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박사학위청구논문

# Possible roles of Sirtuin 1 in decidualization of mouse uterus

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성신여자대학교 대학원

생물학과

황 연 정

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# Possible roles of Sirtuin 1 in decidualization of mouse embryo

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# ABSTRACT

Embryo implantation is a complex process between embryo and endometrium. During implantation, the endometrium undergoes histological and functional modification. Extracellular matrix is the key molecules for maintain the microenvironment Also, supporting the embryo implantation and further development of blastocyst. The decidualized tissue provides a permissive, and on the other hand tightly controlled for invading trophoblast. Sirtuin protein (SIRT) family members are SIRT1~7 involves in physiological and various pathological process including neurodegeneration, cancer growth, aging-related defect and also obesity. One of the most critical Sirtuin family member, SIRT1 has well studied past years expecially cancer model. The function of the SIRT1 still controvercial, because it has ability to acting as either suppressor and promoter in growth of the tumor. On the otherwise, SIRT1 was interesting target gene in female reproductive system. Because previous studies evaluated the significant SIRT1 upregulation in human and baboon endometriosis. These results suggest that proper SIRT1 regulation is important for a healthy female reproductive system. However, SIRT1 function in the uterus has not been directly studied. In here, uterine specific *Sirt1* null mice using immunohistochemistry analysis, SIRT1 expression throughout early pregnancy in mice and found it to be most strongly

expressed at GD4.5 and GD5.5 in decidualized cells and at GD7.5 in secondary decidual cells. 6-month fertility trial revealed that *Sirt1*$^{d/d}$ females were subfertile, delivering significantly decreased numbers of litters/mouse and pups/litter (n=5, *p<0.05). Without decreased the number of blastocysts collected from *Sirt1*$^{d/d}$ uteri at GD 3.5 and the levels of progesterone and estrogen were comparable to controls, indicating normal ovarian function. However, implantation site numbers were significantly decreased in *Sirt1*$^{d/d}$ mice compared to controls at GD5.5 ($p < 0.05$). To more closely assess the cause of the implantation defect, histological characters and marker genes expression were analyzed in the uterus at GD4.5. *Sirt1*$^{d/d}$ implantation sites could be divided into two groups, Group #1 with luminal closure and non-specific cyclooxygenase 2 (COX2) expression compared to controls (14/20, 70%) and Group #2 with an open lumen and no COX2 (6/20, 30%). In control mice, epithelial forkhead box protein O1 (FOXO1) was localized to the nucleus and PGR was limited to the stroma. In *Sirt1*$^{d/d}$ Group #1, nuclear FOXO1 expression in luminal epithelial cells was significantly decreased (20% of cells), and cytoplasmic FOXO1 was increased (80% of cells, $p<0.05$). Semi-quantitative H-score showed that epithelial PGR in *Sirt1*$^{d/d}$ Group #1 and #2 was significantly increased compared to controls ($p<0.001$). In *Sirt1*$^{d/d}$ Group #2, nuclear FOXO1 expression. In epthelium was almost completely absent, and there was strong PGR expression in epithelial cells. At GD5.5, *Sirt1*$^{d/d}$ embryo areas showed

two patterns of E-cadherin staining. Group #1 looked similar to controls with no positive staining for E-cadherin surrounding the embryo. In Group #2 , intact E-cadherin positive epithelium remained surrounding the embryo. Stromal PGR and COX2 were significantly decreased in both Group #1 and Group #2 *Sirt1*$^{d/d}$ embryo areas compared to controls ($p<0.001$), indicating defective decidualization. FOXO1 is not expressed around GD 5.5 implantation sites in natural conditions, and *Sirt1*$^{d/d}$ Group #1 embryo areas were consistent with controls. However, in Group #2 embryo areas, epithelial cells with nuclear FOXO1 are visible, which may indicate delayed molecular signaling in this group. An artificially induced decidualization test revealed that *Sirt1*$^{d/d}$ females showed defective decidualization at decidualization day 5 based on a significantly decreased uterine weight ratio ($p<0.05$). This finding is indicative of a non-receptive endometrial epithelium. Also, important ECM protein including Vimentin and Elastin shows no difference between phenotypes. However, the expression of Integrin β1 shows abnormal in *Sirt1*$^{d/d}$ Group #2 at GD 4.5 and 5.5. In normal conditions, Integrin β1 was not detected in the embryo attachment site but Integrin β1 maintain the expression in same area of *Sirt1*$^{d/d}$ Group #2 at GD 4.5. In Addition, there is a structural defect of ECM in *Sirt1* mice. The result of Masson's trichrome staining for total collagen in GD 4.5 Sirt1 ablated uterus shows that in epithelial ECM, accumulation of collagen bundle. Also GD 5.5 *Sirt1*$^{d/d}$ Group #2, shows dramatically decreased collagen

in all uterine cell layers. Result suggests that SIRT1 can involve normal ECM structure during the implantation window. Altogether, these data suggest that SIRT1 is important for decidualization and contributes to preparing a receptive endometrium for successful implantation.

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# Introduction

Implantation is a critical event in the initiation of a successful pregnancy involving a series of complex mechanisms between the embryo and the maternal endometrium (Carson et al., 2000). Decidualization, a critical components of this process, is the morphological and functional change of uterine stromal fibroblasts into large, epithelial-like, secretory decidual cells (Ramathal et al., 2010). Humans show monthly decidual reactions independently of implantation, Also decidualization is essential for a successful pregnancy. But in mice, decidualization starts when just undergoes embryo attachment in the luminal epithelium(Dey et al., 2004). The murine implantation process involves the apposition of the blastocyst next to the endometrial luminal epithelium, the attachment of the trophectoderm layer of the blastocyst to the epithelium, and the invasion of the blastocyst through the epithelial layer(Carson et al., 2000; PrabhuDas et al., 2015) Upon embryo invasion, the uterine stroma surrounding the embryo undergoes remodeling through proliferation and differentiation as decidualization progresses (Bandzerewicz and Gadomska-Gajadhur, 2022; Lee and DeMayo, 2004; Lee et al., 2007; Tan et al., 2002). The decidual process starts at the antimesometrial region of the endometrium, which becomes the primary decidual zone (PDZ)  In mice, the PDZ starts to form on the

afternoon of gestation day (GD) 4.5 (vaginal plug checked morning=GD 0.5) and becomes fully developed on the morning of GD 5.5 with the completion of cell proliferation(Yuan et al., 2019; Zhou et al., 2017)

The ovarian steroid hormones estrogen (E2) and progesterone (P4) generate dynamic changes in the uterus to regulate endometrial receptivity, embryo implantation, and decidualization of stromal cells, all of which is essential for the establishment of a successful pregnancy (Kelleher et al., 2017). P4 signaling through the progesterone receptor (PGR) is the major activation pathway for decidualization (Jeong et al., 2009), and PGR signaling upregulation requires E2 action in the uterine stroma during the window of receptivity (Marquardt et al., 2019). In mice, the uterus is in a prereceptive condition on GD 0.5–2.5 and becomes receptive to blastocyst implantation on GD 3.5. By the afternoon of GD 4.5, the endometrium is past the receptive window (Burnum et al., 2009). Decidualized stromal cells express a number of decidual marker genes in coordination with simultaneous changes in the composition of the epithelium (Cha et al., 2012).

Sirtuin1 (SIRT1) is a globally expressed member of the sirtuin family of Class III histone deacetylases and has number of important roles in peripheral metabolic tissues, such as liver, muscle, and adipose tissue (Frye, 1999; Pfluger et al., 2008). In the ovary, SIRT1 is expressed in oocyte and granulosa cells in large follicles, and SIRT1

deletion can reduce the size of the ovaries during early-stage follicular development (Tao et al., 2015). The dysregulation of sirtuin family members has been known to result in abnormal reproductive phenotypes for over a decade, but the function of SIRT1 in uterine biology is still unclear (Tatone et al., 2018). In the previous analysis of SIRT1, it was found it to be significantly upregulated in human and baboon endometrium affected by the presence of endometriosis (Yoo et al., 2017). This was consistent in both proliferative and secretory phase human biopsy samples, and it was time-dependent after induction of endometriosis in baboons (Yoo et al., 2017). In early studies, SIRT1 involve in PGR signaling via interacting with a number of proteins especially HDAC3, SOX17, IHH, GATA2 (Li et al., 2021) HDAC gene family contribute to epigenetic regulation of histone and nonhistone substrates, which has ability to mediate the acetylation(Yoon et al., 2005). HDAC1~3 and HDAC8 has critical role in many biological process, including cell proliferation, differentiation and cell survival(Reichert et al., 2012). Epigenetic regulation relative genes have important role in response to endometrial steroid hormone. This process could affect to normal endometrial function(Houshdaran et al., 2016; Munro et al., 2010). Several epigenetic modification genes have been studied in human endometrium as also in animal models.(Meyer et al., 2014; Xue et al., 2007). HDAC1 and 2 expressions in uterine endometrial stromal cells are mediated by

ovarian steroids have been reported in the previous studies(Colon-Diaz et al., 2012). General expression of HDAC3 is like HDAC1 and HDAC2. However, HDAC3 has different contruct domain with other family members, including unusual C terminal. Also, HDAC3 protein localized in not only nuclei, but also cytoplasm, and plasma membrane. This result suggest that HDAC3 has distict function with other members(Zhang et al., 2005).

HDAC3 acts as the corepressor of various transcription factors(Yoon et al., 2003). N-CoR and SMRT are transcription factors that could be corepressors of HDAC3(Shang et al., 2000). In addition, several nuclear receptors including estrogen receptor (ER) and progesterone receptor (PGR) also interact with HDAC3(Zhang et al., 1998). However, the function of HDAC3 in uterine endometrium remains unknown.

SOX17 belongs to the family of sex determination genes (Y related). Also, one of the SOX family members is encoded by Sox17(Abdelalim et al., 2014). Mouse and humans have 20 Sox genes but key regulators are Sox2, Sox9, Sox17, Sox4, and Sox18 (Kamachi and Kondoh, 2013). This gene could involve in cell reprogramming or epigenetic remodeling. Recently studies show SOX17 is the target of PGR (Rubel et al., 2012b), it revealed SOX17 has a potential role in PGR signaling in the pregnant uterus also disease (Kandoth et al., 2013). In addition, Sox17 gene is one of the downstream targets of the

*Pgr-Gata2*-dependent transcriptional pathway. *Pgr-cre* derived uterine conditional *Sox17* knockout female mice shows infertile. This result suggests that SOX17 affect directly in receptive uterine endometrium for embryo implantation (Wang et al., 2018).

Ihh and Gata2 has well established as importatnt gene in *Pgr* signaling(Takamoto et al., 2002; Wang et al., 2018). Ihh included as a member of Hedgehog (Hh) gene family , which is developmentally regulated morphogens (Britto et al., 2000). In mice, there are three Hedgehog(Hh) homologs: Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh) (Britto et al., 2000). Generally, Hh signaling regulates cell proliferation and differentiation by range independent, because Hh protein has diffusible character (Takamoto et al., 2002). This study could suggest Ihh involved in signaling between two distinct compartments, epithelium and mesenchyme (St-Jacques et al., 1999). Gata2 also P4 induced gene in pregnant endometrium, which has transcriptional regulating fuction(Rubel et al., 2012a). These studies reveal that many PGR signaling relative gene has a connection with embryo survival and endometrial environment for successful pregnancy, direct or indirect way. But more critical molecules have to be unveiled. The results of this study suggest that SIRT1 has the potential to important regulator in PGR signaling.

