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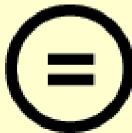
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석사학위 청구논문

Various Cell Death Patterns During Early Pregnant Uterus in Mouse

2017

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논 문 개 요

임신초기에 관찰되는 자궁은 포배가 착상하고 발생할 수 있는 구조적·기능적 환경을 조성하면서 많은 변화를 겪는다. 조직학적 변화는 배아의 발생과 시기적 일치성을 갖는 특징을 보인다. 특히, 착상을 비롯한 태반형성 과정에서 매우 극적인 변화가 관찰된다. 세포의 죽음은 이러한 변화를 진행하는데 있어 중요한 것으로 알려져 왔다. 임신초기에 자궁내막에서 관찰되는 세포의 죽음은 프로그램화된 죽음으로(programmed cell death, PCD) 알려진 세포자멸사(apoptosis)이다. 최근 들어 PCD 에는 세포자멸사, 자식작용에 의한 사멸(autophagy), 네크롭토시스(necroptosis)가 모두 포함되는 것으로 밝혀졌다. Necroptosis 가 기본적인 유형이고 이후 apoptosis 와 autophagy 가 진화적 관점으로 관찰 되는 것으로 알려졌으며, 이와 함께 이들 각 programmed cell death 의 초기경로는 중복되어 있음이 밝혀지고 있다. 그러나, 지금까지의 연구들에서 제시된 결과는 apoptosis 와 관련된 세포 내 신호전달 경로와 그 결과의 일부를 증거로 하고 있어 보다 다양한 특이 마커를 이용하여 분석을 통해 규명하는 것이 필요하다. 따라서 이 연구에서는 프로그램화 된 세포의 죽음의 종류들을 분석하여 관련되어 있는 신호전달매체의 마커들을 통해 구체적인 생리학적 상태를 알아보고자 하였고, mRNA 수준과 단백질 수준, 각 세포의 죽음 과정에서 주된 역할을 하는 단백질의 발현을 억제하는 microRNA 의 수준 그리고 면역 조직학적 방법과 조직학적 방법을 적용하여 확인하였다. RIP1mRNA 와 단백질 양은 임신 4 일째부터 7 일째에 감소하였다. Caspase3 는 임신 1 일부터 7 일까지 점차적으로 감소하고 이후 9 일 12 일에 증가하는 경향을 보였다.miR-378 과 let-7a 는 임신 1 일과 2 일에 높은 발현을 보였고 이후 감소하였다. ULK1 은 임신 1 일부터 증가하는 경향을 보이며 이후 7 일부터 감소 하였고, ULK2 는 점차적으로 감소하였다가 7 일 이후 증가함을 보였다. 이를 억제하는

miR-20 과 miR141-3p 는 임신 1 일과 2 일에 높았다. miR-17 도 임신 1 일과 2 일에 높은 발현 양상을 보였다. LC3B 단백질 발현 수준은 임신 1 일과 2 일에서 상대적으로 높은 발현을 하였다. RIP3 는 mRNA 수준에서 보았을 때 전체적으로 비슷한 양의 발현을 보였다. 그리고 단백질 수준에서는 임신 4 일에서부터 점차적으로 증가하였다. MLKL 은 mRNA 수준과 단백질 수준에서 보았을 때, 임신 1 일과 2 일에 상대적 높은 발현을 보였다. miR-874 의 발현은 임신 1, 2 일에 상대적으로 높았다. 면역조직화학 결과에서 활성형의 capase 3, 인산화된 MLKL, RIP3 가 표지된 조직과 조직학적으로 분석된 세포 죽음 유형은 일치하였다. 비록 apoptosis, autophagy, necroptosis 의 세포 내 신호전달경로에서 서로를 구분하는 중간매체의 발현이나 조직학적인 분포 특성에 대한 연구가 더 필요하나, 이러한 결과는 임신 초기에 지금까지 밝혀진 apoptosis 이외에 necrosis 와 autophagy 가 함께 공존한다는 것을 의미한다. 따라서 초기 임신시기 자궁 조직의 재형성과 조직항상성 유지에 apoptosis, autophagy, necroptosis 가 모두 관여하고 있다고 사려된다.

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INTRODUCTION

Tissue remodeling and homeostasis is maintained through cell proliferation and death (Yaron et al., 2011). In mammals, the pregnancy is accomplished by the proliferation and differentiation of uterine cells. During early pregnancy remodeling and homeostasis of the epithelial tissue are dramatically progressed with the cell proliferation and embryo invasion. During this process epithelial cells undergo cell death, detached from their basement membrane (Favaro et al., 2014). On the other hand, epithelial lining proliferate and differentiate secretory decidual cells (Ramathal et al., 2010). Culminate in the formation of the definitive placenta (Weitlauf et al., 1988).

Programmed cell death (PCD) plays a fundamental role in animal development and tissue homeostasis. PCD is a preceeding the following development program that make use of endogenous molecular machinery. Now, the accepted forms of active cell death are apoptosis, autophagy, and necroptosis. Also it is revealed that these three death pathways are cross talk together. The ratio of Bax and Bcl2 level or caspase3 determines the amount and spatial distribution of apoptosis (Joswing et al., 2003). Caspase (especially caspase-3, -9) play critical roles in apoptosis. So caspase activation (cleavage of procaspase to active caspase) is hallmark of almost all apoptotic system. Caspase3 is a central effector caspase in many cells and mediates the cleavage of itself, other downstream caspase, and other caspase substrates (Allen et al., 2002). Also, apoptosis is morphologically characterized by nuclear shrinkage and formation of apoptotic bodies (Image et al., 2016).

Autophagy is well-controlled biological process that plays essential roles in development, tissue homeostasis (Nikoletopoulou et al., 2013). The autophagy is regulated that involves the following events induction, vesicle nucleation (or formation of a phagophore), vesicle elongation and autophagosome formation, and autolysosome cargo degradation. Several molecular complexes or components are very important for the process of autophagosome formation. These components are the UNC-51-like kinase(ULK)complex, the classⅢ phosphatidylinositol-3 kinase(PI3K) complex, the ATG12 conjugation system and the microtubule-associated protein 1 light chain 3(LC3) conjugation system (Su et al., 2015). Autophagy begins with the formation of a phagophore, an osmophillic double membrane structure (Lamb et al., 2012). LC3 is conjugated to phosphatidylethanolamine and targeted to autophagic membranes. Therefore, changes in LC3 localization have been used to measure autophagy (Akiko et al., 2007).

Necroptosis, post-apoptotic secondary necrosis, is a caspase-independent cell death. Necrotic cell death is carried out by complex signal transduction pathways and execution mechanisms (Berghe et al., 2010). Necroptosis has been recently elucidated, that serine-threonine kinase receptor-interacting protein 1(RIP1) and RIP3 complex and phosphorylate MLKL as a downstream target of RIP3 play pivotal roles in caspase-independent necrosis. The necroptosis is morphologically characterized by rounding of the cell, cytoplasmic swelling (oncosis), and presence of dilated organelles and absence of chromatin condensation (Berghe et al., 2010).

Apoptosis is a suggested main death mechanism in pregnant uterus (Parr et al., 1987). Apoptotic regression at the mesometrial pole in early to mid-pregnancy is related to expression level of Bax and Bcl2 proteins (Joswing et al., 2003).

However, recent studies open the new suggestion that other death patterns could be involved in the uterine endometrial remodeling because the death biomarkers have multifunction in death pathways. In this study, the death related signaling mediators were analyzed to evaluate the death pathways which are specific to the physiological status-specific manners.

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 female mice 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.

Uterus sampling

Female CD-1 mice were mated with fertile male mice of the same strain. The following morning, these mice were examined for the presence of vaginal plug, and this was defined as day 1 of pregnancy. The mice were sacrificed to collect uteri on day 1, 2, 3, 4, 5, 6, 7, 9 and 12 of pregnancy. Pregnancy on day 1-12 was confirmed by the presence of embryos in the reproductive tracts. The embryos were removed from oviducts or uteri by flushing with DEPC-treated PBS. The uteri were frozen in liquid nitrogen and kept at -80℃ until RNA and protein extraction.

