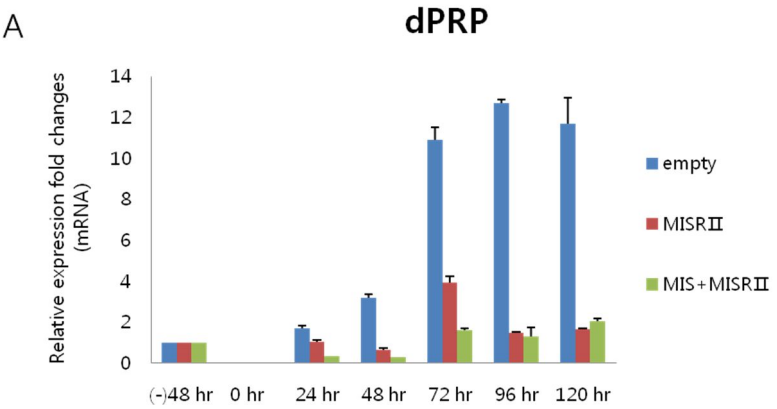
Fig 2. Cell viability test

The mESCs was treated with empty vector (control group), MISRII, combination rhMIS/MISRII. The cells were counted in a total cell count assay. The y-axis represents mean number of viable cell (%).

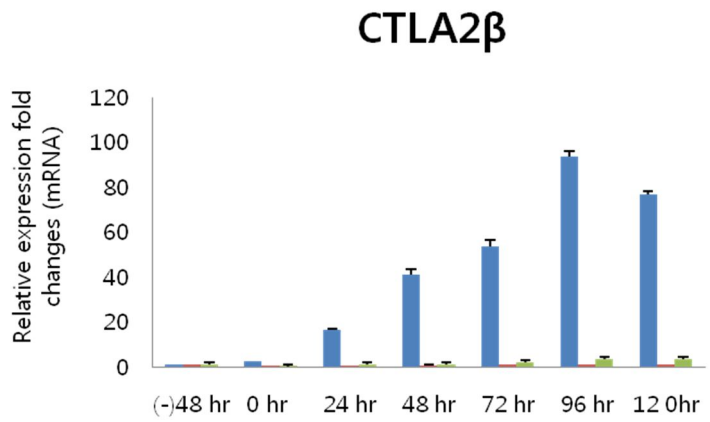
MIS suppresses decidualization of stroma cell

To evaluate the potential role of MIS in stromal cells during decidualization *in vitro,* decidual induction culture system was employed. After *in vitro* decidualization induction, total RNA was extracted from the cells at - 48 hr, 0 hr, 24 hr, 48 hr 72 hr, 96 hr and 120 hr. By the RT-PCR result, the expression levels of decidualization maker gene mRNA significantly decreased in rhMIS treat group at 24 hr post significantly induction. After 48 hr, the expression levels of decidual prolactin related protein (dPRP), Cytotoxic T-lymphocyte antigen-2β (CTLA2β), progesterone receptor (PR) specific mRNA were significantly decreased in rhMIS or MISRII treated group (Fig. 3). It means that the cytokinesis during decidualization mainly happens until 48 hr. These results clearly explain the histological change of the decidualizing cells. These results also mean that MIS derived from decidua can suppress the cell differentiation.

A


dPRP
Relative expression fold changes (mRNA)
14
12
10
8
6
4
2
0
(-)48 hr
0 hr
24 hr
48 hr
72 hr
96 hr
120 hr
empty
MISRII
MIS+MISRII

B


CTLA2β
Relative expression fold changes (mRNA)
120
100
80
60
40
20
0
(-)48 hr
0 hr
24 hr
48 hr
72 hr
96 hr
12 0hr

C

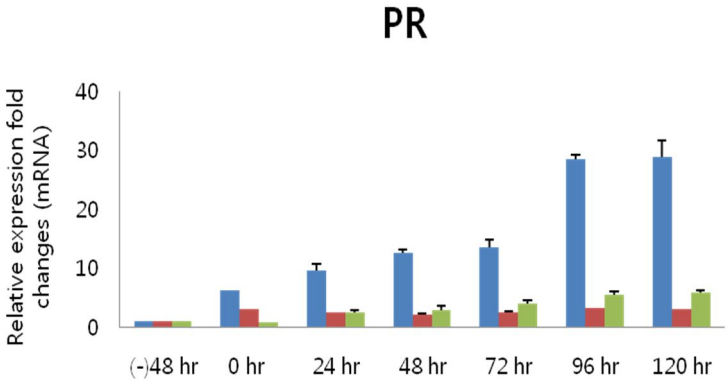

PR
Relative expression fold changes (mRNA)
40
30
20
10
0
(-)48 hr
0 hr
24 hr
48 hr
72 hr
96 hr
120 hr

Fig 3. The MIS suppresses the decidualization of endometrial stromal cell

The expression levels of dPRL (A), CTLA2β (B), PR (C) mRNA expression. Mouse endometrial stromal cells (mESCs) were isolated of CD-1 mice in PMSG primed. During decidualization induction, control group; empty vector and 1 nM β-estradiol (E_2) and 1 μM progesterone (P_4) treatment, MISRII and 50ng/ml rhMIS+ MISRII in the presence of E_2 and P_4. After 120 hr, total mRNA was extracted from the cells. Analysis was repeated three times, and mRNA level normalized using Rplp0 mRNA level as the housekeeping control. Values represent the mean ± SD. Statistical analysis was performed by Student's t-test with concentration matched. Statistically significant change ($P<0.05$).

The MIS suppresses the glycogen and lipid of endometrial stromal cell decidualization induction

To evaluate the potential role of MIS in stromal cells during decidualization *in vitro,* decidual induction culture system was employed. Decidualized cells have cytoplasmic accumulation of glycogen and lipid droplets. To test the decidualized cell, After *in vitro* decidualization induction, The cells were fixed and then stained with PAS and Oil-Red-O (Fig. 4 ,6). PAS signal intensity was significantly lower both in MISRII overexpression and rhMIS+MISRII than the control (Fig. 5).