Molecular interaction between *Sirt1* and other genes are well established in variety cell model. Previous study shows that in human

hepatocellular carcinoma cell (Hep3B), Smad2 relative interaction was confirmed to be enforced upon acetylation, its active form localized in nucleus(Garcia-Vizcaino et al., 2017). Sirt1 has minor role in Smad2 acetylation. However, Anti-*Sirt1* ChIP results suggest that increased promoter activity of *Sirt1* after TGFβ-stimulation. Smad2-dependent transcriptomic complex on DNA has ability to modulate gene expression. Also, *Sirt1* overexpression mice shows decreased expression of Smad2-driven TGFβ-dependent reporter gene(Garcia-Vizcaino et al., 2017; Warburton et al., 2013). Another research identified deacetylate function of SIRT1. SIRT1 activators SRT501 and SRT2183 combine with malignant lymphoid cell lines and induce cell cycle arrest and apoptosis which is STAT3, and NF-kB induced. relative cell growth arrest and apoptosis. This result shows SIRT1 has a role in the deacetylation of essential genes that relative to normal uterine function (Scuto et al., 2013).

Cyclooxygenase-2 (COX2), an inducible proinflammatory gene, is an important marker for decidualization, and is highly expressed around the embryo invasion site. It was previously shown that COX2-deficient females have decidualization failure when fertilized embryos invade the endometrium (Lim et al., 1999). E-cadherin, a critical cell adhesion molecule, constitutes epithelial cell-cell contact sites. In natural conditions, E-cadherin is strongly expressed in the GD 4.5 luminal epithelial cells but is nearly absent at

GD 5.5 due to entosis of epithelial cells by invading trophoblast (Li et al., 2015). Activation of signal transducer and activator of transcription 3 (STAT3) via phosphorylation is required for decidualization, directly interacting with PGR in the uterus at GD 5.5. Additionally, mice lacking STAT3 in PGR positive cells fail to support embryo implantation and decidualization, indicating that crosstalk between endometrial STAT3 and PGR signaling is required for these critical events during early pregnancy (Lee et al., 2013). Furthermore, previous studies have shown that tightly controlled spatiotemporal expression of PGR and forkhead box O1 (FOXO1) is critical for successful implantation and decidualization of stromal cells (Takano et al., 2007; Vasquez et al., 2015; Vasquez et al., 2018). FOXO1 expression also increases with the reciprocal decrease of PGR in the nuclei of endometrial epithelia of both humans and mice during the time of embryo implantation, and FOXO1 plays an important role in uterine receptivity through regulation of epithelial integrity (Vasquez et al., 2018). In natural conditions, at GD 5.5, cell death occurs in the mouse embryo as preparation for trophoblast invasion (Wilson, 1963) At the time, signaling of degradation of invasive inner cellular masses migrating into uterine luminal epithelium (Potts, 1968).

SIRT1 has specific role in endometriotic stromal cell which is regulate the inflammatory cytokine expression (Taguchi et al., 2014). However, the role of SIRT1 in uterine biology or defect model, such as

endometriosis are not clear. The character of endometriosis is the presence of uterine-like tissue, including glands and stroma outside of uterus (Mehedintu et al., 2014). Recently many groups are focus on SIRT1 function of endometriosis. Generally, the expression of SIRT1 was upregulated in endometriosis, also shows same pattern with baboon endometriosis model (Kim et al., 2022). There is 2 major gene which is tightly interact with *Sirt1*. The KRAS, has been suggested as a strong candidate gene in the therapeutic mechanism of endometriosis, also previous study identified that the overexpression of KRAS and SIRT1 in eutopic endometrial tissue from endometriosis patient (Yoo et al., 2017). In addition, Bcl6 identified as a transcriptional repressor in B cell development also oncogenesis. Another role of BCL6, it has ability to involved in the recruitment of SIRT1 deacetylase (Tiberi et al., 2014) In early study reported that the over-expression of BCL6 in eutopic endometrium of infertile women with endometriosis (Evans-Hoeker et al., 2016).

Extracellular matrix (ECM) is many of fibrous proteins complex in tissues and acting as a substrate for many biological processes including cell adhesion, the reaction of mechanical stimulation, maintaining the structure, immune response, angiogenesis, and tissue repair (Frantz et al., 2010; Rozario and DeSimone, 2010; Saldin et al., 2017). Ablation of ECM homeostasis can cause the fibrotic diseases: cardiovascular disease, systemic sclerosis, liver cirrhosis, and also the

progression of many cancer (Cox and Erler, 2011). The ECM has a special character in each tissue including uterine endometrium, the mucous layer on the luminal epithelial cell, and under goes scar-free tissue remodeling which follows the menstrual cycle (Lopez-Martinez et al., 2021). To successful implantation and placentation, the ECM of uterine is dramatically changed, along with modification of luminal epithelium (Hernandez-Vargas et al., 2020). ECM microenvironment can be remodeled by intracellular proteins which is regulate the signaling and cargos of cell secretion (Baker et al., 2015). As the important nicotinamide adenine dinucleotidedependent protein deacetylase, SIRT1 is generally involved in many cellular process and metabolism (Chang and Guarente, 2014). Previous study of other group shows that SIRT1 has critical role in genome stability, DNA damage reaction, autophage (Lee et al., 2008; Wang et al., 2008; Zhang et al., 2020). That result shows SIRT1 not just effect cellular homeostasis but also could involve ECM microenvironment modification (Wang et al., 2021). Extracellular matrix is composed with variety of fibrous proteins and viscous proteoglycans. These molecules provide 3D structural scaffold with adherent cell (Muiznieks and Keeley, 2013; Yanagishita, 1993). In many of previous study demonstrate the ECM component as passive substrate(Piez, 1997) However, now days ECM becomes key mediator of embryonic development, therapeutic clue and organ growth (Vining and Mooney, 2017). ECM has ability to

regulate the cell differentiation and morphogenesis during embryo development is well established (Mammoto and Ingber, 2010). The critical role of ECM in maternal-fetal interaction in uterine tissue still not fully understand (O'Connor et al., 2020).

Collagen family members are well established critical ECM molecule. It can support the tissue with strength and structure(Heino, 2007; White et al., 2004). also, collagen involve a number of biological function include cell growth, differentiation(Heino, 2007). addition, collagen has a role in membrane receptors of cell. All subtypes characterized by morphological or protein structure: collagen type I: fibril formation, collagen type VI: bead like filament, collagen type VII, collagen type IX: fibril-associated collagens with interruptions in triple helix, collagen type X: hexagonal network forming, collagen type XIII: transmembrane localized form and the collagen include multi domain, multiplexins type XVIII(Bandzerewicz and Gadomska-Gajadhur, 2022). A number of studies shows that in implantation time, several types of collagen has spatiotemporal expression patterns(Clark et al., 1993; Hurst et al., 1994; Hurst et al., 1997; Spiess et al., 2007). in rat model, subtype COL I, III, and V are decreased in invasion site compared to inter-implantation site(Spiess et al., 2007). The expression pattern of collagen in non-decidualized stroma and myometrial region has no dramatic change during Day 5.5 to 7.5(Day 0 as mating midnight)(Spiess et al., 2007). COL4 is well known to major

endometrial ECM component, number of study established about normal function of collagen type 4. COL4 is major endometrial ECM component(Blankenship and Given, 1995). Also its very low level in luminal epithelial basement membrane of embryo invasion site.In addition, COL4 was degradation at rat stromal cell in decidual reaction period(Rahima and Soderwall, 1977). All these result suggest that abnormal collagen accumulation might accuse uterine malfunction by disrupted vascularization or incorrectly activate remodeling mechanism in implantation(Mulholland et al., 1992).

Hydroxyproline is protein amino acid which has neutral heterocyclic structure. It is found in collagen or other major ECM protein. also, it is common in many gelatin products. Hydroxyproline is generally used as a diagnostic marker of fibrosis or cancer (Xu et al., 2019). In many previous study shows, dissected tissue compartments include various forms of hydroxyproline in uterus (Welsh and Enders, 1987). Elastin is one of most commonly detected molecule in uterine tissue, concentration and total amount of Elastin of critical event in normal tissue(Welsh and Enders, 1983). but function and role of elastin is still unclear in uterine endometrium. Vimentin is stromal cell marker that contribute to maintain the cell integrity and structural filament (Type III). Also involve in cell migration, adhesion, and motility. In cancer biology, upregulated Vimentin expression was detect in solid cancer (Chakraborty et al., 2019). In addition, Vimentin identified as

epithelial to mesenchymal transition (EMT) marker and has ability to drives metastasis. But a number of Vimentin function still unveiled (Le et al., 2017). Vimentin is highly expressed in pre-implantation period uterine stromal cell and maintain the elevated level in primary decidual cell in implantation period(Wu et al., 2018).

In human, uterine glandular epithelial cell produces the of some integrin family, such as integrin α1β1, α4β1 and αvβ3 (Lessey, 1998). Integrin β1 is known to be expressed during the human menstrual cycle also, ablation of mouse integrin β1 shows implantation failure due to attachment defect and the abnormal invasion to the subepithelial area (Brakebusch et al., 1997). Some integrins are well established as biomarkers in endometrial receptivity and a number of studies show the critical function of integrins (Aplin, 1997). The insights of the early study suggest that there is the mechanism of integrins β1 and β3 which has some specific role in uterine receptivity, driven by ovarian estrogen and progesterone (Chen et al., 2016). Interaction between SIRT1 and Integrins is not fully elucidated. However, IHC data in *Sirt1*$^{d/d}$ shows abnormal integrin β1 distribution and suggest that the important role of SIRT1 is in maintaining a normal endometrial environment.

So far, the possible roles of SIRT1 has been suggested in endometrium, but it is controversy. In this study, the role of SIRT1 was examined with *Sirt1* conditional knockout mice. The characters of

fertility, decidualization was detected with immunohistochemistry and histological methods.

# Materials and methods

### Mouse procedures and tissue collection

All mice are supported from Michigan State University and all experiment was performed under the guideline of animal committee. Mice were bred on a mixed background of the mouse strains C57Bl/6 and SV129 under controlled humidity and temperature. Female 8-week-old mice were used for sample collection. *Sirt1*$^{f/f}$ (*Sirt1*$^{flox/flox}$ , Strain #:029603) is genetic flox construct was inserted for activation with *Pgr-cre* from Jackson Laboratory(ME, USA). *Sirt1*$^{f/f}$ mice were used as controls, and *Sirt1*$^{d/d}$ (*Sirt1* knockout, *Pgr*$^{cre/+}$*Sirt1*$^{f/f}$) mice were generated by mating *Sirt1*$^{f/f}$ mice with the *Pgr-cre* drived mice resulting in the ablation of the floxed gene in uterine tissue(Cheng et al., 2003; Soyal et al., 2005) To collect uterine tissue from specific stages of pregnancy, female mice were mated with fertile wild-type males and designated as GD 0.5 when a vaginal plug was found in the morning.

### Mouse fertility test

For fertility test, tracking 6 months for collect fertility data more than 5 mice couple used. If females were separated with their pups until weaning. After weaning time at postnatal day 21–28, male and female littermates were isolated and housed in groups at 5 mice/cage maximum until use in breeding or experiments.