Total RNA extraction

Total RNAs of uterine stroma cells were extracted using TRIzol reagent (Invitrogen, Cat #: TRI18, San Diego, CA, USA) according to manufacturer's instruction with modification. Briefly, the samples 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, Cat #: 200436, 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㎍/μl), 1.0 μl random primers (0.1㎍/μl), 2 μl dNTP mix (100 mM), and RNase-free water. Reaction mixture was incubated at 65℃ for 5 min, placed the tube at RT to allow the primers to

anneal to RNA for 10 min, after then added 4.0 μl DTT(100 mM), 2.0μl RNase block ribonuclease inhibitor (40 U/ml, Cat # 300152-51), and1.0μl 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 mRNA 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, Cat #:RR420, 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 $\Delta\Delta$Ct method with the housekeeping gene, ribosomal protein, Rplp0, as the internal control.

Micro-RNA expression assay

First strand cDNAs were synthesized using microRNA cDNA synthesis kit (Sigma,Cat #: LN Q23484 CA, USA) according to the manufacturer's instruction. Briefly, reaction reagents were 1μg total RNA, 1.0 μl Poly(A) polymerase, Poly(A) tailing buffer 2 μl, and Nuclease- free water in total volume

up to 10 μl. Reaction mixture was incubated at 37℃ for 1hr and then 70℃ for 5min, hold the reaction at 4℃.To first-strand cDNA synthesis reaction, add Poly(A) tailing reaction 10 μl, Mysticq microRNA cDNA reaction Mix 9μl, and Ready Script Reverse Transcriptase 1.0 μl. Reaction mixture was incubated at 42℃ for 20min and then 85℃ for 5 min

Uterus extraction and Western blotting

Before protein extraction, tissues were washed using cold Y-PBS (0.7mM PMSF, 1 mM Benzamidine-HCl, 4 μg/ml Leupeptin, 2 μg/ml Aprotinin, and 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, and 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 (GE Healthcare Life science, Cat #: 10600021, Germany) in transfer buffer (25 mM Tris base, 192 mM Glycine, 0.1% SDS, and 20% Methanol, pH 8.3). The membranes were blocked in 5% skimmed dry milk in TBST buffer (10 mM Tris-HCl, 150 mM NaCl, and 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, Cat #: RPN2232, Little Chalfont, UK) by Kodak image station.

Immunohistochemistry and H.E analysis

Collected uterus was washed in PBS and followed by 4% paraformaldehyde for overnight and transferred to 70% Et-OH. And then samples put in the 80% Et-OH, 90% each for 1hr at 4℃. And change 95% Et-OH for 1hr in RT, 100% Et-OH same condition but followed 2 times. Put in the (xylene : 100% Et-OH=1 :1) solution, 100% xylene for 1hr at RT and soft xylene(xylene : paraffin =1:1) for 1hr at 60℃. Move the tissue quick to hard paraffin 100% for overnight and then embedded in paraffin blocks, serially sectioned at 4μm. Immunohistochemical staining was performed on manufacturer’s manual of and HRP-conjugated rabbit and ABC kit (Vector Labaratories, Cat #: PK-6101, Burlingame, CA). Briefly, 4μm thickness paraffin embedded tissue keep in primary Ab 24hr at 4℃ after antigen retrieval. The specific antibodies for cleaved caspase3 (Merck, Germany), phsporylated MLKL (Abcam, Camgridge, USA), LC3B (Abcam, San Francisco, USA) were diluted 1:40 in 0.1%BSA in PBS solution. DAB was used for final color reaction (SK-4100, Vector lab USA). After dehydration, tissue was mounted with and microscopy analyzing. To calculate morphological characteristic of dead cells, H-E staining analyzed the shape of nucleus and cell membrane. Analysis was performed of x1000.

Statistics

All experiments were conducted at least in triplicate. The one way anova test and 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(mRNA level)

Step		Temperature (℃)	Time
Hold	Hold	95	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’)
Apoptosis marker	Caspase3	NM_009810.3	S AS	GTGGACTCTGGGATCTATCTGGACA AGAATCACACACACAAAG ATGCTC
TNFRSF interacting kinase	RIP1	NM_009068.3	S AS	GCTGGTGATGGAGTACATGGAGAA TGGTGCTGCTCACTTCTTTCTG
NecroptosisⅡb RIP complex	RIP3	NM_019955.2	S AS	CGTGAACTCGAAGAAGATATCCTGG AGAATGTTAGAGGGCTTGAGGTCC
Necroptosis marker	MLKL	NM_029005.2	S AS	CTTCCACGCTAATTTGCAACTGC GCAGTTGCAAATTAGCGTGGAAG
Autophagy initiation, ULK complex	ULK1	NM_009469.3	S AS	AACCTTGCCAAGTCCCAAACAC AGGATGTTTTGGGGCTTCAGGT
Autophagy initiation, ULK complex	ULK2	NM_013881.4	S AS	CATCGTAGCGCTCTATGATGTTCAG CACTGTGGATTATCCCTTTGCT

Table 3. Thermal cycler schedule(micro-RNA)

Step		Temperature (℃)	Time
Hold	Hold	95	2 min
3 step PCR (40 cycle)	Denaturation	95	5 sec
	Annealing	60	15 sec
	Extension	70	15 sec
Hold		4	Indefinitely

Table 4. Sequence- specific of primers

Gene	Symbol	miRbase gene reference	Primer sequence (5'-3')
Mmu-miR-19a	miR-19a	NM_007475	CCGTAGTTTTGCATAGTTGCACTAC
Mmu-miR-378a	miR-378	MIMAT0000742	CCTGACTCCAGGTCCTGTGTA
Mmu-miR-7a-1-3p	Let-7a	MIMAT0004670	CCAACAAATCACAGTCTGCCATA
Mmu-miR-20a	miR-20	MIMAT0000529	CCCTAAAGTGCTTATAGTGCAGGTAG
Mmu-miR-141-3p	miR-141-3p	MIMAT0000153	GGTAACACTGTCTGGTAAAGATGGA
Mmu-miR-106b	miR-17	MIMT0000386	GGTAAAGTGCTGACAGTGCAGAT
Mmu-miR-7661-5p	Mir-512-3p	MIMAT0029828	CCAAGAAAGAAACCTGGAGTTTAAAGT
Mmu-miR-874-5p	Mir-874	MIMAT0017268	CCACGCACCAGGGTAAGAA

Table 5. Antibodies information

Name		company
Cleaved caspase3	-Rabbit polyclonal	Merck
LC3B	-Rabbit polyclonal	Abcam
MLKL	-Rabbit monoclonal	Abcam
β-Actin	-Mouse monoclonal	Sigma

RESULTS

Expression of RIP1, Caspase3, ULK1, ULK2, RIP3 and MLKL mRNA in mouse uteri during early pregnancy

mRNA levels of RIP1 were decreased from day 4 to day 7 of pregnancy (Fig. 1A). Caspase3 were steadily decreased from day 1 to day 7 of pregnancy after that increased day 9 to day 12(Fig. 1B). ULK1 expression was increased up to day 7 and then dropped from day 9 (Fig. 1C). ULK2 expression was steadily reduced to expression from day 1 to day7 and then increased up to day12 (Fig. 1D). RIP3 expression was similar expression pattern from day 1 to day 12 (Fig. 1E). MLKL were relatively high expression day 1 and 2 (Fig. 1F).