Oil-red-O stain signal intensity was also significantly low both in MISRII overexpression and rhMIS+MISRII than the control (Fig. 7).

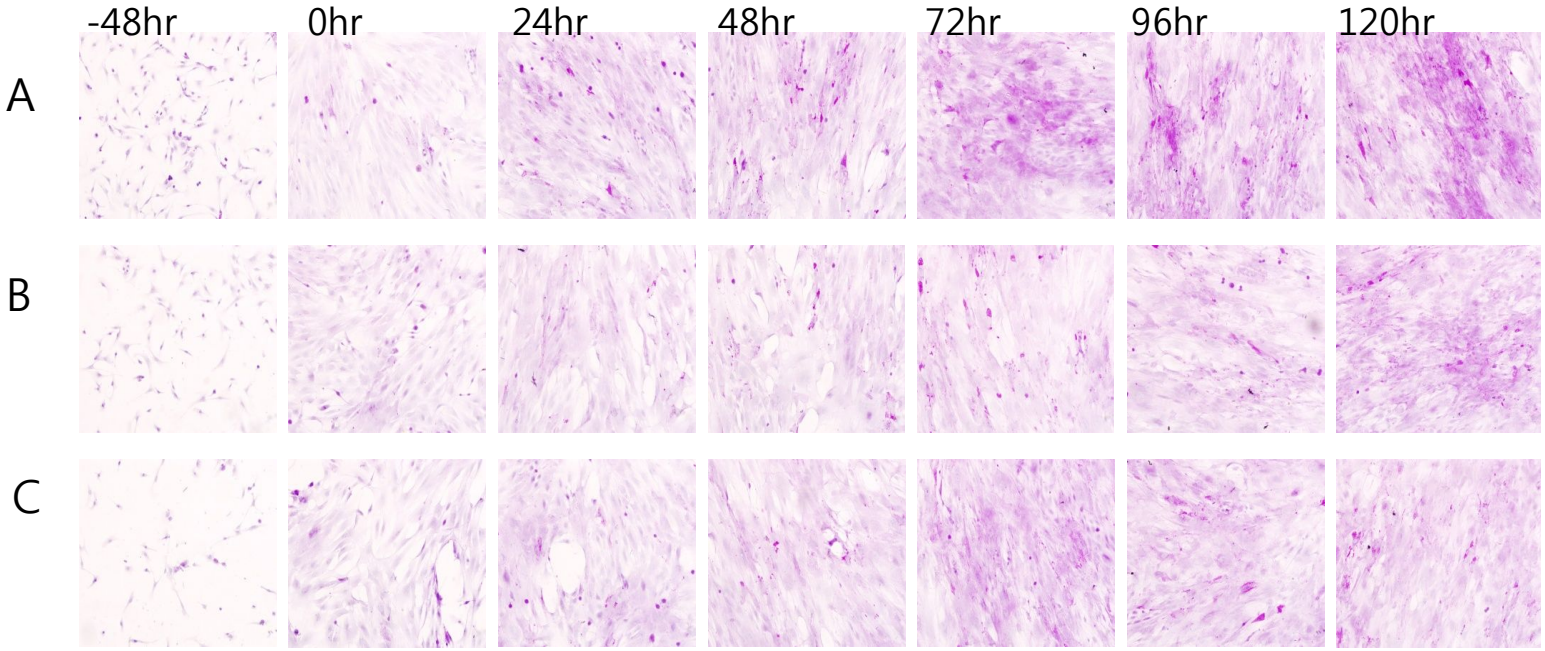


Fig 4. The MIS suppresses the glycogen of endometrial stromal cell decidualization induction

(A) Control group; empty vector and 1nM β-estradiol (E_2) and 1 μM progesterone (P_4) treatment (B) MISRII (C) 50ng/ml rhMIS+ MISRII in the presence of E_2 and P_4. Magnification (A), (B), (C) x200.

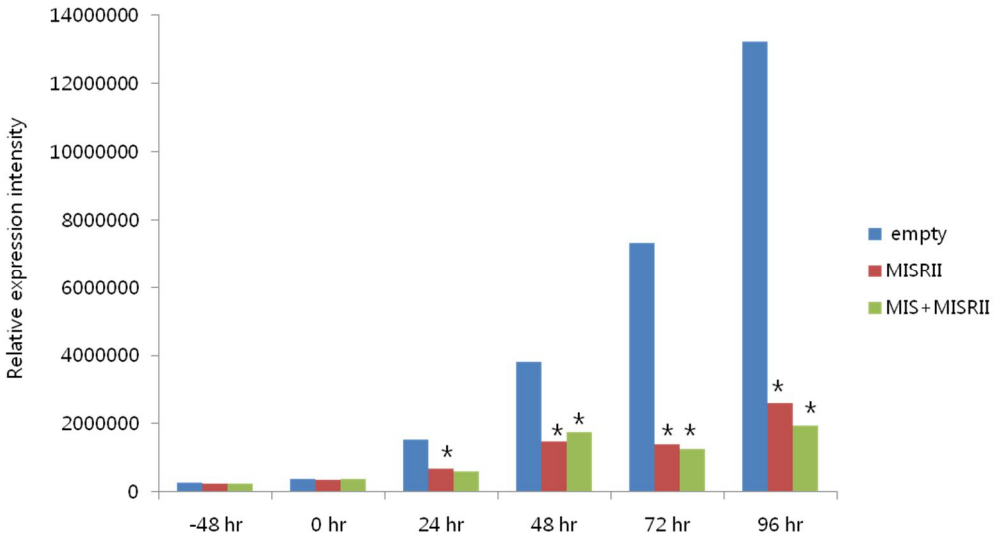


Fig 5. The relative PAS staining intensity

The glycogen significantly decreased in MISR II , combination rhMIS/MISRII treat group at 48hr post significantly induction. The images were analyzed using NIH Image J software for cell stain intensity. Statistical analysis was performed by Student's t-test with concentration matched. *, Statistically significant change (p<0.05) compared with control and experimental group.