**RT-qPCR**

To quantification of expression levels, specific designed primer for genes was used (Table 2-1). Also information of thermal cycle in Table 2-2. Total RNA was isolated from frozen uterine tissue with QIAGEN RNeasy Mini Kit (QIAGEN, 74106). cDNA was synthesized with M-MLV Reverse Transcriptase (Invitrogen, 28025-013) by using 1 μg of total RNA primed with random hexamer primer according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed in a Step One Plus Real-Time PCR System (Applied Biosystem), and quantification was performed with SYBR green (PowerUp™ SYBR™ Green Master Mix, Applied Biosystems, A25742) Dissociation curves were run on all reactions to ensure amplification of a single product with the appropriate melting temperature. The fold change in gene expression was calculated using the ΔΔCt method with the housekeeping gene, ribosomal protein, *Rpl7*, as the internal control.

**Western blot analysis**

Before protein extraction, tissue was maintain in -80 ℃. Frozen uterine tissue was homogenized in cold buffer with proteinase inhibitor. The homogenate was centrifuged to remove insoluble materials. The protein concentration was determined using protein dye reagent (Bio-Rad Laboratories, Inc., Richmond, CA) by bradford assay. Five μg of proteins were separated on SDS-PAGE gels and transferred

to PVDF membrane (Millipore, Bedford, MA). The membranes were blocked by incubation for 1 h in 0.5% (w/v) casein (Sigma Aldrich, St. Louis, MO) in PBS with 0.1% Tween 20, and incubated overnight at 4°C with primary antibodies against each target. After washing with PBST, the membranes were incubated with horseradish peroxidase-linked secondary antibody.

**Histology and Immunohistochemistry**

Whole mouse uterine tissue was isolated and fixed in 4% formaldehyde for 24hr and embedded in paraffin. For histological analysis, 5μm of endometrial sections were stained with Hematoxylin (Hematoxylin Stain, GILL's solution, Vector) and Eosin (Eosin-Y 0.5% solution, Merk). Immunohistochemistry was performed after deparaffinization, boil the sample slide with 10mM sodium sitrate buffer for antigen retrieval. Endogenouse peroxidase activity was blocked with 0.3% hydrogen peroxide. performed with specific commercially available primary antibodies for COL1 (1:250, Santa cruz, SC-293182), COL4 (1:250, Abcam, ab6586), Integrin B1(1:250, Abcam, ab28100) at 4 C° overnight incubation. Vectastain kit (Rabbit IgG, PK-4001/ Mouse IgG, PK-4002) was used after administration of primary antibodies at Room temperature 1~2 hour. positive immunoreactivity was detected with the Vectastain Elite DAB kit (Vector Laboratories, Burlingame, CA).

**H-Scoring**

Each measuring sample number is more than 3 by group, tissue area selected randomly for counting positivity in ImageJ (imagej.net, NIH). For compare the immune positive staining, a semiquantitative grade (H-score) was measured by adding the percentage of intensively stained nuclei (3×), the percentage of moderately stained nuclei (2×), and the percentage of weakly stained nuclei (1×) in a tissue region of approximately 100 cells; the score range is 0 to 300(Ishibashi et al., 2003).

**Artificially induced decidualization**

Mice were treated with 3 daily injections of 100 ng of estradiol-17 (E2) per mouse (3 mice per genotype). After 2 days of rest, mice were then treated with 3 daily injections of 1 mg of progesterone (P4) and 6.7 ng of E2 per mouse by s.c. injection. The uteri were received the mechanical trauma by a scratch of the antimesometrial lumen with needle. 6 h after the last hormone injection. Mice were given daily s.c. injections of 1 mg of P4 and 6.7 ng of E2 per mouse for 5 days after stimulation to observe the induction of the uterine decidual response.

**Picro Sirius Red staining**

Picro Sirius Red staining performed as manufacture protocol (ab150681, Abcam). Deparaffinize the sample slide with xylene and

rehydrate using alcohol with a progressively lower concentration. Apply adequate Picro Sirius red solution to completely cover the tissue section and incubate for 60 minutes at RT (Room temperature) humid chamber. Rinse the slide with Acetic Acid Solution and absolute alcohol in order. Clear the sample slide and mount with permount solution.

**Statistical analysis**

The results represent means ± SED. The data were analyzed using one-way analysis of variance (ANOVA) and t-test between control and experimental group. In all cases, values of $p<0.05$ were deemed to indicate statistical significance.

**Table 1.** Primer sequences for quantitative real-time PCR

| Gene | Symbol | NCBI gene reference | | Primer sequence(5'-3') |
|---|---|---|---|---|
| Sirtuin 1 | *Sirt1* | NM_019812.3 | S<br>AS | ACAGAACGTCACACGCCAG<br>TTGAGGGTCTGGGAGGTCTG |
| Ribosomal protein L7 | Rpl7 | NM_011291.5 | S<br>AS | TCAATGGAGTAAGCCCAAAG<br>CAAGAGACCGAGCAATCAAG |

**Table 2. Real-time RT-PCR Thermal cycler schedule**

| Step | | Temperature (℃) | Time | cycles |
|---|---|---|---|---|
| **Hold** | | 94 | 30 min | 1 |
| **3 steps PCR** | Denaturation | 95 | 1 min | 45 |
| | Annealing | 59 | 30 sec | |
| | Extension | 72 | 1 min | |
| **Dissociation** | Denaturation | 95 | 15 sec | 1 |
| | Annealing | 60 | 30 sec | |
| | Extension | 95 | 15 sec | |
| **Hold** | | 4 | indefinitely | 1 |

**Table 3. Antibody information**

| Antibody | Description | Cat # | Company |
|---|---|---|---|
| COX2 | Mouse IgG | 160,112 | Cayman |
| FOXO1 | Rabbit IgG | 2880 | Cell Signaling |
| PGR | Rabbit IgG | 8757 | Cell Signaling |
| E-cadherin | Mouse IgG | 610,181 | BD Science |
| SIRT1 | Rabbit IgG | 9475S | Cell Signaling |
| ACTIN | Rabbit IgG | SC-1616 | Santa Cruz |
| COL1 | Mouse IgG | SC-293182 | Santa Cruz |
| COL4 | Rabbit IgG | ab6586 | Abcam |
| Vimentin | Rabbit IgG | ab92547 | Abcam |

| Antibody | Description | Cat # | Company |
|---|---|---|---|
| Elastin | Mouse IgG | 58756 | Santa Cruz |
| Integrin B1 | Rabbit IgG | MEM-101E | Abcam |
| Goat Anti-Rabbit IgG Biotinylated antibody | Secondary Antibody | BA-9100 | Vector Laboratories |
| Goat Anti-Mouse IgG Biotinylated antibody | Secondary Antibody | BA-9200 | Vector Laboratories |

# Result

**Fertility defect of mice with ablation of *Sirt1* in the PGR-expressing cells**

Global deletion of *Sirt1* in mice results in perinatal lethality in inbred backgrounds (McBurney et al., 2003; Vasquez et al., 2018) To assess the function of *Sirt1* in the female reproductive tract but avoid this lethal phenotype, It was generated uterine-specific *Sirt1* knockout mice by crossing *Pgr-cre* ($Pgr^{cre/+}$) and *Sirt1* floxed female mice ($Sirt1^{f/f}$) (Figure 1A) (Soyal et al., 2005). We confirmed the ablation of uterine SIRT1 at GD 5.5 when SIRT1 is normally strongest, with RT-qPCR showing that *Sirt1* mRNA was significantly decreased in the uterus of $Sirt1^{d/d}$ mice compared with control mice (Figure 1B). Western blot analysis demonstrated loss of SIRT1 proteins in the uteri of $Sirt1^{d/d}$ mice (Figure 1C). To confirm the loss of SIRT1 in all uterine compartments, we performed immunohistochemistry analysis in the implantation site region of GD 5.5. In $Sirt1^{d/d}$ mice, there was weak SIRT1 expression except for in the embryo (Figure 1D). These results demonstrate the successful ablation of *Sirt1* in the murine uterus.

To investigate the impact of ablation of *Sirt1* on female fertility, female control ($Sirt1^{f/f}$) and $Sirt1^{d/d}$ mice were mated with wild-type C57Bl/6 male mice for 6 months. $Sirt1^{d/d}$ mice had only 2.40 ± 0.60 litters/mouse compared with 5.40 ± 0.30 litters/mouse from control mice (Table 4). Control mice had an average 6.63 ± 0.13 pups/litter, whereas female $Sirt1^{d/d}$ mice had an average 4.20 ± 0.81 pups/litter. $Sirt1^{d/d}$ mice produced significantly fewer litters per mouse *(*p <0.05*) and pups per litter (***p <0.001), revealing that $Sirt1^{d/d}$ female mice are subfertile.

To test for an ovarian cause of subfertility, female *Sirt1*$^{d/d}$ mice were examined for proper ovarian histology, ovulation, and steroid hormone production at GD 3.5. Blastocysts generated by natural mating were flushed from the uterus at GD3.5. We isolated 7.00 ± 0.71 and 6.20 ± 0.49 embryos from uteri of *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ mice, respectively (n = 5; Figure 3A). The serum levels of E2 and P4 were 16.22 ± 3.05 pg/ml and 10.53 ± 1.85 ng/ml, respectively, in control mice, meanwhile 17.58 ± 4.08 pg/ml and 8.44 ± 1.75 ng/ml, respectively, in *Sirt1*$^{d/d}$ mice, not significantly differing between the mice at GD 3.5 (n = 5/genotype) (Figure 3B and C). In addition, histological analysis of the intact ovaries from *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ mice revealed no morphological defect (Figure 3D). These results demonstrate that ovarian morphology and function were not affected in the *Sirt1*$^{d/d}$ females suggesting that the subfertility is primarily due to a uterine defect.

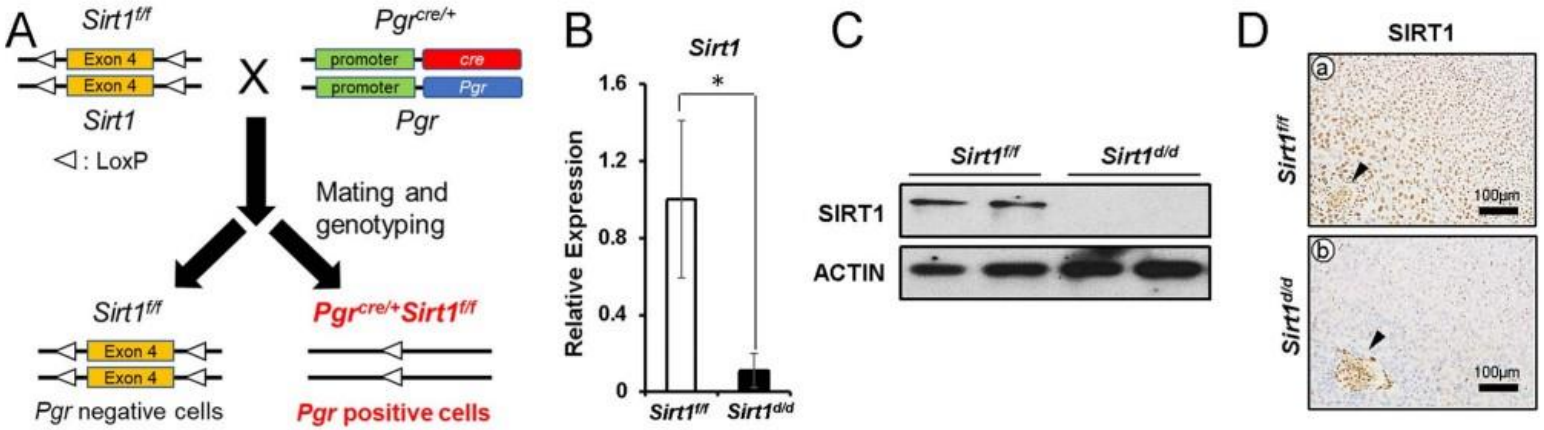



**Figure 1. Generation of *Sirt1*$^{d/d}$ mice.**

For *Sirt1*$^{d/d}$ mice generation. *Sirt1*$^{d/d}$ female mice do not express SIRT1 in any uterine cell layer. (A) The schematic shows the strategy for generating *Pgr*$^{cre/+}$*Sirt1*$^{f/f}$ (*Sirt1*$^{d/d}$) mice. (B) Relative expression of uterine Sirt1 at GD 5.5, mRNA normalized to Rpl7 (n = 5, ∗p <0.05). The results represent the mean ± SEM. ∗p < 0.05. (C) Uterine SIRT1 protein expression at GD 5.5 normalized to ACTIN. (D) Representative images of SIRT1 IHC in uterine tissue at GD 5.5 (n = 3).