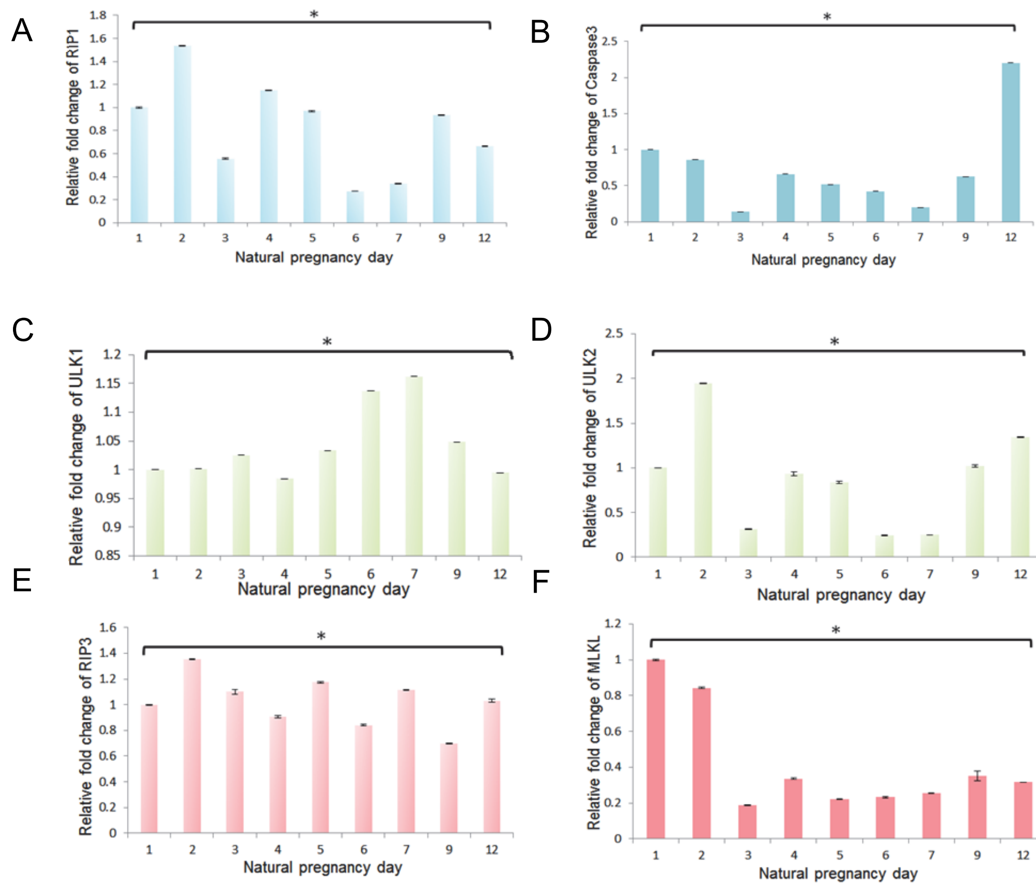


Fig 1. Expression profiles of RIP1, Caspase3, ULK1, ULK2, RIP3 and MLKL mRNA expression level in mouse uteri during early pregnancy.

(A) RIP1, (B) Caspase3, (C) ULK1, (D) ULK2, (E) RIP3, (F) MLKL. NPD; Natural pregnancy day. Values represent the mean ± SD. Statistical analysis was performed by Student's t-test with concentration matched. Statistically significance (one way anova test, $P<0.05$).

Expression of miR-19a, miR-378, Let-7a, miR-20, miR-141-3p, miR-17, miR-874, and miR-512-3p microRNA in mouse uteri during early pregnancy

MiR-19a was expressed highly day 1, 4, and 7 (Fig. 2A). MiR-378 and Let-7a steadily decreased from day 1 to day 12 (Fig. 2B). MiR-20 and miR-141-3p expression steadily declined day1 up to day6 and then increased day7 of gestation (Fig. 2C-a,b). Mir-17 was significantly reduced from day 3 to day 12 (Fig. 2C-c). MiR-874 and miR-512-3p expressed highly on day 1 and day 2 after that decreased until day 6 and increased on day7 temporarily and then decreased day9, and day12 (Fig. 2D-a,b).

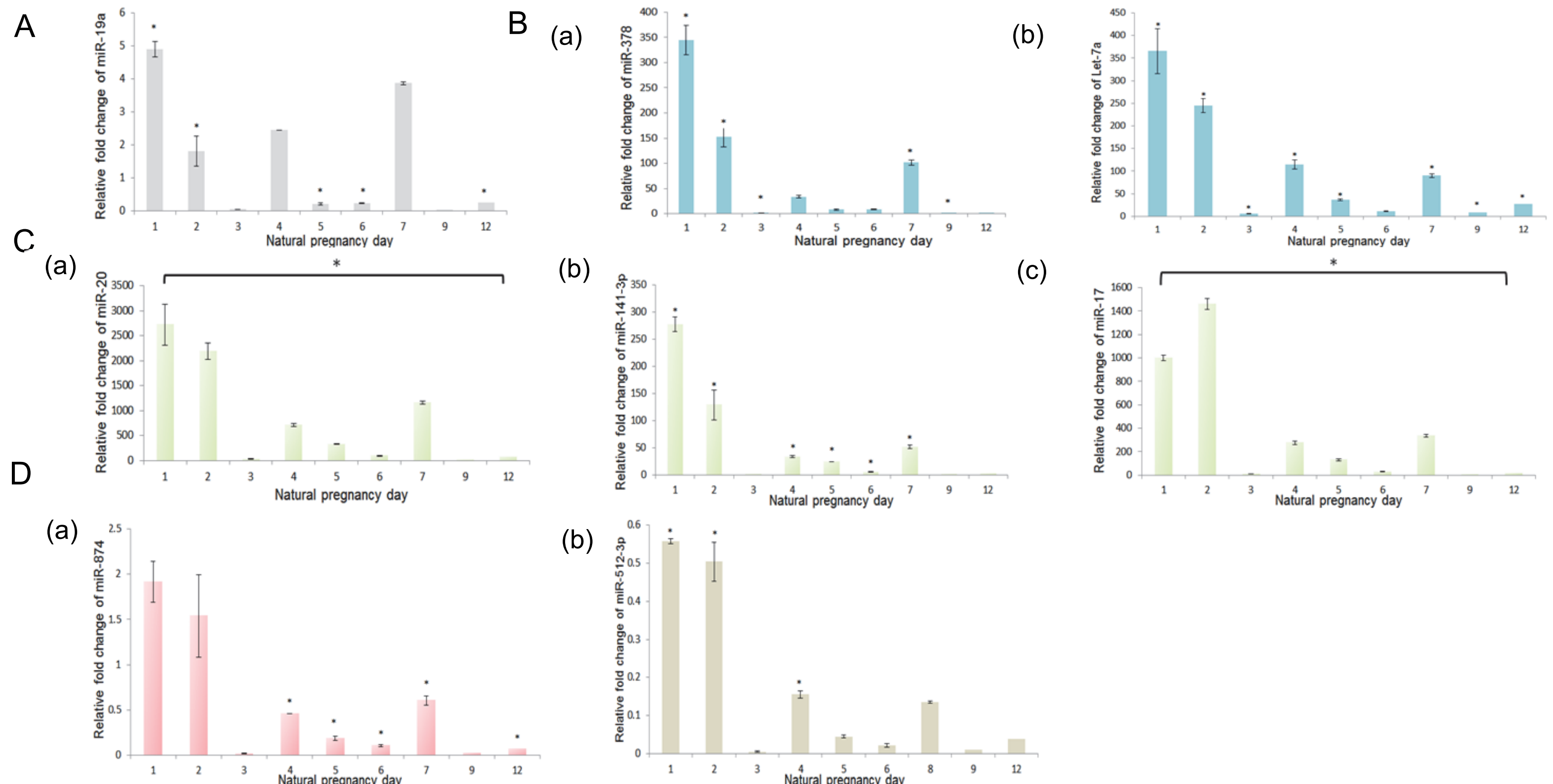
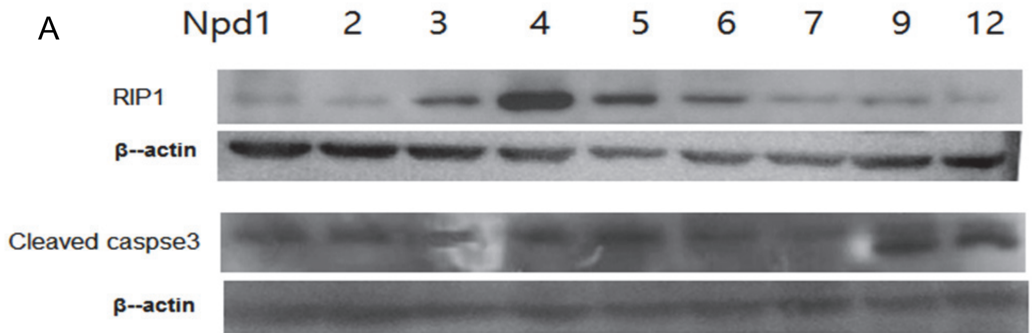


Fig 2. Expression profilies of miR-19a, miR-378, Let-7a, miR-20, miR-141-3p, miR-17, miR-874 and miR-512-3p. **A** miR-19a; Surviaval factor suppresor, **B** (a) miR-378 and (b) Let-7a; targeted caspase3, **C** (a) miR-20;ULK1, (b) miR-141-3p;ULK2 and (c) miR-17; Atg16L targeted Autophagy. **D** (a) miR-874; targeted caspase8, (b) miR-512-3p; targeted cFLIP. Values represent the mean ± SD. Statistical analysis was performed by Student's t-test with concentration matched. Statistically significance (one way anova test, P<0.05).