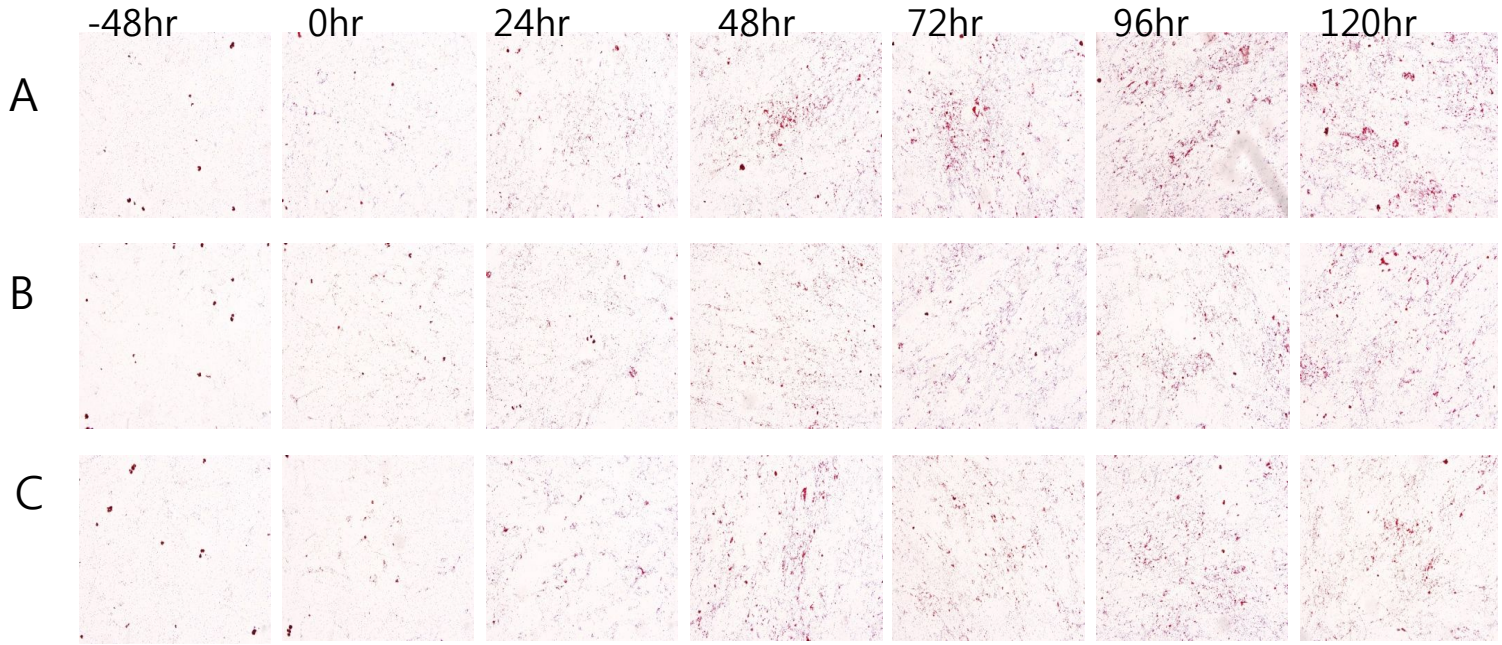


Fig 6. The MIS suppresses the lipid of endometrial stromal cell decidualization induction

(A)Control group; empty vector and 1nM β-estradiol (E_2) and 1 μM progesterone (P_4) treatment (B) MISRII (C) 50ng/ml rhMIS+ MISRII in the presence of E_2 and P_4. Magnification (A), (B), (C) x200.

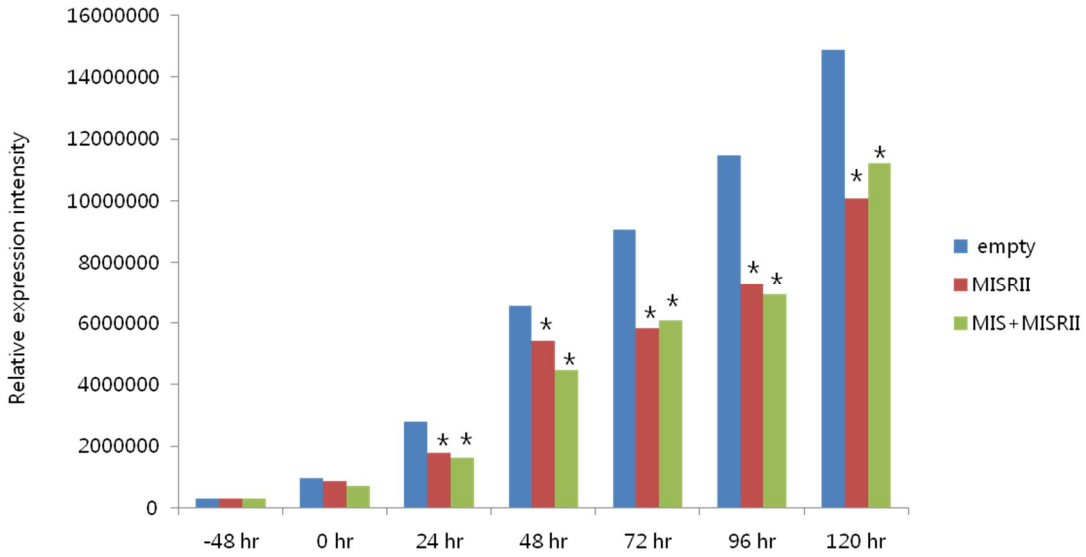


Fig 7. The relative Oil-red-o staining intensity

The Oil-red-O stain significantly decreased in MISR II , combination rhMIS/MISRII treat group at 48hr post significantly induction. The images were analyzed using NIH Image J software for cell stain intensity. Statistical analysis was performed by Student's t-test with concentration matched. *, Statistically significant change (p<0.05) compared with control and experimental group.