**Table 4. Subfertility of *Sirt1*$^{d/d}$ female mice**

| | Number of Mice Tested | Number of Litters | Number of Pups | Average of Pups/Litter | Average Number of Litters/Mouse |
|---|---|---|---|---|---|
| ***Sirt1***$^{f/f}$ | 5 | 26 | 167 | 6.42 ± 0.18 | 5.20 ± 0.43 |
| ***Sirt1***$^{d/d}$ | 5 | 10 | 33 | 4.20 ± 0.68 | 2.00 ± 0.63 |

## Increased SIRT1 during early pregnancy in the mouse uterus

To examine the function of SIRT1 in mouse uterine tissue, we profiled the SIRT1 protein level during early pregnancy. SIRT1 was weakly expressed in uterine stromal cells and luminal and glandular epithelial cells at GD 0.5 to 3.5 (Fig. 2c). At GD 4.5, SIRT1 expression increased in nuclei of decidual stromal cells, mainly in the PDZ (Fig. 2d, e). At GD 5.5, SIRT1 expression is strongest, with high expression levels in both the PDZ and secondary decidual zone (SDZ) (Fig. 2f, g). At GD 7.5, expression of SIRT1 was similar to GD 5.5, but it was weakly localized to nuclei of decidual cells and weaker in the PDZ than the SDZ (Fig. 2h-j).

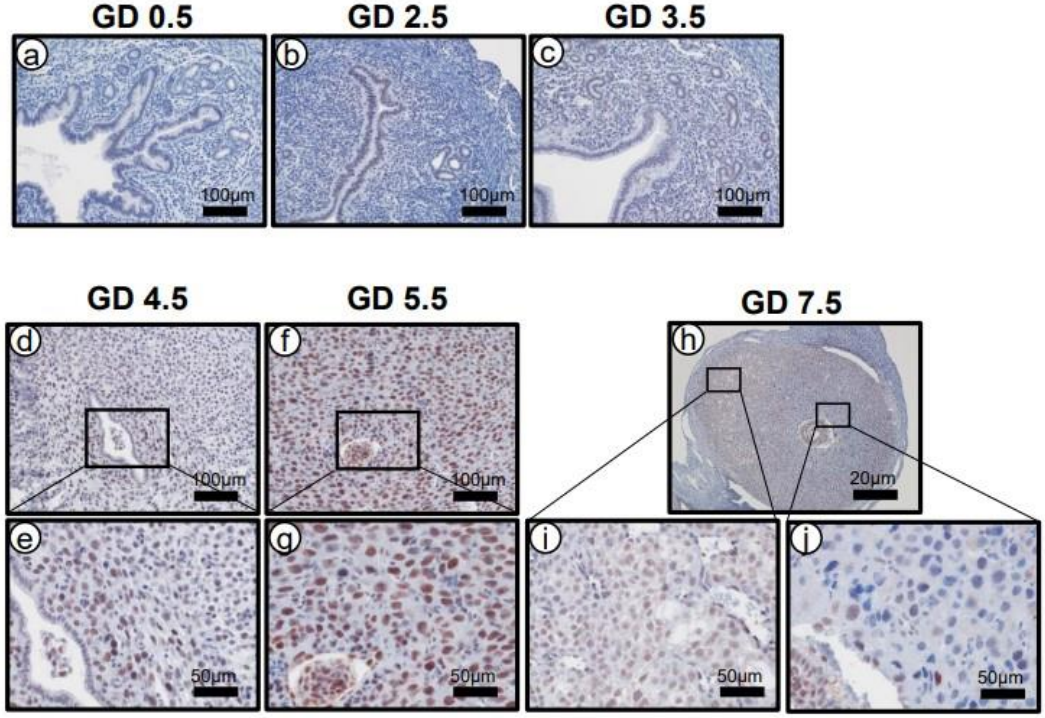



**Figure 2. Increased uterine SIRT1 during early pregnancy in the mouse uterus.**

SIRT1 IHC in uterine tissue of natural pregnant mice (n=3). At GD 0.5 to 3.5 SIRT1 shows low expression (a-c), but at GD 4.5 (d-e) and 5.5 (f-g) SIRT1 expression was strong in decidualized cells. At GD7.5 SIRT1 was also expressed in secondary decidual cells (h-j).

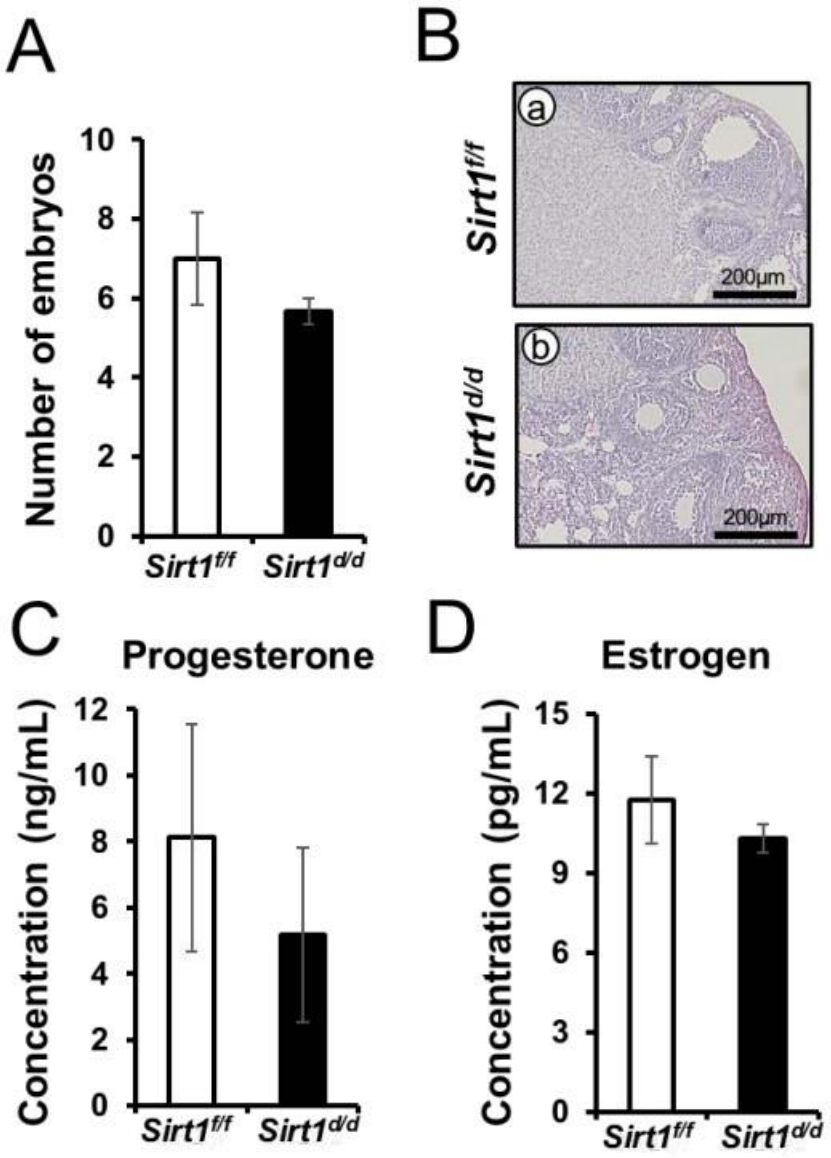

A
Number of embryos
10
8
6
4
2
0
Sirt1f/f
Sirt1d/d
B
a
Sirt1f/f
200μm
b
Sirt1d/d
200μm
C
Progesterone
Concentration (ng/mL)
12
10
8
6
4
2
0
Sirt1f/f
Sirt1d/d
D
Estrogen
Concentration (pg/mL)
15
12
9
6
3
0
Sirt1f/f
Sirt1d/d

**Figure 3. GD 3.5 *Sirt1*$^{d/d}$ mice show no ovarian function**

(A) The average number of blastocysts from *Sirt1*$^{f/f}$ uteri was 7.00 ± 1.15, and the number from *Sirt1*$^{d/d}$ uteri was 5.65± 0.33 (n=3 per group, $p > 0.05$, no significance). (B) Ovarian histology by H&E staining shows no difference between *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ mice. Serum progesterone (C) and estrogen (D) levels were no significantly different between *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ mice (*$p > 0.05$).