Western blot analysis of RIP1, cleaved caspase3, LC3B, RIP3, and MLKL in mouse uteri during early pregnancy

RIP1 expression was steadily increased day1 up to day5 and then was decreased until day 12. Expression of cleaved caspase3 was increased early pregnancy day 1 and day 2 after then it was steadily decreased (Fig.3). LC3B expression was relatively high on day 1 and day 2 (Fig. 4). RIP3 expression was steadily increased up to day 9. MLKL expression was increased day 1 and day 2 after then detected similar amount (Fig. 5). It means that these markers for apoptosis, autophagy, and necroptosis were expressed in some amount in a pregnant day specific manners.



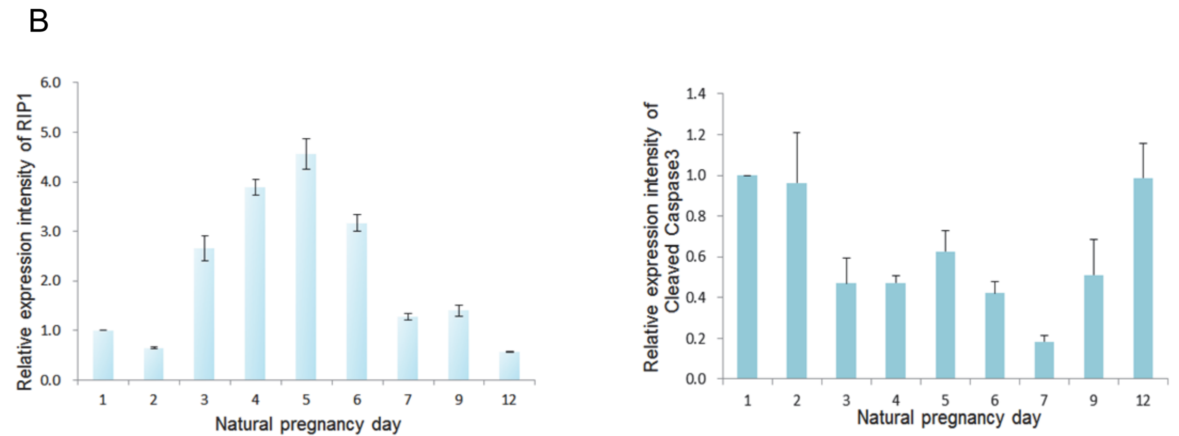


Fig 3. Expression profiles RIP1, cleaved caspase3 and β-actin
(A) RIP1 and cleaved caspase3 protein expressed in the mouse uterus during early pregnancy.
(B) Normalization of the RIP1 and cleaved caspase3 protein.

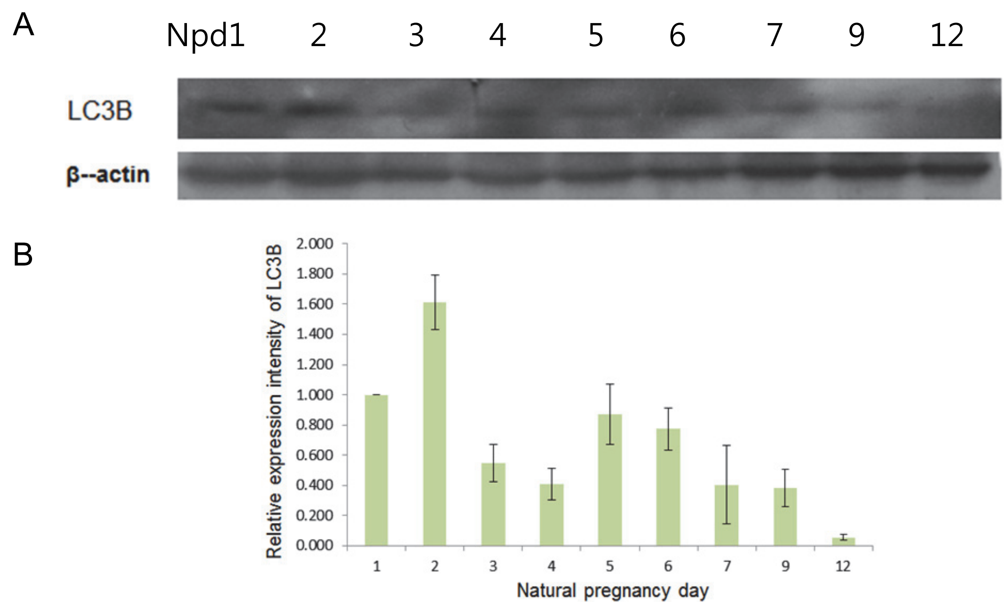
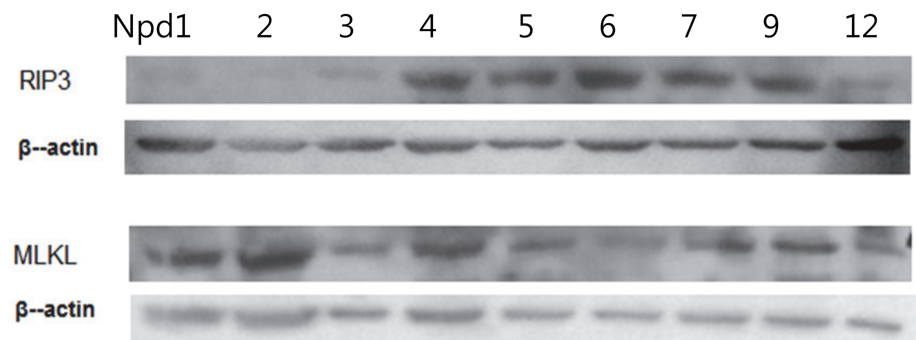


Fig 4. Expression profiles of LC3B and β--actin
(A) LC3B protein expressed in the mouse uterus during early pregnancy.
(B) Normalization of the LC3B protein.



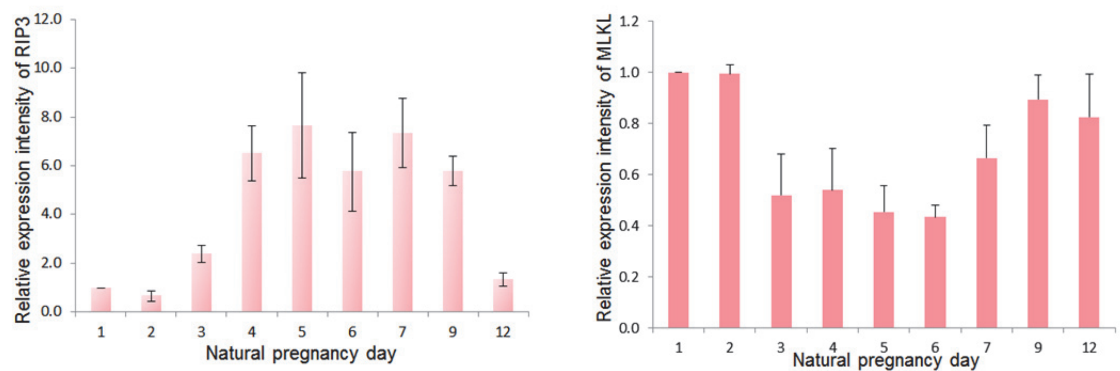


Fig 5. Expression profiles of RIP3, MLKL and β--actin
(A) RIP3 and MLKL protein expressed in the mouse uterus during early pregnancy.
(B) Normalization of the RIP3 and MLKL protein.

Immunohistochemical localization of markers for cell death types in early pregnancy uteri

Cleaved caspase3, apoptosis specific marker, was localized in luminal epithelium from day 1 to day3 (Fig.6 B-D) and stroma on day 4, day 5 (Fig.6E-F). After that embryo implantation closely site was primary decidual zone stained until day7 (Fig. 6G-H). Day9, stroma was stained (Fig.6I). And finally day 12, it was localized in placenta (Fig. 6J). Positive control was used by mouse small intestine (Fig. 6A). LC3B was localized lumen and any part of the whole until day 9. On day 12, was localized in placenta (Fig.7B-J). Positive control was used by mouse brain (Fig.7A). Phosphorylated MLKL was localized in luminal epithelium and glandular epithelium (Fig.8B-F). After that it was localized in primary decidaul zone for a wide range day 6, day 7 (Fig.8G-H) and on day 9 of gestation (Fig.8I). It was localized in placenta on day 12 (Fig.8J). These results mean that expression of markers has specific localization pattern differently during early pregnancy.