Alkaline phosphatase (ALP) activity decrease by MIS treatment

Decidual inductions were performed in containing control group; empty vector and 1 nM β-estradiol (E_2) and 1 μM progesterone (P_4) treatment, MISRII and 50ng/ml rhMIS+ MISRII in the presence of E_2 and P_4. Alkaline phosphatase activity was very low in MISRII and 50ng/ml rhMIS+ MISRII containing induction medium (Fig. 8 A,B).

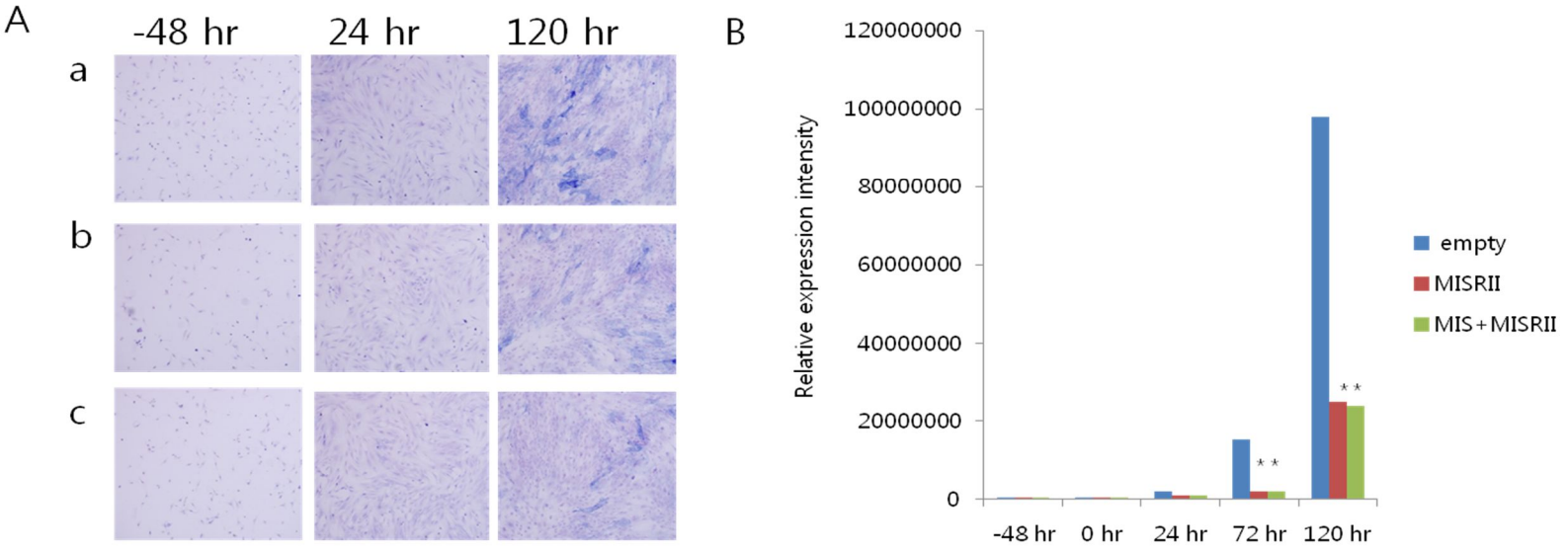


Fig 8. The MIS suppresses the ALP of endometrial stromal cell decidualization induction

(A) (a)Control group; empty vector and 1 nM β-estradiol (E_2) and 1 μM progesterone (P_4) treatment. (b) MISRII (c) 50ng/ml rhMIS+ MISRII in the presence of E_2 and P_4. Magnification (a), (b), (c) x100.

(B) The relative ALP staining intensity

The ALP stain significantly decreased in MISR II, combination rhMIS/MISRII treat group at 72hr post significantly induction. The images were analyzed using NIH Image J software for cell stain intensity.

Suppressed progesterone receptor expression by MIS

Progesterone receptors have work for decidualized cell. So the changes of the expression levels of RPs were analyzed with western blotting methodology. All of PRs, PR-α and PR-β, were decreased by MISRII overexpression (Fig. 9). PR-α and PR-β were detected from 0hr in control but from 72 hr both in MISRII over expression and MIS + MISRII overexpression (Fig.10 A,B).

The statistical difference between MISRII overexpression and MIS+MISRII overexpression was no both PR-α and PR-β except 72 hr and 120 hr of induction (Fig. 10 A,B).

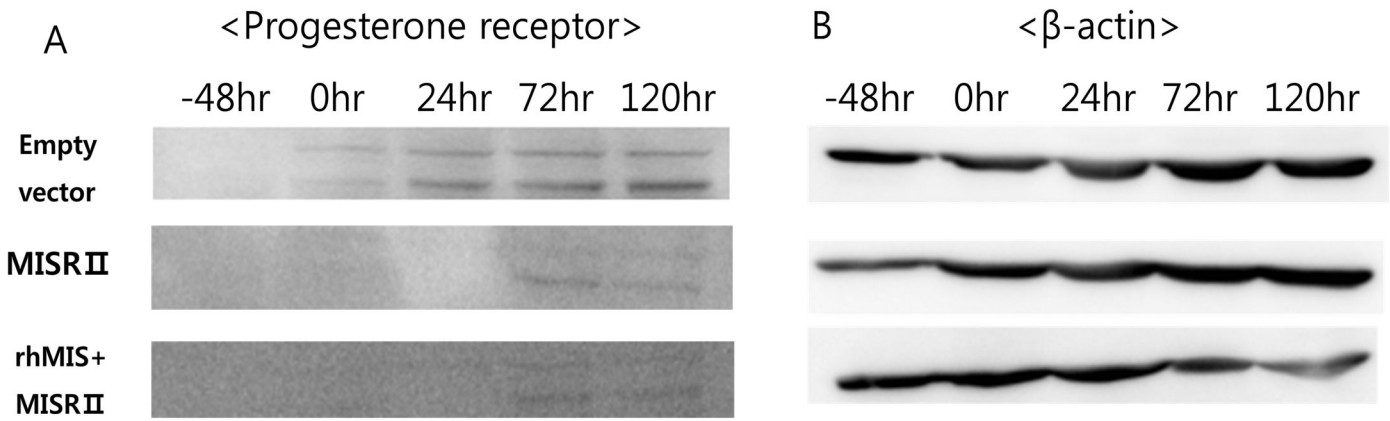


Fig 9. The MIS suppresses the progesterone receptors of endometrial stromal cell

(A) The PR protein was detected by western blot.