## Implantation defect in *Sirt1*$^{d/d}$ mice

To determine the cause of subfertility in *Sirt1*$^{d/d}$ mice, 8-week-old female *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ mice were mated with fertile control male mice. The uteri were examined at GD 5.5 to examine the ability of embryos to implant. *Sirt1*$^{f/f}$ female mice showed average 7.57 ± 0.43 implantation sites per mouse (n = 7). However, *Sirt1*$^{d/d}$ uteri contained significantly fewer implantation sites (5.00 ± 1.04) than control mice (n = 8) (Figure 4A and B). To understand the molecular basis of the implantation defect in *Sirt1*$^{d/d}$ mice, we examined the uterine histology and COX2 expression (Lim et al., 1997) at GD 4.5. The endometrium in control mice normally undergoes luminal epithelial closure at the time of embryo implantation (Matsumoto et al., 2018), and COX2 is strongly and specifically expressed in stromal cells that are beginning to decidualize near the embryo invasion region (Chakrabarty et al., 2007). However, our histological and immunochemistry analysis revealed two types of abnormal implantation sites in *Sirt1*$^{d/d}$ mice (Figure 5). Group #1 (14/20, 70%) showed a partially closed luminal epithelial layer, decreased COX2 expression in stromal cells near implantation sites, and abnormal COX2 expression in luminal epithelial cells (Figure 5h). Group #2 (6/20, 30%) had a more severe phenotype compared with Group #1, exhibiting a fully opened luminal epithelial layer with floating embryos (Figure 5f). In addition, COX2 expression was nearly absent (Figure 5i). To determine if the two different phenotypes in *Sirt1*$^{d/d}$ mice originated due to regionally differing *Sirt1* knockout efficiency, we examined the expression of SIRT1 at implantation sites of two

different groups. However, SIRT1 was absent around implantation sites in both groups of *Sirt1*$^{d/d}$ mice (Figure 5k and l)

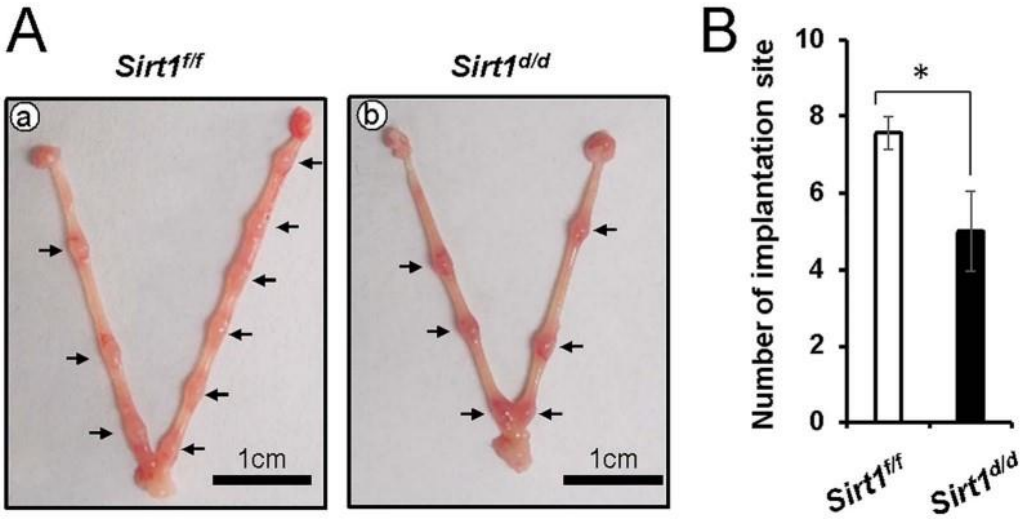



**Figure 4. Decreased implantation site number in GD 4.5 *Sirt1*$^{d/d}$ uteri**

Representative images of H&E (a-f), COX2 IHC (g-i), and SIRT1 IHC (j-l) at GD 4.5 *Sirt1*$^{f/f}$ (a, d, g, and j), *Sirt1*$^{d/d}$ Group #1 (b, e, h, and k), *Sirt1*$^{d/d}$ Group #2 (c, f, i, and l) implantation sites.

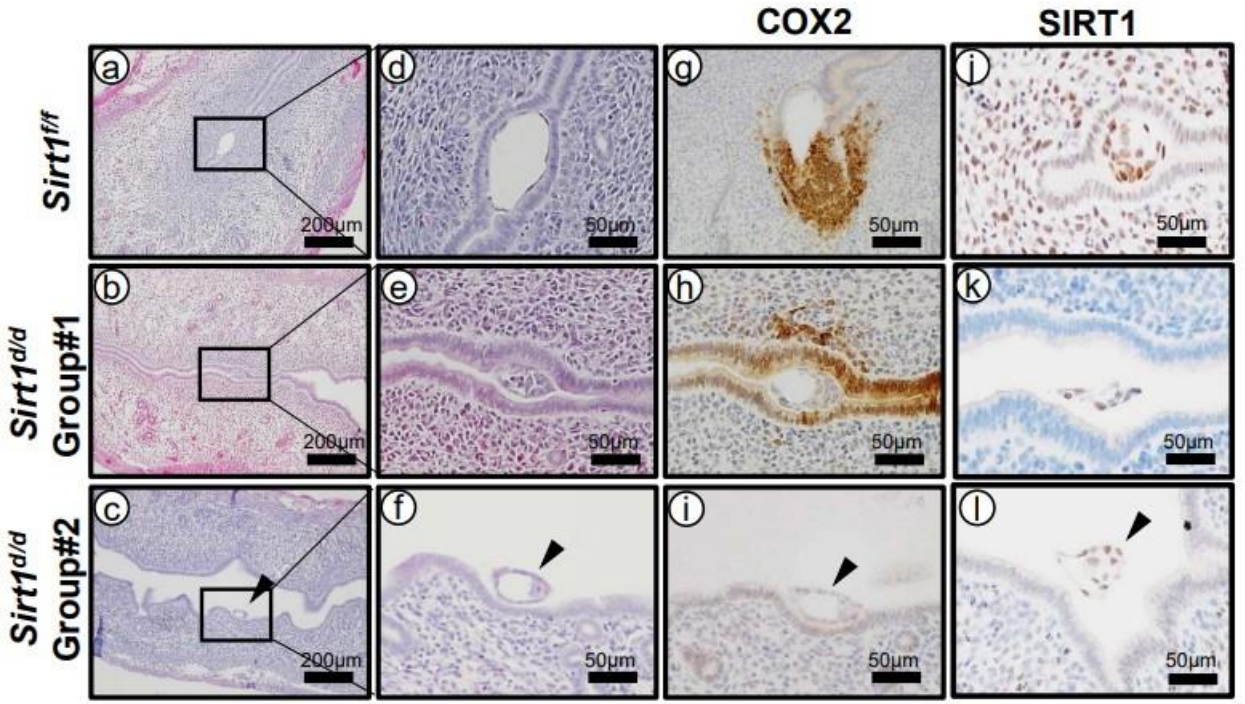
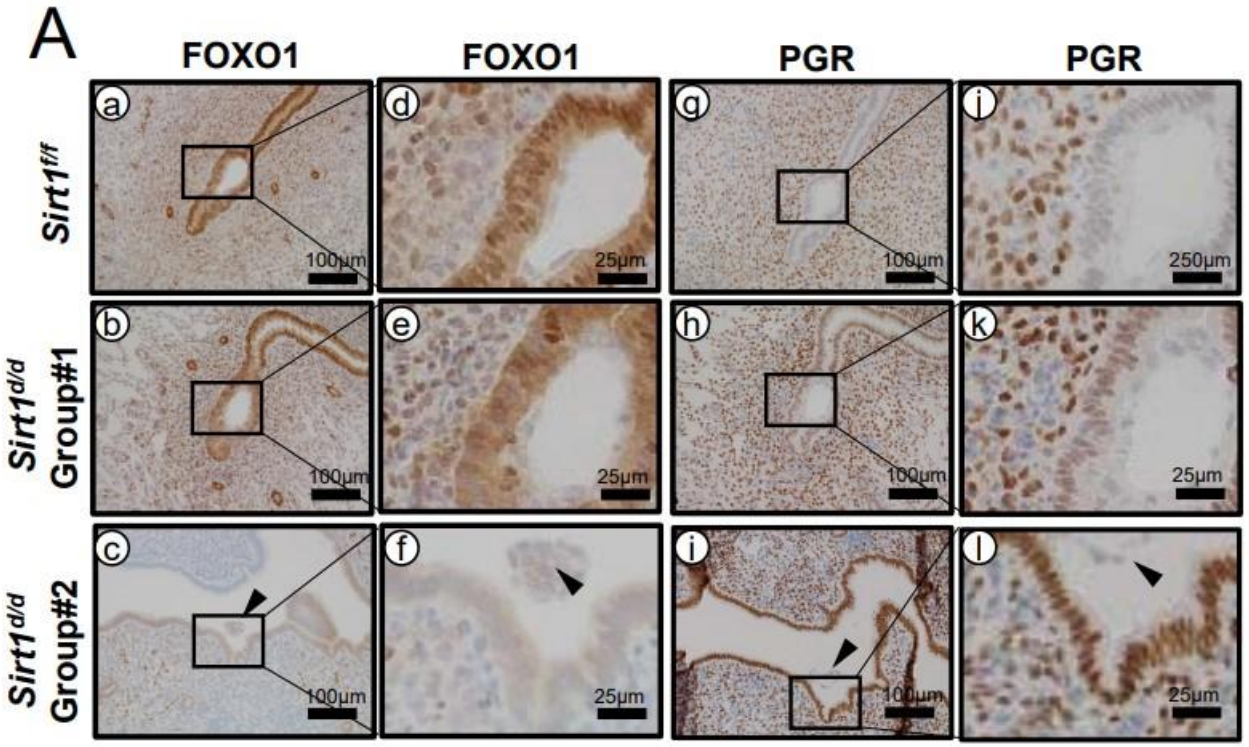

COX2
SIRT1
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
a
b
c
d
e
f
g
h
i
j
k
l
200μm
50μm

**Figure 5. Altered COX2 expression in GD 4.5 *Sirt1*$^{d/d}$ uteri**

Representative images of H&E (a-f), COX2 IHC (g-i), and SIRT1 IHC (j-l) at GD 4.5 *Sirt1*$^{f/f}$ (a, d, g, and j), *Sirt1*$^{d/d}$ Group #1 (b, e, h, and k), *Sirt1*$^{d/d}$ Group #2 (c, f, i, and l) implantation sites


A
FOXO1
FOXO1
PGR
PGR
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
a
b
c
d
e
f
g
h
i
j
k
l
100μm
25μm
100μm
250μm
100μm
25μm
100μm
25μm
100μm
25μm

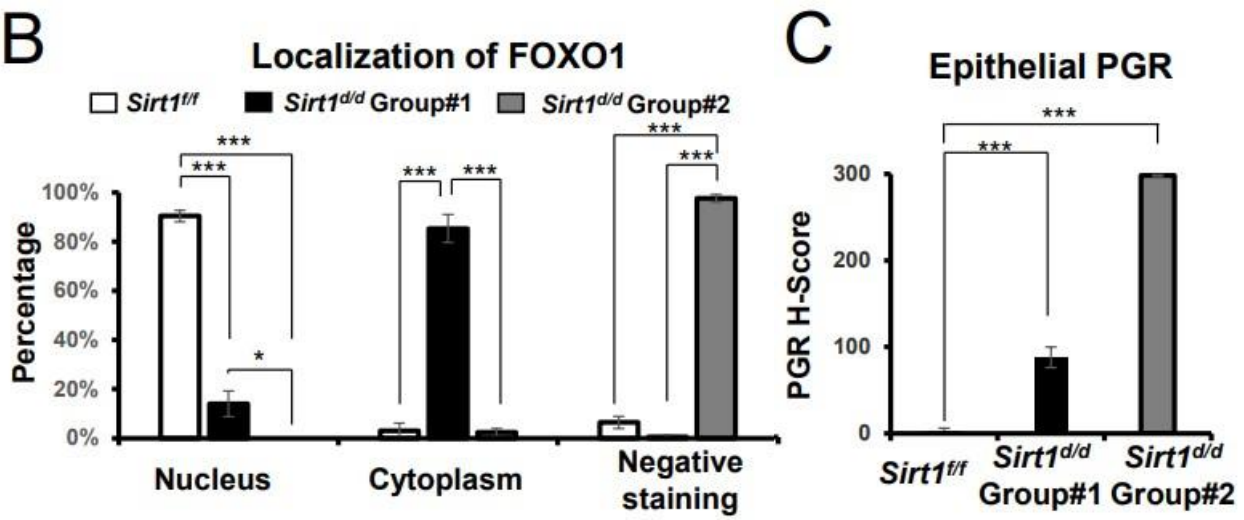

B
Localization of FOXO1
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Percentage
100%
80%
60%
40%
20%
0%
***
***
*
***
***
***
***
Nucleus
Cytoplasm
Negative staining
C
Epithelial PGR
PGR H-Score
300
200
100
0
***
***
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2

**Figure 6. Altered FOXO1 and PGR expression in GD 4.5 *Sirt1*$^{d/d}$ uteri**

(A) Expression of FOXO1(a-f) and PGR (g-l) IHC at GD 4.5 *Sirt1*$^{f/f}$ (a, d, g, and j), *Sirt1*$^{d/d}$ Group #1 (b, e, h, and k), *Sirt1*$^{d/d}$ Group #2 (c, f, I, and l) implantation sites. (B) Quantification of the localization of FOXA2 at *Sirt1*$^{f/f}$, *Sirt1*$^{d/d}$ Group #1, and *Sirt1*$^{d/d}$ Group #2 implantation sites. (C) Semi-quantitative H-score for epithelial PGR at *Sirt1*$^{f/f}$, *Sirt1*$^{d/d}$ Group #1, and *Sirt1*$^{d/d}$ Group #2 implantation sites. The results represent the mean ± SEM., *; $p < 0.05$; ***; $p < 0.001$.