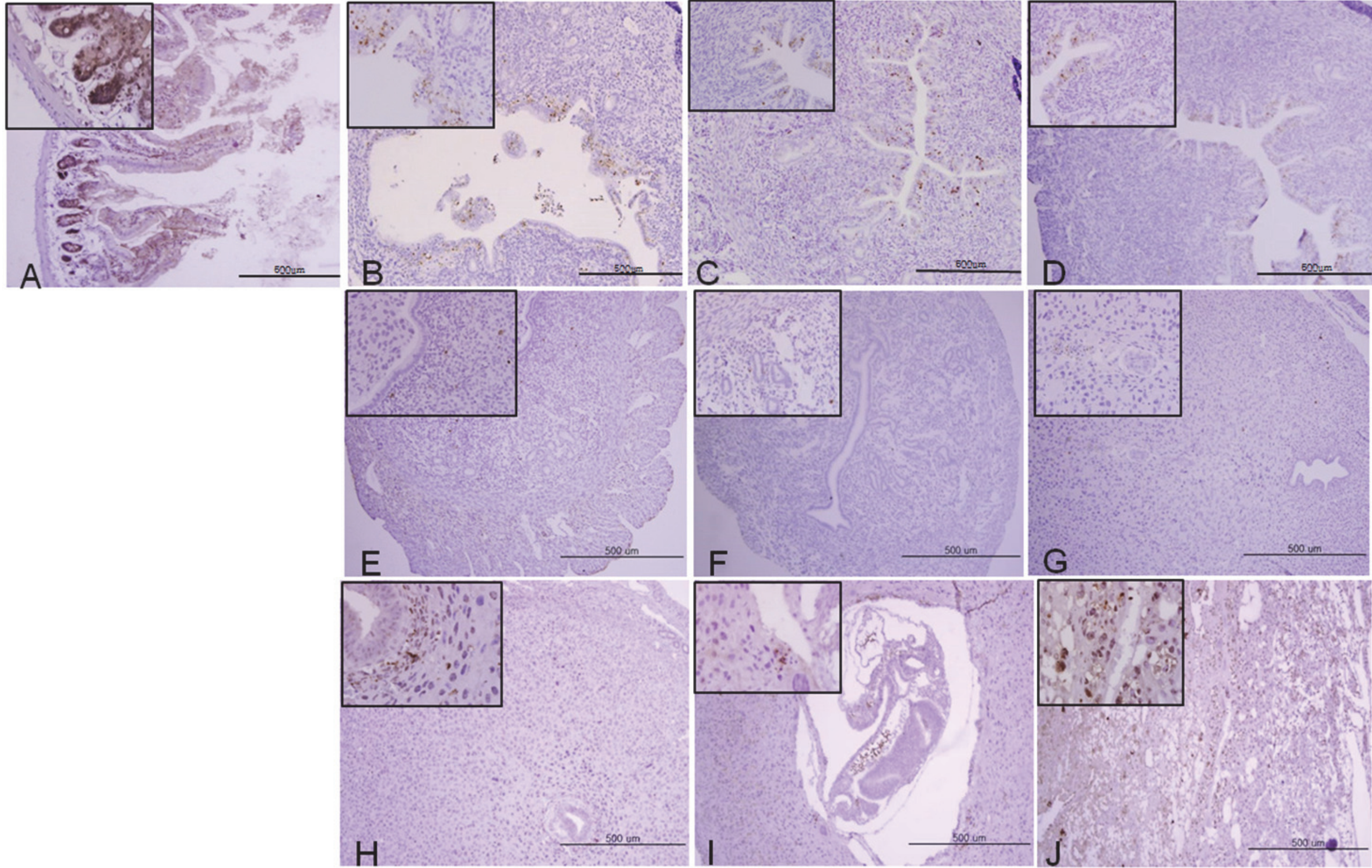

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Fig 6. Cleaved caspase localization in natural pregnancy mouse uterus tissue.
Immunohistochemistry assay DAB staining region is cleaved caspase3 positive (A) mouse small intestine; x100, inside x600. (B) natural pregnancy day 1; x100, inside x400. (C) natural pregnancy day 2; x100, inside x400. (D) natural pregnancy day 3; x100, inside x 400.(E) natural pregnancy day 4; x100, inside x400. (F) natural pregnancy day 5; x100, inside x 400. (G) natural pregnancy day 6; x100, inside x400. (H) natural pregnancy day 7; x100, inside x600. (I) natural pregnancy day 9; x100, inside x600. (J) natural pregnancy day 12; x100, inside x600.

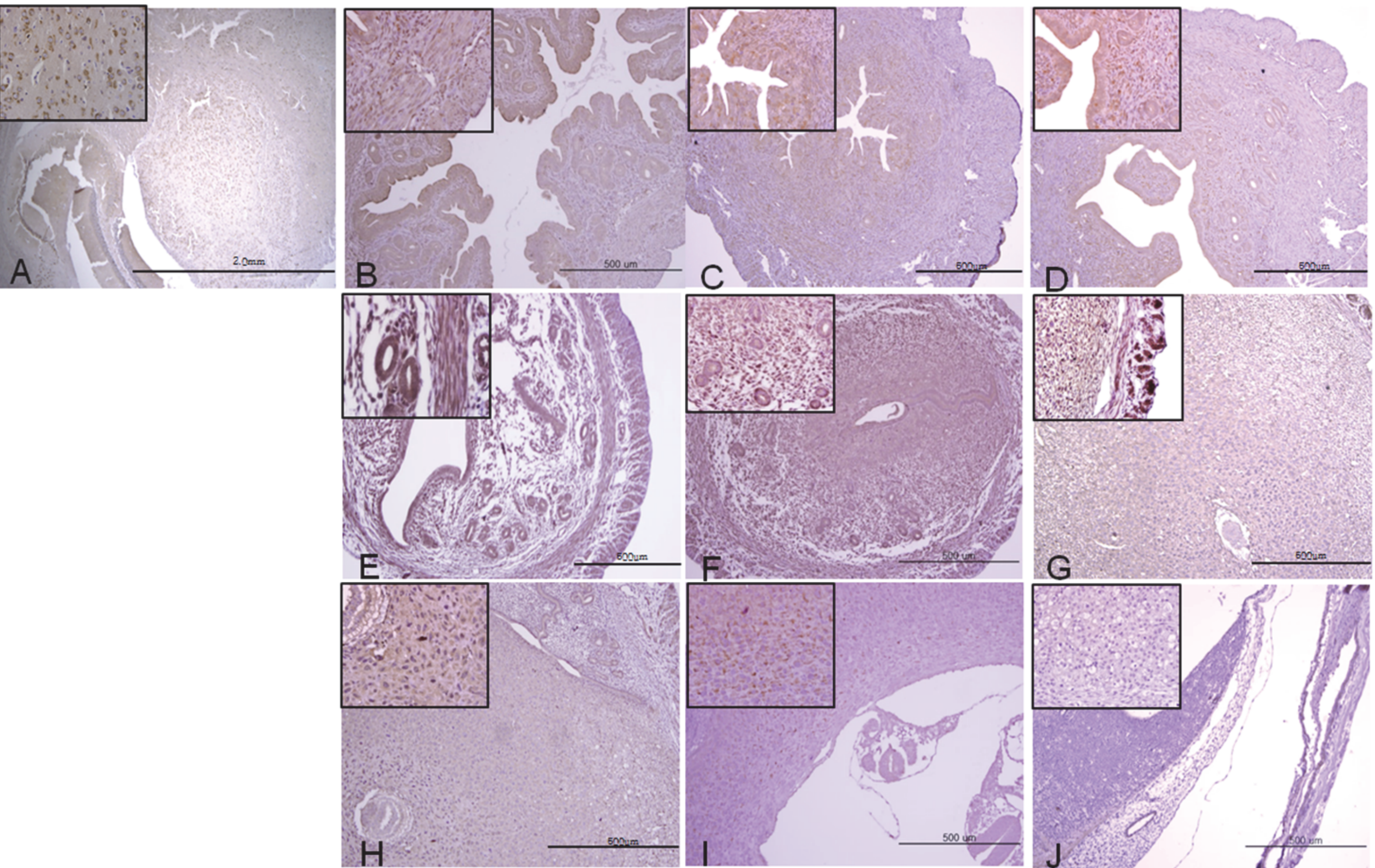
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Fig 7. LC3B localization in natural pregnancy mouse uterus tissue.
Immunohistochemistry assay DAB staining region is LC3B positive (A) mouse brain; x40, inside x600. (B) natural pregnancy day 1; x100, inside x400. (C) natural pregnancy day 2; x100, inside x400. (D) natural pregnancy day 3; x100, inside x 400.(E) natural pregnancy day 4; x100, inside x400. (F) natural pregnancy day 5; x100, inside x 400. (G) natural pregnancy day 6; x100, inside x400. (H) natural pregnancy day 7; x100, inside x600. (I) natural pregnancy day 9; x100, inside x600. (J) natural pregnancy day 12; x100, inside x600.