(B) β-actin protein produced in the endometrial stromal cell during decidualization induction was detected by western blot.

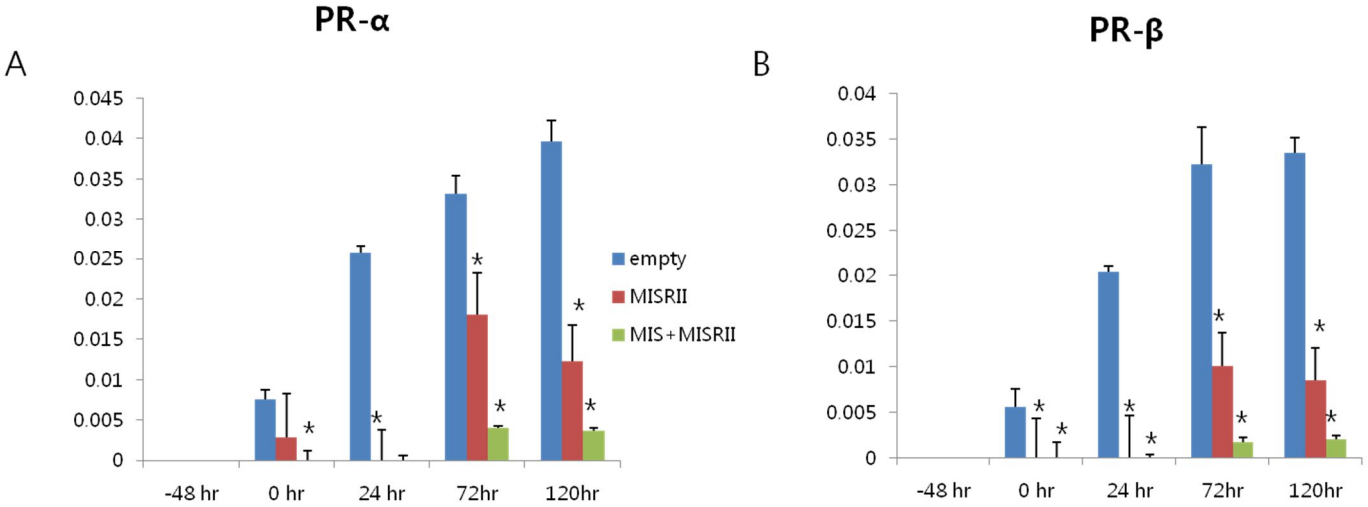


Fig 10. Quantification of protein level

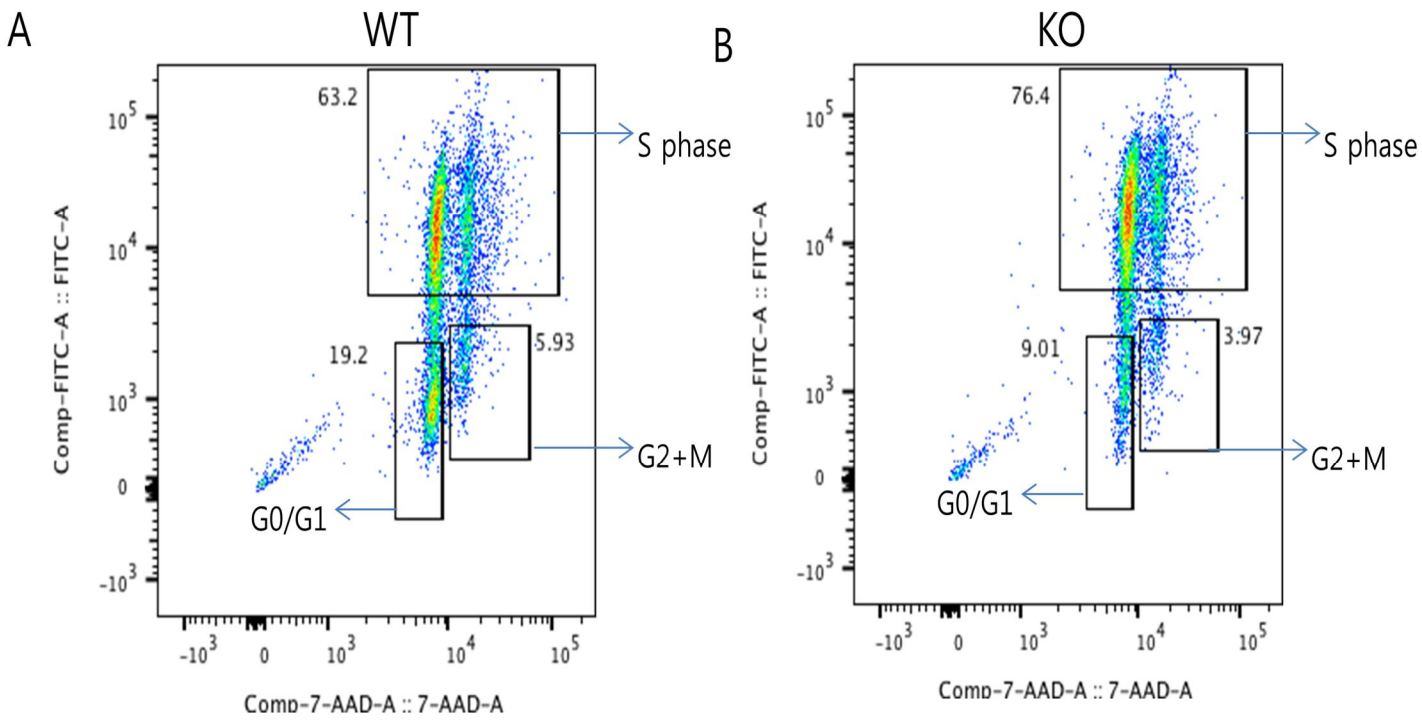
(A) PR-a protein level normalized using β-actin protein level as the housekeeping control.