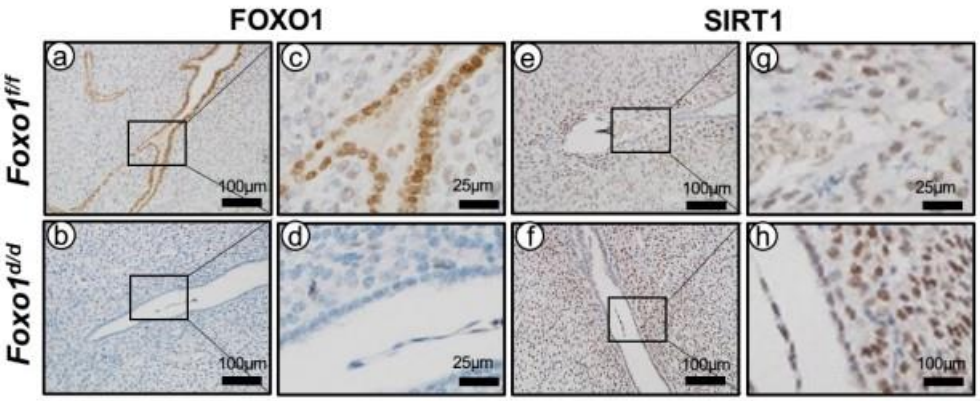



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**Figure 7. The expression of FOXO1 and SIRT1in GD 4.5 *Foxo1*$^{d/d}$ uteri**

FOXO1 (a-d) and SIRT1 (e-h) staining in GD 4.5 *Foxo1*$^{f/f}$ (a, c, e, and g) and *Foxo1*$^{d/d}$ (b, d, f, and h) implantation sites.

**Altered E-cadherin, COX2, FOXO1 and PGR expression in GD 5.5 *Sirt1*$^{d/d}$ uteri**

To further characterize the role of SIRT1 in implantation and decidualization, we analyzed uterine histology and screened important molecular markers at GD 5.5 (Figure 8A). In normal control mice, the luminal epithelium at the embryo invasion site undergoes degradation by this time (Li et al., 2015), and therefore we observed no E-cadherin-positive epithelium at implantation sites (Figure 8A a and d). FOXO1 was also not expressed (Figure 8Ag), but PGR and COX2 were abundant in nuclei of decidual stromal cells in the PZD (Figure 8Ba and d) (Lim et al., 1999). In contrast, the *Sirt1*$^{d/d}$ uterus showed structural and molecular defects. Like at GD 4.5, *Sirt1*$^{d/d}$ implantation sites exhibited two distinct phenotypes. Group #1 showed normal implantation structure, and E-cadherin (Figure. 8Ab and e) and FOXO1 (Figure 8Ah) were negative. However, the expression of COX2 and PGR was significantly downregulated in the PZD (Figure 8Bb and e; C). Group #2 had a more critical defect. Immunohistochemistry results revealed intact epithelial cells positive for E-cadherin and FOXO1 (Figure 8Af and i). This phenotype matches that of GD 4.5 control mice (Li et al., 2015). Similar to Group #1, PGR and COX2 were significantly decreased in the PDZ (Figure 8Bc and f; C). However, strong E-cadherin staining was constantly detected at inter implantation sites of control and both groups of *Sirt1*$^{d/d}$ mice (Figure 8Aj-l). All these data underscore the importance of SIRT1 in the mechanisms of epithelial

degradation and stromal decidualization during implantation.

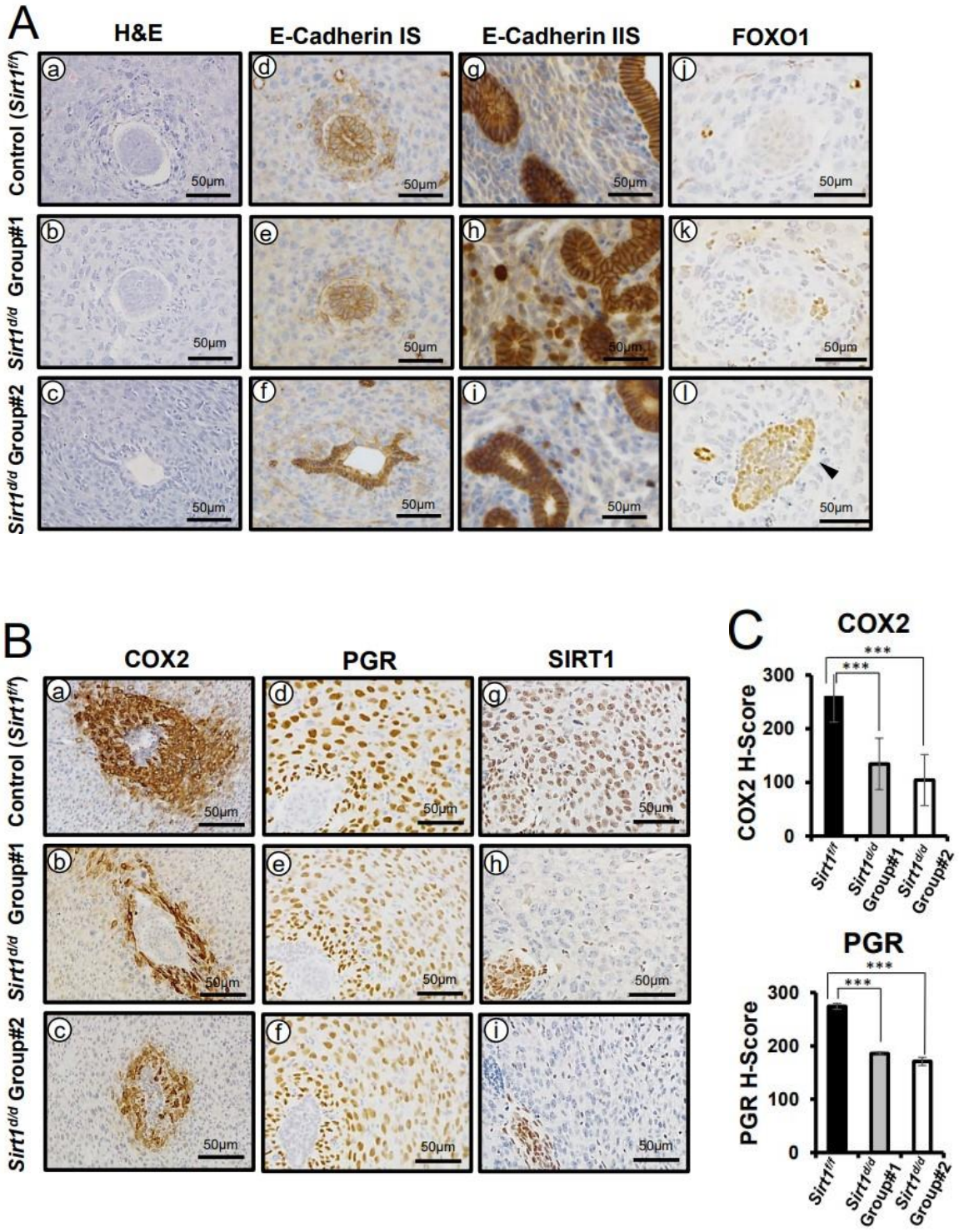

A
H&E
E-Cadherin IS
E-Cadherin IIS
FOXO1
Control (Sirt1f/f)
Sirt1d/d Group#1
Sirt1d/d Group#2
50μm
B
COX2
PGR
SIRT1
C
COX2
COX2 H-Score
PGR
PGR H-Score
***
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2

**Figure 8. Altered E-Cadherin, FOXO1, COX2, and PGR expression in GD 5.5 *Sirt1*$^{d/d}$ uteri**

E-Cadherin, FOXO1, COX2, and PGR expression was altered in GD 5.5 *Sirt1*$^{d/d}$ uteri (A) Representative images of H&E (a-c), E-cadherin (d-f and j-l), and FOXO1 (g-i) IHC at implantation sites (IS) and inter implantation sites of *Sirt1*$^{f/f}$ (a, d, g, and j), *Sirt1*$^{d/d}$ Group #1 (b, e, h, and k), and *Sirt1*$^{d/d}$ Group #2 (c, f, i, and l). (B) Representative images of COX2 (a-c), PGR (d-f), and SIRT1 (g-i) IHC at GD 5.5 *Sirt1*$^{f/f}$ (a, d, and g), *Sirt1*$^{d/d}$ Group #1 (b, e, and h), *Sirt1*$^{d/d}$ Group #2 (c, f, and i) ISs. (C) Semi-quantitative H-score for COX2 and PGR at *Sirt1*$^{f/f}$, *Sirt1*$^{d/d}$ Group #1, and *Sirt1*$^{d/d}$ Group #2 ISs. The results represent the mean ± SEM. ***$p < 0.001$.

## Defect of decidualization response in *Sirt1*$^{d/d}$ mice

To determine the effect of SIRT1 ablation on decidualization, we used a mouse model of artificially induced decidualization. In the stimulated uterine horn of control mice, the decidual response was fully induced after hormone administration and mechanical trauma (Fig. 9Aa). However, the stimulated uterine horn of *Sirt1*$^{d/d}$ mice showed a partial defect of decidual response (Fig. 9Ab), and the stimulated/control horn weight ratio was significantly decreased (Fig. 9B). Additionally, histological analysis of control uteri showed abundant decidual cell morphology, but it was decreased in *Sirt1*$^{d/d}$ uteri (Fig. 9C). These data reveal t hat SIRT1 is required for a complete decidualization response.