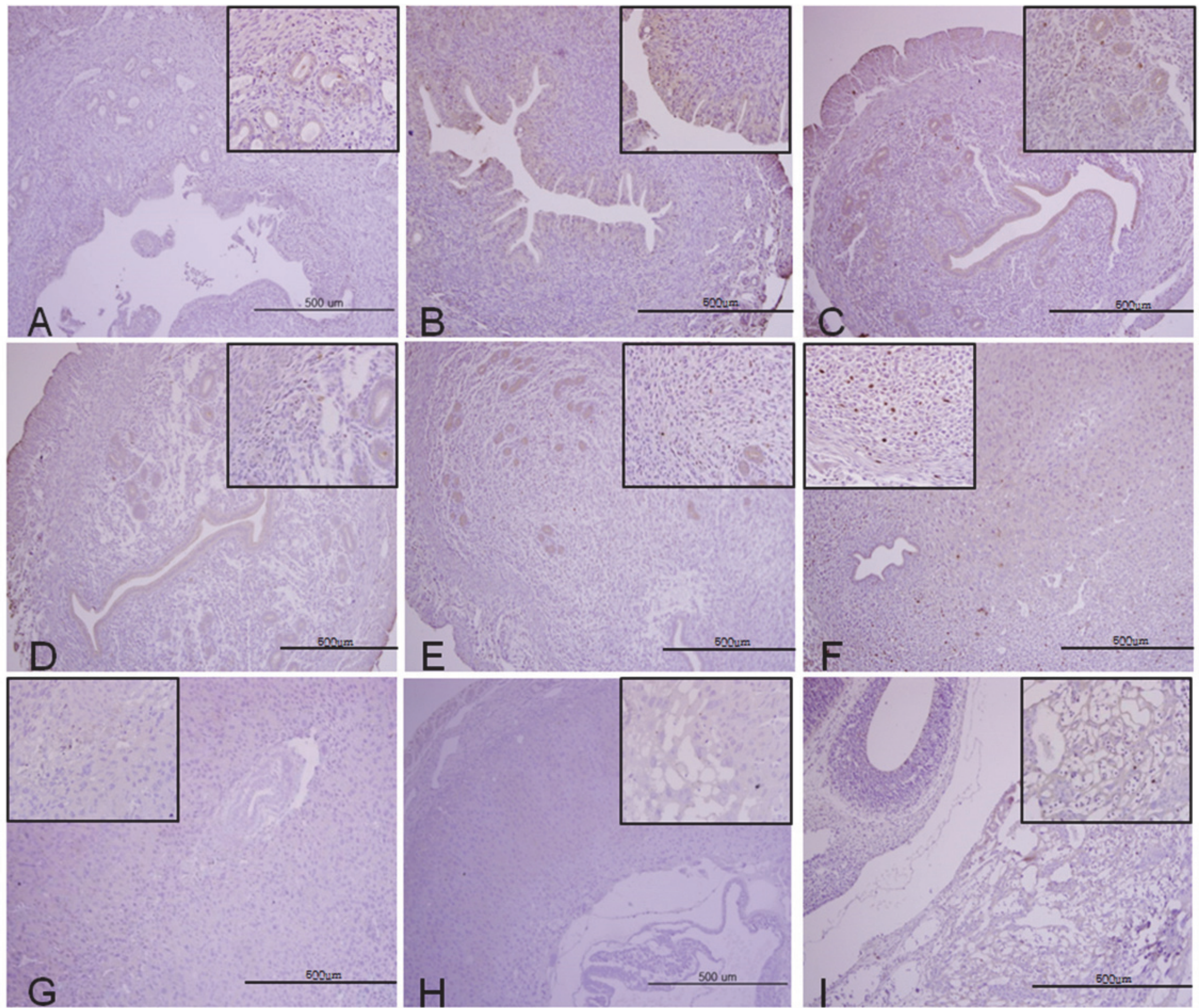
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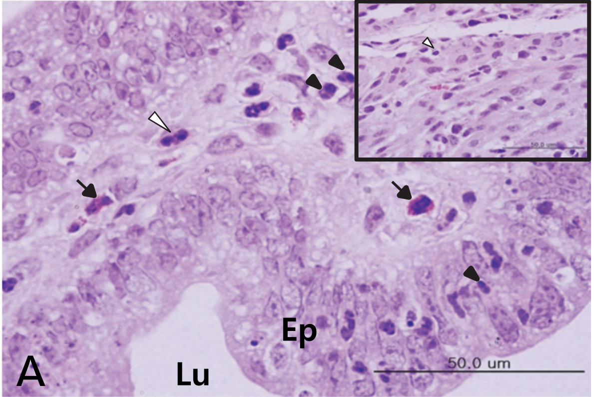
Fig 8. Phosphorylated MLKL localization in natural pregnancy mouse uterus tissue.
Immunohistochemistry assay DAB staining region is phsporylated MLKL positive (A) natural pregnancy day 1; x100, inside x400. (B) natural pregnancy day 2; x100, inside x400. (C) natural pregnancy day 3; x100, inside x 400.(D) natural pregnancy day 4; x100, inside x400. (E) natural pregnancy day 5; x100, inside x 400. (F) natural pregnancy day 6; x100, inside x400. (G) natural pregnancy day 7; x100, inside x400. (H) natural pregnancy day 9; x100, inside x400. (I) natural pregnancy day 12; x100, inside x400.

Death related histological characterization of uterine tissues during early pregnancy uteri