(B) PR-b protein level normalized using β-actin protein level as the housekeeping control.
Values represent the mean ± SD. Statistical analysis was performed by Student's t-test with concentration matched.

Statistically significant change ($P<0.05$).

Proliferation analysis in MIS knockout stromal cells

To evaluate the role of MIS in stromal cell proliferation during decidualization induction, MIS knockout mice (6-8 Wks) were used. Uterine stromal cells were cultured and induced as described in Materials and Methods. Stromal cell proliferation is activated after decidual induction. Stromal cell proliferation was significantly higher in MIS knockout stromal cells than the wild stromal cells. At 24 hr of induction, percentage of S phase was 76.4% in MIS knockout mice and 63.2% in wild type (Fig. 11).


A
WT
63.2
S phase
19.2
5.93
G2+M
G0/G1
Comp-FITC-A :: FITC-A
Comp-7-AAD-A :: 7-AAD-A
B
KO
76.4
S phase
9.01
3.97
G2+M
G0/G1
Comp-FITC-A :: FITC-A
Comp-7-AAD-A :: 7-AAD-A

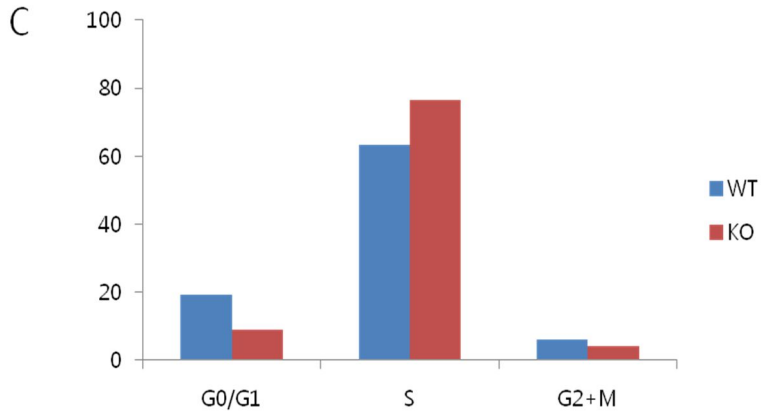


Fig 11. Cell cycle analysis of endometrial stromal cell

(A) Representative FITC-BrdU/7-AAD cell cycle analysis of Wild Type CD-1mouse endometrial stromal cell in vitro decidualization induction 24hr.

(B) Representative FITC-BrdU/7-AAD cell cycle analysis of MIS Knock out mouse endometrial stromal cell in vitro decidualization induction 24hr.

(C) Cell cycle analysis at different phases (%).

DISCUSSION

In mammals, a hormonally primed receptive uterus is a prerequisite for attachment of the embryo to the uterine epithelium to initiate the process of implantation (Bazer et al., 2009). The mouse model has been used extensively to study the molecular signaling mechanisms underlying the process of embryo implantation (Dey et al., 2004). The main feature of decidual event is the transformation of endometrial fibroblasts into a new type of cells, the decidual cells which exhibit epithelial characteristics (Teodoro et al., 2003). Endometrial stromal cells transform into large, polyhedral, and often polyploidy in character (Zaytsev et al., 1987). Ultrastructurally decidualized cells have cytoplasmic accumulation of glycogen and lipid droplets (Gellersen et al., 2007). Decidualized cell express decidual markers, such as dPRP, CTLA2β and PR.

MIS detected in the endometriosis tissues (Carrarelli et al., 2014). In adult human endometrium the functional MIS signal transduction system is existed and suggested a negative regulating factor in cellular viability (Wang et al.. 2009). Using in vitro decidualization model, the uterine stromal cell was well proliferated and induced to decidual cells. To overexpress MISRII adenoviral MISRII particles were transfected but it was not induced cell death. In vitro decidual induction of uterine stromal cell or overexpression of MISRII did not work as negative regulator of cellular viability. From the MTT assay there were no differences between control and MISRII and rhMIS+MISRII groups (data did not show). In addition, it is revealed that the dead cell number was not increased in all groups during decidual induction in Trypan blue assay. These results are different from the Wang's report (2009). Wang et al (2009) suggested that MIS may regulate negatively endometrial cellular viability. It may be cause of the difference of physiological status and the difference of species.

Adenoviral MIS infection increased about two fold the MISRII proteins. In adenoviral MISRII transfected and decidual induced stromal cell, the mRNA levels of dPRP, CTLA2β, PR were significantly decreased. The amount of accumulated

glycogen and lipids were also decreased. In addition MISRII overexpression suppressed the decidual induction and the number of the decidualized cells was decreased. It means that MIS suppress the dicidual differentiation of stromal cell through MISRII.

MIS is a known suppressor of the cell cycle in various cells (Mac Laushlin and Donahoe., 2010). P16, a member of the INK4 family of cyclin-dependent kinase (CDK) inhibitors, mediated MIS effects in cell cycle. It inhibits the kinase activity of cyclin/CDK 4/6 complexed and disrupt cell cycle progression (Sherr and Roberts., 1995). Interestingly, the stromal cell proliferation was increased in MISKO mice during decidual induction. On the other hand, MIS and MISR II was express in decidualized cells. Those means that MIS is suppress the stromal cell proliferation through its receptors.