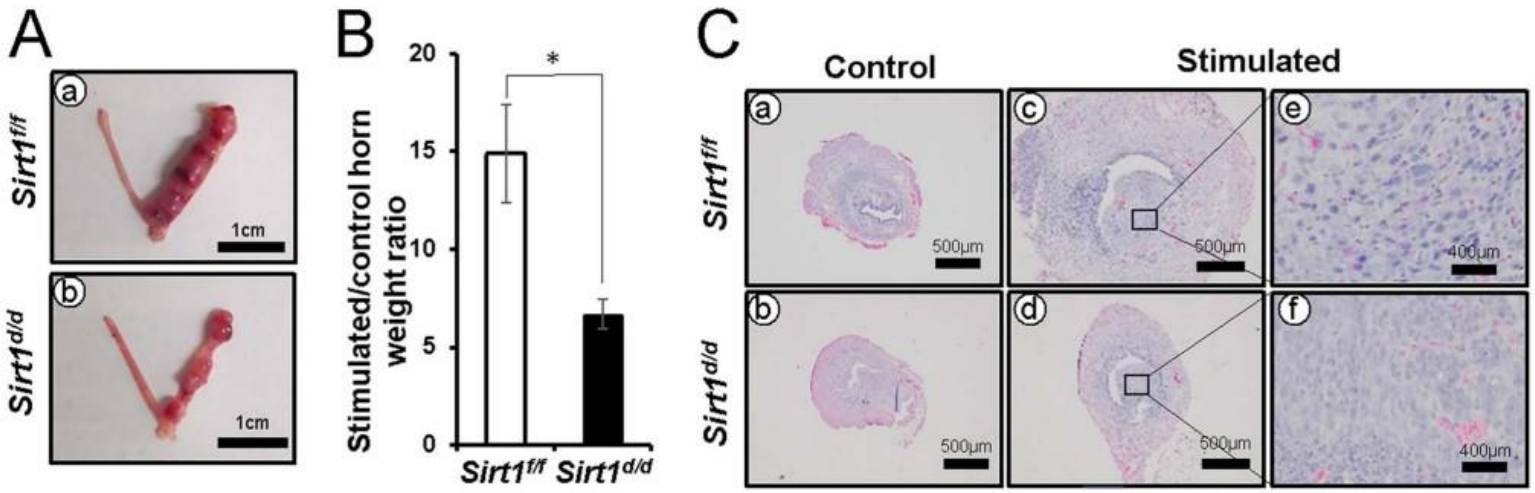

A
a
b
Sirt1f/f
Sirt1d/d
1cm
1cm
B
Stimulated/control horn weight ratio
20
15
10
5
0
*
Sirt1f/f
Sirt1d/d
C
Control
Stimulated
a
c
e
b
d
f
500μm
500μm
400μm
500μm
500μm
400μm

**Figure 9. Defect of decidualization response in *Sirt1*$^{d/d}$ mice**

(A) Uterine morphology of artificially induced decidualized uteri of *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ mice. The right-side horn received mechanical stimulation. (B) A significant decrease in stimulated/unstimulated (control) horn weight ratio in *Sirt1*$^{d/d}$ mice as compared with *Sirt1*$^{f/f}$ mice. (C) H&E staining of control and stimulated horns in *Sirt1*$^{f/f}$ (a and c) and *Sirt1*$^{d/d}$ (b and d) mice at day 5 of decidualization. The results represent the mean ± SEM., *; $p < 0.05$.

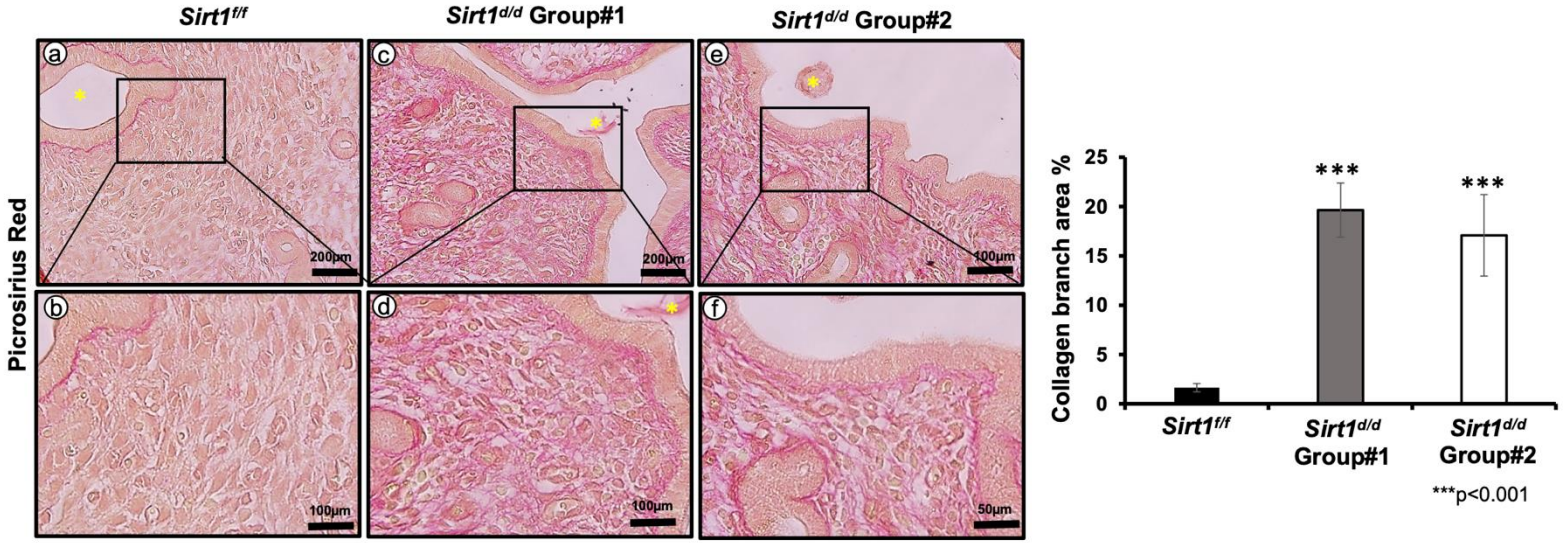
## Abnormal collagen distribution of *Sirt1*$^{d/d}$

Collagen is one of major ECM protein which has contributed to tissue structure and cellular microenvironment (Kisling et al., 2019). To identify the distribution of collagen in *Sirt1* ablated uterus during implantation period, we perform the Masson's trichrome staining. In GD 4.5 *Sirt1*$^{f/f}$, blue color stained with collagen fibers in luminal, glandular epithelium and stromal cell also endometrial ECM region (Figure 10Aa, b, c). In GD 4.5 *Sirt1*$^{d/d}$ Group #1, 2 shows similar pattern with *Sirt1*$^{f/f}$. However, collagen fiber was decreased in ECM of implantation (black dotted circle) (Figure 10Ad~ i). In GD 5.5 *Sirt1*$^{f/f}$ collagen fiber detected in decidualized cell and endometrial ECM region Also myometrial (Figure 10Ba, b, c). In *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$ Group #1 there is no collagen fiber in embryo implantation site. However, in *Sirt1*$^{d/d}$ Group #2, collagen was dramatically decreased in all uterine cell layer (Figure 10Bg~ i). These results suggest that *Sirt1* could involve in maintain normal structure of endometrial ECM.

Collagen subtype I and IV is important component in pregnant uterus (Spiess et al., 2007). To determine the distribution of COL1 and COL4, we perform immunohistochemistry in GD 4.5, 5.5 *Sirt1* mice. In GD 4.5 *Sirt1*$^{f/f}$ COL1 localized in ECM region of stromal cell, except for embryo invasion site (Figure 11Aa,b). However, there is no positive signal in both *Sirt1* ablated group (Figure 11Ac-f). In GD 5.5 COL1 expression was very weak in inter-implantation site and myometrial all *Sirt1* mice group (Figure 12B). The expression of Collagen Subtype IV was similar to COL1 in GD 4.5. COL4 detected in ECM of stromal cell except embryo

invasion site (Figure 12Aa,b). In both *Sirt1*$^{d/d}$ group shows there is no positive signal at GD 4.5 In GD 5.5, the expression of COL4 in *Sirt1*$^{f/f}$ was positive in implantation site (Figure 12Ba,b), and there is no detection in all uterine cell layer of *Sirt1*$^{d/d}$ Group #2 (Figure 12Bc-f). These results demonstrate that the *Sirt1* has relation with normal collagen distribution.

A


Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Picrosirius Red
a
b
c
d
e
f
200μm
100μm
200μm
100μm
100μm
50μm
Collagen branch area %
25
20
15
10
5
0
***
***
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
***p<0.001