Apoptotic form was detected in apoptotic bodies and necrotic cells detected dilation of membrane in luminal epithelium on day 1. Autophagic cell were detected in stroma (Fig.9A). Chromatin condensation in apoptotic cell and dilation of necrotic cell membrane observed in luminal epithelium and apoptotic bodies and autophagic cells that have double membrane structure were detected in stroma from day 2 (Fig.9B). Apoptotic bodies, cytoplasm dilation, and cells have double membrane were showed in luminal epithelium and stroma. Cytoplasmic swelling of necrotic cells detected in glandular epithelium from day 3 to day 5 (Fig. 9C-E). In implantation site, cells had apoptotic bodies, chromatin condensation and few cells were detected in primary zone. In stroma, apoptotic and necrotic cells were observed from day 6 to day7 (Fig. 9F-G). Day9, apoptotic bodies and dilation of cells were detected in stroma (Fig 9H). Cells observed apoptotic bodies, chromatin condensation and cytoplasmic dailation of necrotic chracterisic in placenta of ueturs on day 12 (Fig 9I). These results mean that not only apoptosis but other types of cell deaths are related in early pregnancy uterus.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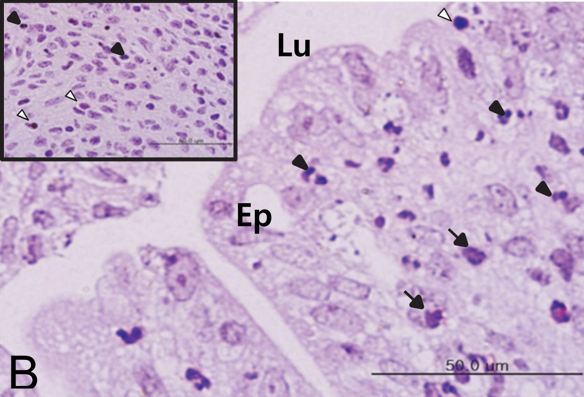
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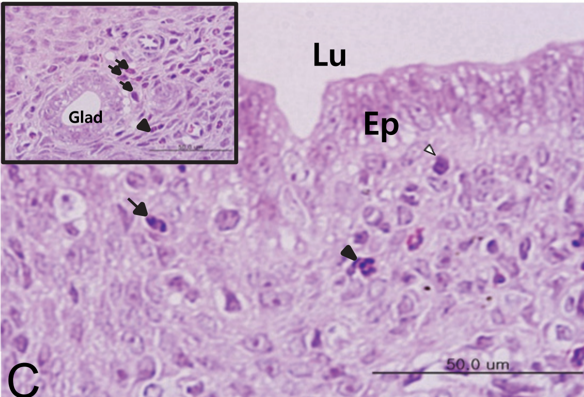
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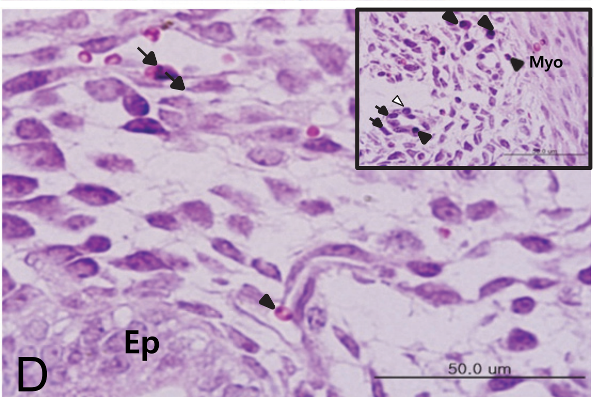
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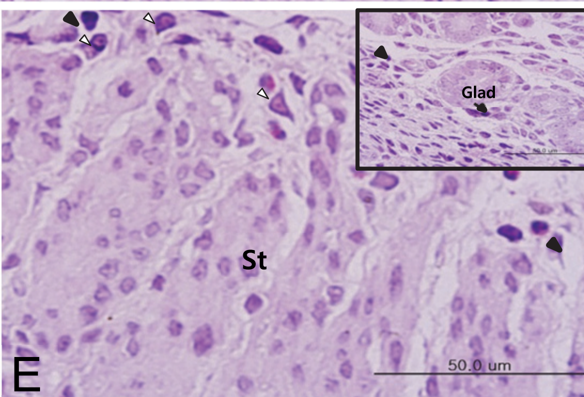
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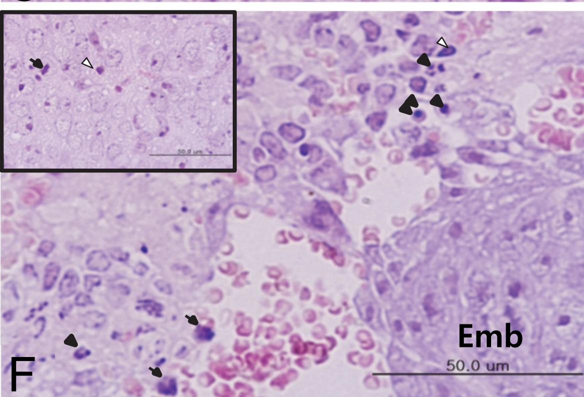
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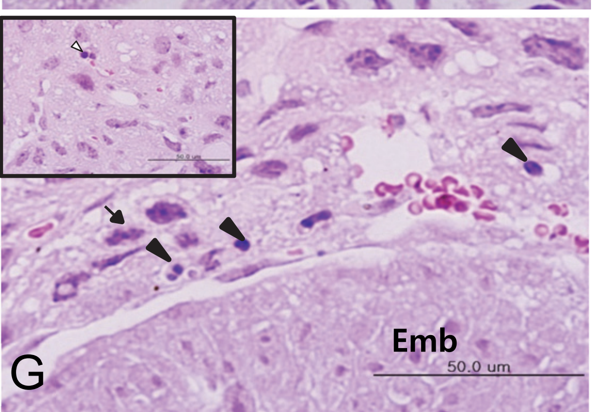
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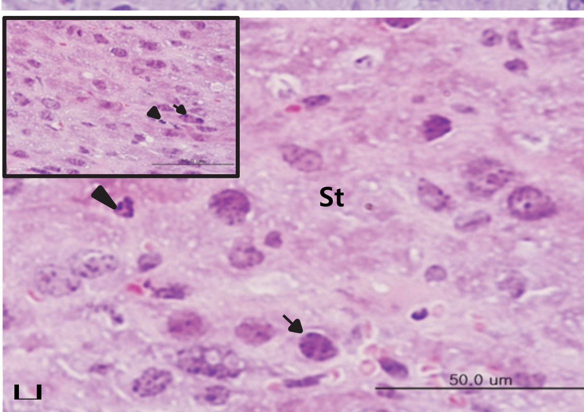
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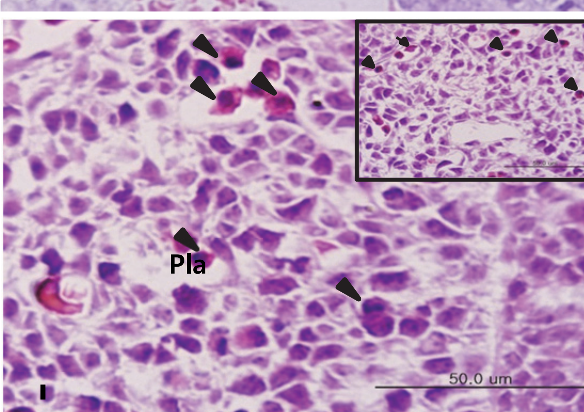
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Fig 9. The histological analysis of cell death types in natural pregnancy day in mouse uterus tissue.

H.E stain analysis of cell death types using 0.2μm paraffin section x1000 (A) natural pregnancy day 1 (B) natural pregnancy day 2 (C) natural pregnancy day 3 (D) natural pregnancy day 4 (E) natural pregnancy day 5 (F) natural pregnancy day 6 (G) natural pregnancy day 7 (H) natural pregnancy day 9 (I) natural pregnancy day 12.Lu : lumen, Ep : luminal epithelium, St : stroma, Glad : gland, Emb : embryo, Pla : placenta. (Balck arrow head : apoptosis, white arrow head : autophagy, and arrow : necroptosis)

DISCUSSION

In mammals, Tissue remodeling and homeostasis is maintained through cell proliferation and death (Fuchs et al., 2011). The pregnancy is accomplished by the proliferation and differentiation of uterine cell. Development of receptive uterus involves structural remodeling (Weitlauf et al., 1988) and homeostasis of the epithelial tissue is dramatically progressed with the cell proliferation and embryo invasion (Favaro et al., 2014). During this process epithelial cells undergo cell death and it was essential process in early pregnant mouse uterus. Apoptosis is a suggested main death progress in pregnant uterus (Parr et al., 1987). But, in recent years, PCD discovered including apoptosis, autophagy and necroptosis. They are crosstalk each other (Nikoletopoulou et al., 2013). Meanwhile, necroptosis was basic type also apoptosis and autophagy observed an evolutionary point of view. So, types of PCD were analyzed through signaling mediators to evaluate specific physiological status. Apoptosis in mouse uterine epithelium and decidua during early pregnancy day 4.5-7.0 post coitum (Joswing et al., 2003). Using caspase3 detected apoptosis in mRNA, that expressed were steadily decreased from day 1 to 7 of pregnancy after that increased day 9 to 12. Micro RNA expressions, mir-378 and Let-7a were increase from day 1 and day 2 and then steadily decreased.

In protein level, cleaved caspase3 which is final execution markers in apoptosis was increased early pregnancy day 1 and day 2 after that steadily decreased.. The cleaved caspase3 was localized in luminal epithelium from day 1 to day 3 and stroma on day 4, day 5 of gestation. On day 7, it was mainly localized in primary decidual zone. On day 9 of gestation, it was localized on stroma cells. On day 12 of gestation, it was localized in placenta.

During pregnancy, autophagic response is high on days 1 and when the uterus shows an inflammatory response to mating, but it subsides around the time of implantation (Choi et al., 2014). ULK1 and ULK2 are initiation marker in autophagy. ULK1 were declined from day 4 to day 7 and then increased until day12. ULK2 were high expression day2 but, overall expression decreased from day 1 to day7. MiR-20 and miR-141-3p expression steadily declined day1 up to day6 and then increased on day7. MiR-17 was significantly reduced from day3 to day12. LC3B protein, a final execution marker in autophagy, was relatively high expression on up to day 2. LC3B pattern in histology was observed around lumen and any part of the whole until day 9. On day 12, was stained in placenta but, day 1, 2 and 3 were strong expression relatively.