MISRII overexpression was the cause of suppression of decidualization without MIS. In rhMIS + MISRII overexpression group, the proliferation rate was not different form the MISRII overexpression. It may be the results of endogenous secretion of MIS in decidualizing cells and the MIS in FBS.

In summary, the decidualization markers were dramatically decreased by MISR II overexpression. Its level showed similar patterns with that of the mRNA levels of it. The intensity of PAS, Oil-Red-O and ALP staining was dramatically decreased. In MIS knockout stromal cell, the cell proliferation was increased by MISRII overexpression. Based on them it is suggested that MIS suppress the stromal proliferation and decidual differentiation along with its receptor.

REFERENCE

Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G. 2009. Comparative aspects of implantation. Reproduction. 138 :195-209

Barbie TU, Barbie DA, MacLaughlin DT, Maheswaran S, Donahoe PK. 2003. Müllerian Inhibiting Substance inhibits cervical cancer cell growth via a pathway involving p130 and p107. Proceedings of the National Academy of Sciences.100:15601–15606.

Carralelli P, Rocha AL, Belmonte G, Zupi E, Abrao MS, Arcuri F, Piomboni P, Petraglia F. 2014. Increased expression of anti müllerian hormone and its receptor in endometiosis. Fertil Steril 101:1353-1358.

Catlin, E. A., Tonnu, V.V., Ebb, R. G., Pacheco, B. A., Manganaro, T. F., Ezzell, R. M., Donahoe, P. K. and Teixeira, J. 1997. Müllerian inhibiting substance inhibits branching morphogenesis and induces apoptosis in fetal rat lung. Endocrinology 138,790-796.

Cheon YP, Li Q, Xu X, DeMayo FJ, Bagchi IC, Bagchi MK. 2002. A genomic approach to identify novel progesterone receptor regulated pathways in the uterus during implantation. Mol Endocrinol 16:2853-71

Clarke TR, Hoshiya Y, Yi SE, Liu X, Lyons KM, Donahoe PK. 2001. Müllerian inhibiting substance signaling uses a bone morphogenetic protein (BMP)-like pathway mediated by ALK2 and induces SMAD6 expression. Mol Endocrinol 15:946-959.

Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H . 2004. Molecular cues to implantation. Endocr Rev 25:341-373

Donahoe PK, Fuller AF Jr, Scully RE, Guy SR, Budzik GP. 1981. Müllerian inhibiting substance inhibits growth of a human ovarian cancer in nude mice.

Ann Surg 194:472-80.

Dimitriadis E, Menkhorst EM, Salamonsen LA, Paiva P. 2012. Review: LIF and IL11 in trophoblast-endometrial interactions during the establishment of pregnancy. Placenta 24: S99-S104.

Donahoe PK, Swann DA, Hayashi A, Sullivan MD. 1979. Müllerian duct regression in the embryo correlated with cytotoxic activity against humanovarian cancer. Science 205:913-915.

Durlinger ALL, Visser JA, Themmen APN. 2002. Regulation of ovarian function: the role of Anti-Müllerian Hormone. Reproduction 124:601–609.

Farrar JD, Carson DD. 1992. Differential temporal and spatial expression of mRNA encoding extracellular matrix components in decidua during the peri-implantation period. Biol Reprod 46:1095-1108.

Godkin JD, Doré JJE. 1998. Transforming growth factor b and the endometrium. Reproduction 3:1–6.

Gellersen B. Brosens IA, Brosens JJ. 2007. Decidualization of the humen endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med. 25:445-53

Ha TU, Segev DL, Barbie D, Masiakos PT, Tran TT, Dombkowsk D, Glander M, Clarke TR, Lorenzo HK, Donahoe PK, Maheswaran S. 2000. Müllerian Inhibiting Substance inhibits ovarian cell growth through an Rb-independent mechanism. J Biol Chem 275:37101–37109.

Herington JL, Underwood T, McConaha M, Bany BM. 2009. Paracrine signals from the mouse conceptus are not required for the normal progression of decidualization. Endocrinology 150:4404– 4413.

Huan Y, Yi X, Rong Y, Sha-Li W, XI. 2008. Expression of p16 INK4a in mouse endometrium and its effect during blastocyst implantation. Acta Physiol Sin 60:547-552.

Jeff Wang, Cary Dicken, Joyce W. Lustbader, Drew V. Tortoriello. 2009. Evidence for a Müllerian-inhibiting substance autocrine/paracrine system in adult human endometrium. Fertility and Sterility. Vol 91, no 4.

Joswig A, Gabriel HD, Kibschull M, Winterhager E. 2003. Apoptosis in uterine epithelium and decidua in response to implantation: evidence for two different pathways. Reprod Biol Endocrinol 1:44.

Kim JH, Seibel MM, Maclaughlin DT, Donahoe PK, Ransil BJ, Hametz PA, Richards CJ. 1992. The inhibitory effects of müllerian-inhibiting substance on epidermal growth factor induced proliferation and progesterone production of human granulosa-luteal cells. J Clin Endocrinal metab. 75:911-7

Lee KY, Jeong JW, Wang J, Ma L, Martin JF, Tsai SY, Lydon JP, DeMayo FJ. 2007. Bmp2 is critical for the murine uterine decidual response. Mol Cell Biol 27: 5468-5478.

MacLaughlin DT, Donahoe PK. 2010. Mullerian inhibiting substance/anti-Mullerian hormone: a potential therapeutic agent for human ovarian and other cancer. Future Oncol. 391-405.

Masiakos PT, MacLaughlin DT, Maheswaran S, Teixeira J, Fuller AF Jr, Shah PC,Kehas DJ, Kenneally MK, Dombkowski DM, Ha TU, Preffer FI, Donahoe PK. 1999. Human ovarian cancer, cell lines, and primary ascites cells express the human Müllerian inhibiting substance (MIS) type II receptor, bind, and are responsive to MIS. Clin Cancer Res 5:3488-3499.

Paria BC, Ma W, Tan J, Raja S, Das SK, Dey SK, Hogan BLM. 2001. Cellular and molecular responses of the uterus to embryo implantation can be elicited by

locally applied growth factors. Proceedings of the National Academy of Sciences 98:1047–1052.

Ramathal CY, Bagchi IC, Taylor RN, Bagchi MK. 2010.Endometrial decidualization: of mice and men. Semin Reprod Med.28:17-26.

Rodolfo Favaro, Paulo A, Abrahamsohn and M, Telma Zorn. 2014. Decidualization and endometrial extracellular matrix remodeling.The Guide to Investigation of mouse pregnancy,125-142

Shuya LL, Menkhorst EM, Yap J, Li P, Lane N . 2011. Leukemia Inhibitory Factor Enhances Endometrial Stromal Cell Decidualization in Humans and Mice. PLoS ONE : e25288.

Sherr CJ, Roberts JM. 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev.1149-63

Takahashi M, Koide SS, Donahoe PK. 1986. Müllerian inhibiting substance asoocyte meiosis inhibitor. Mol Cell Endocrinol 47:225-34.

Tan J, Paria BC, Dey SK, Das SK. 1999. Differential uterine expression of estrogen, progesterone receptors correlates with uterine preparation forimplantation and decidualization in the mouse. Endocrinology 140:5310-5321.

Teodoro WT, Witzel SS, Velosa APP, Shimokimaki M, Abrahanmsohn PA, Zorn TMT. 2003. Increase of Interstitial Collagen in the Mouse Endometrium During Decidualization. Connective Tissue Research. 44: 96-103

Tran TT. 2006. Müllerian inhibiting substance regulates androgen-induced gene expression and growth in prostate cancer cells through a nuclear factor-{kappa} B-dependent Smad-independent mechanism. Mol Endocrinol 20:2382-91.

Visser JA. 2003. AMH signaling: from receptor to target gene. Mol Cell Endocinol 211:65-73.

Visser JA, Durlinger ALL, Peters IJJ, Heuvel ER, Rose UM, Kramer P, Jong FH, Themmen APN. 2007. Increased oocyte degeneration and follicular atresia during the estrous cycle in Anti-Müllerian Hormone null mice. Endocrinology 148;2301–2308.

Vigier B, Picard JY, Tran D, Legeai L, Josso N. 1984. Production of anti-Müllerianhormone: another homology between sertoli and granulosa cells. Endocrinology 114:1315-20.

Wallen JW, Cate RL, Kiefer DM, Riemen MW, Martinez D, Hoffman RM, Donahoe PK, Von Hoff DD, Pepinsky B, Oliff A. 1989. Minimal antiproliferative effect of recombinant Müllerian Inhibiting Substance on gynecological tumor cell lines and tumor expiants. Cance Res 49:2005-2011.

Wang J, Dicken C, Lusbader Jw, Tortoriello DV. 2009. Evidence for a Mullerian-inhbiting substance autocrine/paracrine system in adult human endometrium. Fertil Steril 91:1195-1203.

Zaytsev p, Taxy JB. 1987. Pregnancy-associated ectopic deciduas. Am J surg pathol. 526-30.

ABSTRACT

Roles of müllerian inhibiting substances in stromal basal function during implantation

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The endometrium is one of the most complex tissues; it undergoes dynamic change because it has to remodel in response to implantation and pregnancy processes. Decidualization describes the differentiation and proliferation of endometrial stromal cells into morphologically and functionally distinct decidual cells. During that period uterus undergoes decidualization, functional and histological changes including cell proliferation, differentiation, apoptosis. This delicate condition is regulated by various factors such as steroid hormones, cytokines, and growth factors. The transforming growth factor-β (TGF-β) superfamily constitutes more than 40 members of structurally related cell regulatory proteins including TGF-β, inhibins, activins, born morphogenetic proteins (BMP), and Müllerian inhibiting substance (MIS). This essential in developmental and physiological processes which involve in the regulation of cell proliferation, differentiation, immunoregulation, angiogenesis and the

remodelling of extracellular matrix. Also, this family is associated with reproductive processes. From previous our studies, we show that the spatio-temporal expression of MIS and MISR II in mouse uteri during pregnancy. After implantation, MISR II was mainly localized in the decidual cell surrounding the implanting embryo and in the deep stroma cells adjacent to the myometrium. These result suggested MISR II signal pathway works as autocrine and paracrine fashion after decasualization. Put together, we hypothesized that MIS/MISR II regulate the functional status of deep stroma layer's. In present study aimed to investigate that using i*n vitro* primary endometrial stromal cell culture model for decidualization we evaluate the decidual inhibitory role of MISR II in proliferation during implantation. The aim of study is to clarify the physiological role of MIS action during pregnancy. Decidualization was induced by 1nM E and 1uM P. Control samples received empty vector and 1nM E and 1uM P. Experimental samples received MISR II virus and 50ng/ml rhMIS and 1nM E and 1uM P. The cells were harvested at -48hr, 0hr, 24hr, 48hr, 72hr, 96hr, and 120hr respectively. The expression levels of panel of decidual marker genes were analyzed with real-time RT-PCR, western blotting, and biochemical staining. The decidualization markers were dramatically decreased during treated rhMIS and MISR II virus. The profiles of those proteins were analyzed by Western blot. The normalization was performed with β-actin protein. Its level showed similar patterns with that of the mRNA levels of it. The intensity of PAS, Oil-Red-O and ALP staining was

dramatically decreased during treated rhMIS and MISR II virus. We have provide evidence that an MIS/ MISR II regulate the differentiation of stroma cell during decidualization. MIS suppress the stromal proliferation and decidual differentiation along with its receptor.

감사의 글

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