MLKL is a specific marker for necroptosis and its mRNA expression was analyzed. Its expression levels were relatively high on day 1 and 2 of gestation. MiR-874 expressed highly on day 1 and day 2 after then it was decreased until day 6 and increased on day7 temporarily and then decreased until day12. In protein level, MLKL expression was increased day 1 and day 2 after that detected similar pattern. Also, in IHC, phosphorylated MLKL was localized in luminal epithelium but, additionally detected glandular epithelium unlike apoptosis from day 1 to day 5. After that phosphorylated MLKL expression was visualized in primary decidaul zone for a wide range day 6, day 7 and on day 9. It was detected in stroma and then was stained in placenta on day 12.

Although PCD signaling path in cell needs further study, death markers for apoptosis, autophagy, and necroptosis are overall expression during early pregnancy in mouse uterus. Also these results mean that not only apoptosis but other types of cell deaths are related in early pregnancy remodeling and homeostasis.

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ABSTRACT

Various Cell Death Patterns During Early Pregnant Uterus in Mouse

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Department of Biology
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Tissue remodeling and homeostasis is maintained through cell proliferation and death. In mammals, the pregnancy is also accomplished by the proliferation and differentiation of uterine cells. During early pregnancy remodeling and homeostasis both of the epithelial tissue are dramatically progressed with the cell proliferation and embryo invasion. Now, the accepted forms of active cell death are apoptosis, autophagy, and necroptosis. Also, initial path of PCD was duplicated each other. In the pregnant uterus, programmed cell death, apoptosis, is a suggested main death mechanism. However, so far, it is controversy because some of the apoptotic markers are expressed with complex in uterus. In this study, the death related signaling mediators were analyzed to evaluate the death pathways which are specific to the physiological status. The expression profiles of mRNA and proteins of the key regulators in each cell death processes were analyzed using general PCR,

real-time PCR, and Western blot. Tissue or cell specific expressions of them were analyzed with immunohistochemistry methodology. The expression levels both in mRNA and protein levels were fluctuated by the physiological status specific manners. Apoptosis specific protein, expression of cleaved caspase3 was increased day1, 2 and then was steadily decreased until day7 and increased on day9, 12. The expression patterns were similar in physiological level. Autophagy specific protein, expression of LC3B was relatively increased on day 1, 2. In physiological level, LC3B expression was increased from day1 up to day3 relatively so these results were significant. Necroptosis specific protein, phosphorylated MLKL was high expression on day 1, 2 relatively and then was decreased until day7 and increased on day9, 12. The patterns were significant. Although PCD signaling path in cell needs further study, death markers for apoptosis, autophagy, and necroptosis are overall expression during early pregnancy in mouse uterus. Also these results mean that not only apoptosis but other types of cell deaths are compiled in early pregnancy remodeling and homeostasis.

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지금까지 저와 함께 해주신 하나님 아버지께 먼저 감사 드립니다. 제 삶의 주체이시며 앞으로도 주의 자녀로서 힘차게 살아 갈 수 있게 하실 주님께 영광 돌려 드립니다. 먼저 2 년간 많은 가르침을 주신 지도교수님이자 저의 삶의 스승님이신 전용필 교수님께 진심으로 감사드립니다. 스승과 제자의 사이는 어떠한 연보다 중하다고 말씀하시면서 아버지처럼 곁에서 북돋아 주시고 환하게 웃어주시는 교수님의 모습을 항상 잊지 못할 것 같습니다. 그리고 교수님께서 항상 보내주시는 그 신뢰에 보답하는 제자로 살아가겠습니다. 감사합니다. 또한 바쁘신 시간 내주시어 학위 논문을 심사해 주신 서울여자대학교 김해권 교수님, 찾아 뵐 때마다 항상 따뜻하게 맞아주시고 커피 한 잔 내어주셔서 정말 따뜻했습니다. 그리고 차의과학대학교 이동률 교수님 항상 멋진 분이라고 느끼지만 찾아 뵈면 많은 조언으로 마음까지 따뜻한 분이신 것을 더 느낄 수 있었습니다. 진심으로 감사드립니다. 그리고 학부 때부터 지금까지 항상 뒤에서 응원해주시고 웃으시며 반겨주시는 저의 요새이자 전임교수님이신 전민영 교수님께도 감사드립니다. 연구를 하면서 쉴 새 없는 일정에 지칠 때 즐길 수 있는 시간도 있다는 것을 보여주신 서울여자대학교 양현원 교수님께도 감사드립니다. 또한 연구하는 삶과 실험에 있어서 많은 도움을 주신 성은언니와 많은 선배님들께 감사드립니다. 또한 지나가며 마주치면서 따뜻한 말로 격려해주시고 힘주신 강혜순 교수님, 윤진호 교수님, 김상태 교수님, 강창수 교수님, 최상철 교수님 감사드립니다.

2 년간 지내면서 희로애락을 함께 한 발생생리학 연구실 식구들에게 감사합니다. 먼저 어떻게 해야 할 지 몰라 혼자 힘들 때 자신의 시간까지도 내 주시는 지은언니께 감사합니다. 도도한 모습으로 그러나 따뜻하게 챙겨주신 지현언니 그리고 많은 조언과 저의 정신적 지주였던

순영언니 언니가 있어 부족하지만 앞으로 나아갈 수 있었습니다. 저의 사수이자 많이 예뻐 해 주시고 가르쳐 주신 민지언니. 후배의 어떠한 질문에도 따뜻하게 가르쳐 주신 큰 혜진언니. 누구보다 마음 따뜻하고 힘들 때 먼저 와서 자신의 일처럼 챙겨주시는 정원언니 모두 감사합니다. 그리고 사랑하는 동기 채림언니와 연정언니 준희오빠. 같은 동기였지만 부족한 동생과 함께 해줘서 너무나 고맙고 채림언니 박사 된 언니의 모습을 기대하고 그리고 연정언니 매번 말하지만 언니가 있어 많이 웃을 수 있었고 동기 중에 나의 요새였습니다. 언니 역시 황박사의 모습 기대합니다. 준희오빠 도와달라는 말에 옆에서 묵묵히 도와줘서 너무나 고맙고 간간히 맛있는거 먹으러 찾아가겠습니다. 그리고 은혁아 조교일 하느라 수고 많았고 기수로는 후배이지만 친구가 있어 속마음을 이야기 할 수 있어서 너무 좋았습니다. 언제든지 연락하면 힘이 되겠습니다. 그리고 우리 민영이 학부실험을 가르칠 때 만나 너를 이렇게 후배로 맞이할 수 있어 너무 기쁩니다. 힘든 일이 있거나 도움이 필요할 때 언니가 가장 먼저 생각날 수 있는 선배이길 기대합니다. 지영이도 얼른 들어와서 정착했으면 좋겠고 모두들 감사합니다.

무엇보다도 나의 우선순위이자 사랑하는 우리 가족들에게도 감사드립니다. 어느 한 순간에도 빼먹지 않고 우리 큰 딸이 우선인 아빠. 학창시절 때부터 지금까지 영원한 버팀목이자 꽃처럼 예쁘게 키워주셔서 감사합니다. 그리고 매일마다 기도로 그리고 정신적으로 지원해 힘든 순간순간들을 이겨낼 수 있게 해주는 우리 엄마. 엄마가 없다면 이 세상의 내 존재 역시 없었을 겁니다. 사랑하고 감사합니다. 그리고 틱틱거리지만 언니라고 많이 생각해주는 선영이 앞으로도 함께 힘내서 부모님 잘 모시면서 같이 살아갑시다. 서울에 손녀 보냈다고 걱정해주시는 친할머니 할아버지 감사드립니다. 또한 우리 작은 아빠들 재천, 재록, 재열 작은아버지 첫 조카라고 항상 넘치도록 사랑해주고 한편으로는 친구처럼 옆에 있어줘서 너무나 고맙습니다.

그리고 내 사랑하는 글로벌의과 동기들 미진, 애리, 영인이 내가 먼저 한 발 내 딛게 됩니다. 애들아. 너희도 지금 서 있는 그곳에서 잘 할 거라 믿고 지금까지 못난 친구와 함께해줘서 고맙고 사랑합니다.

이 외에도 지면에 싣지 못한 많은 분들께 감사의 말씀을 드리며 논문을 마치겠습니다. 감사합니다.