B

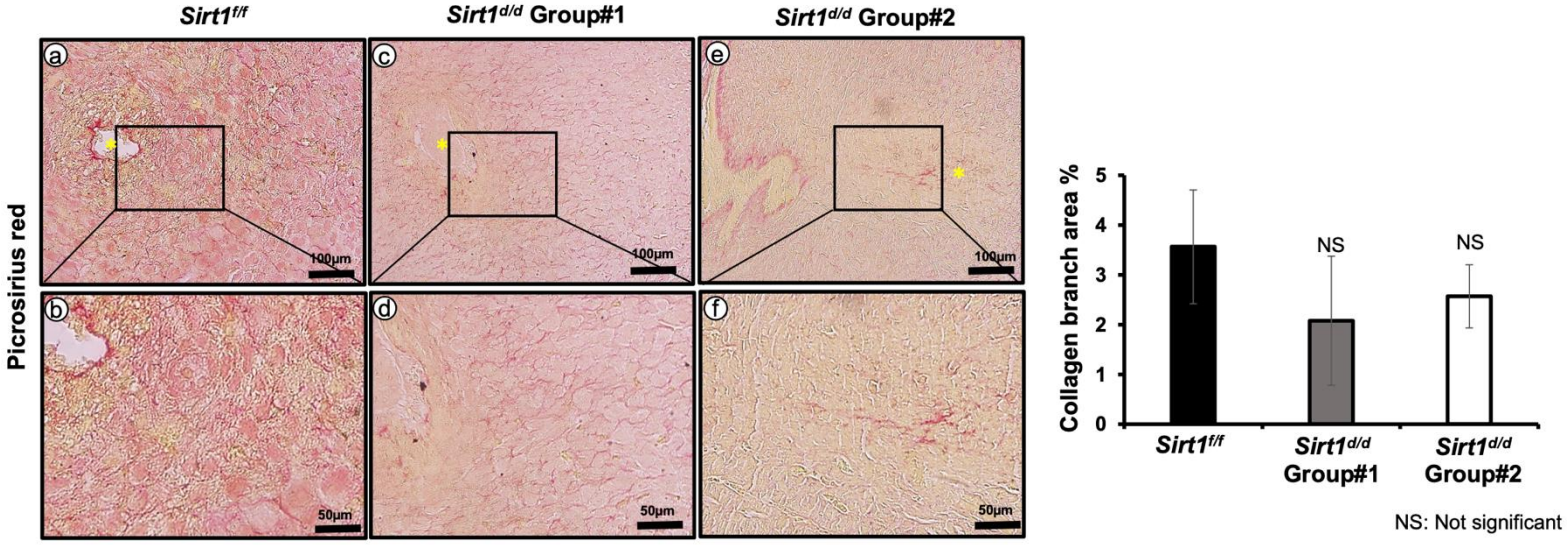
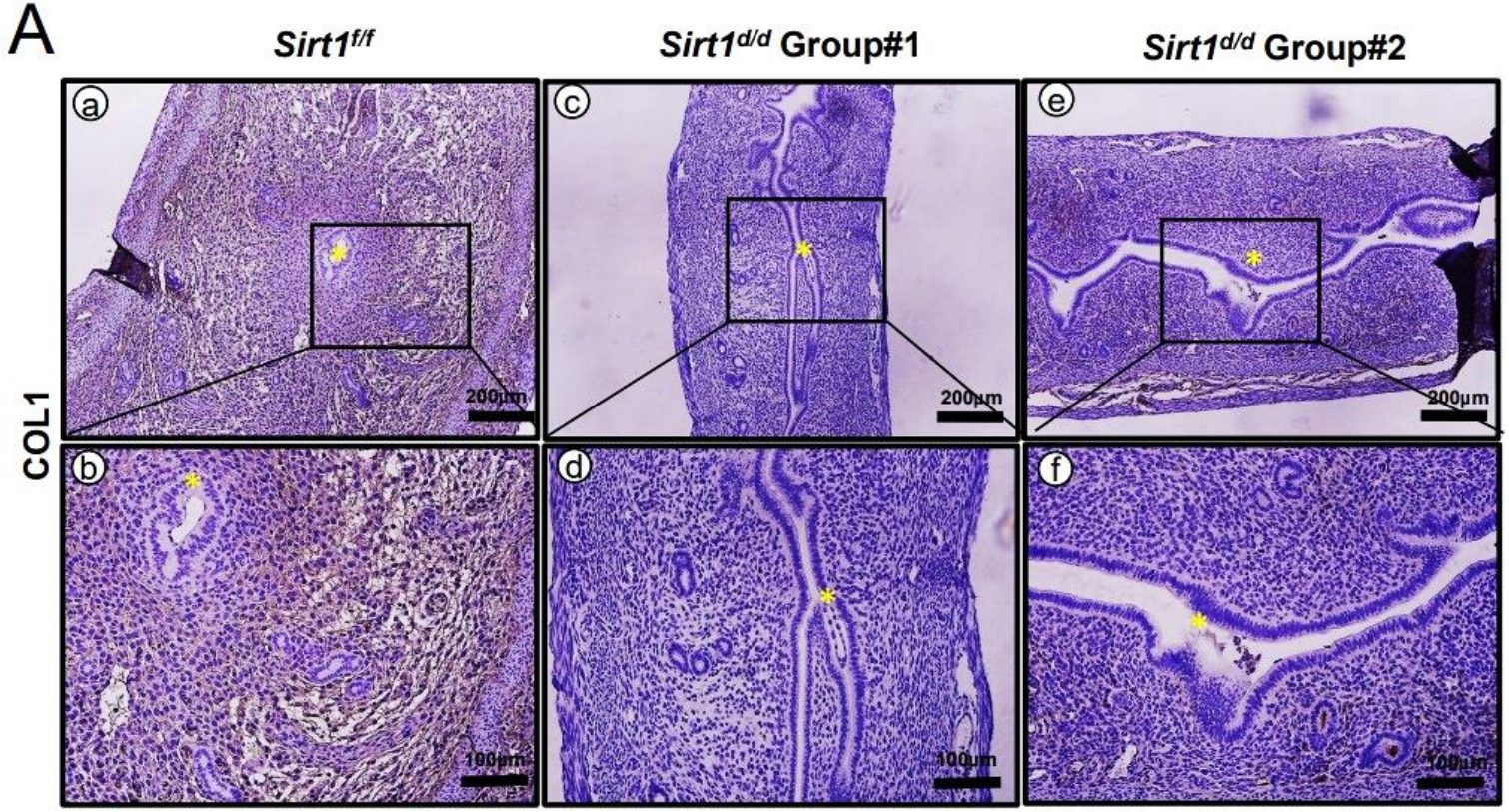
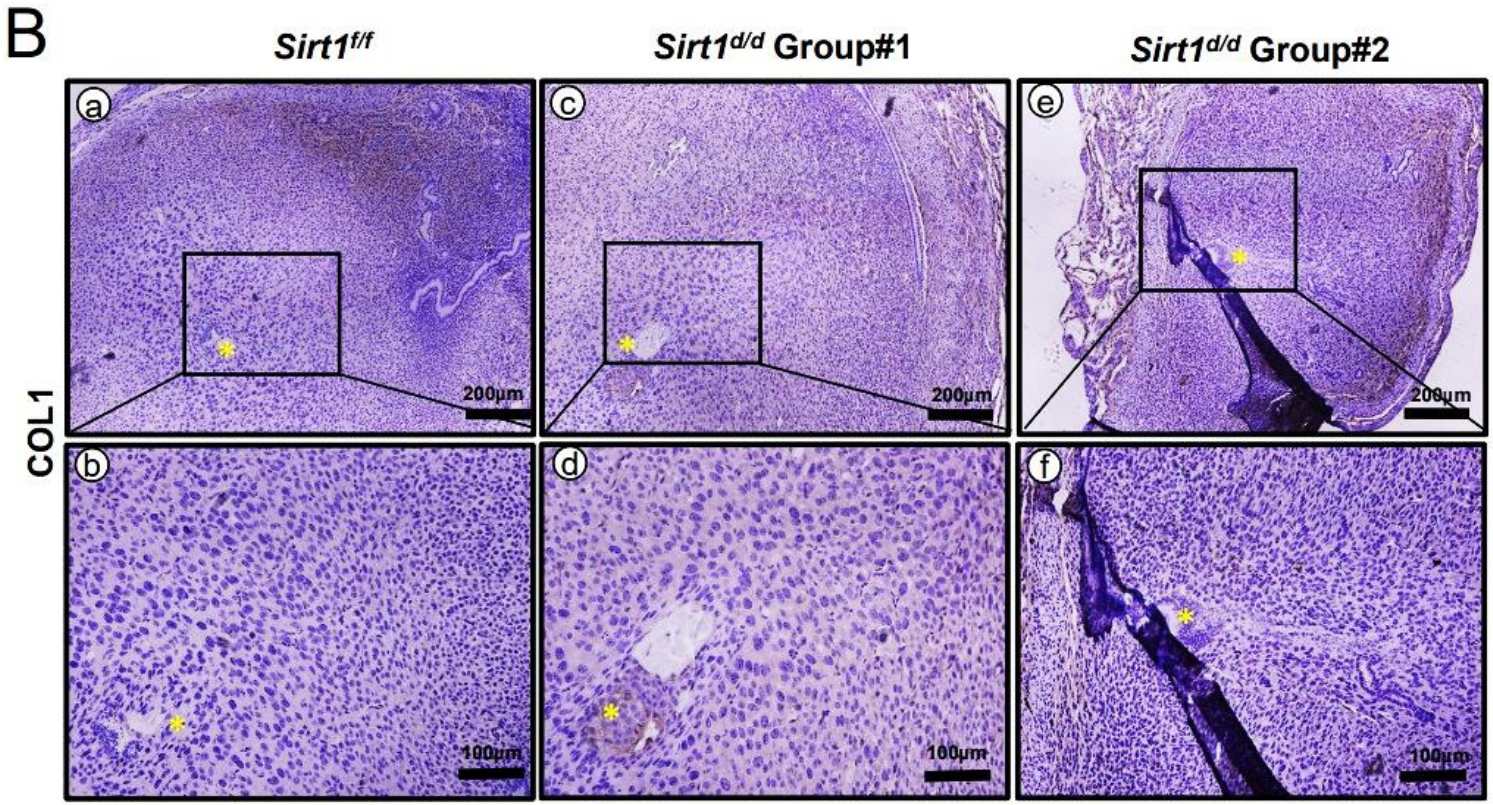

Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Picrosirius red
a
c
e
b
d
f
100μm
50μm
Collagen branch area %
5
4
3
2
1
0
NS
NS
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
NS: Not significant

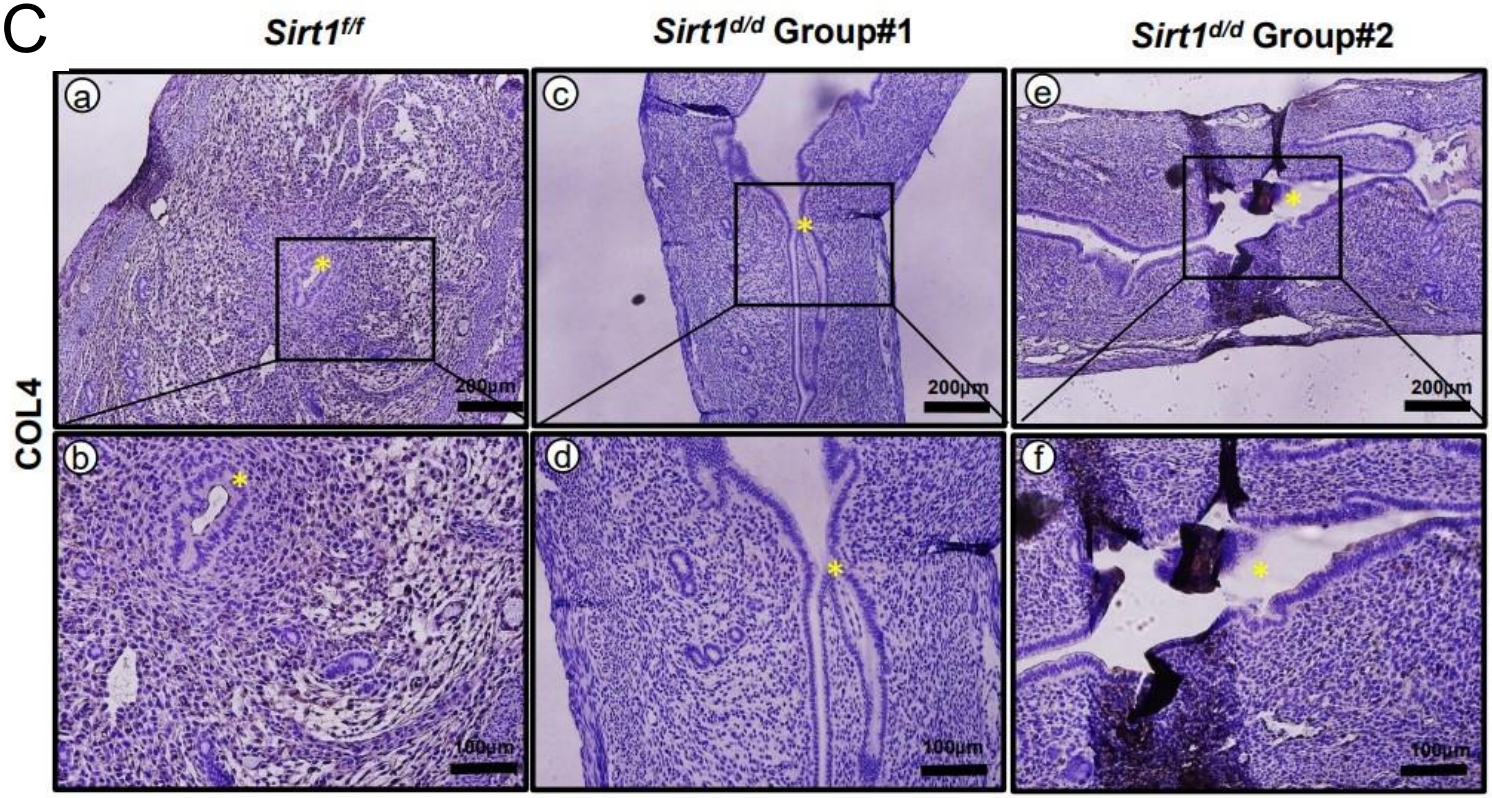
**Figure 10. Abnormal distribution of collagen in *Sirt1*$^{d/d}$**

A. Representative image of Picrosirius red staining in GD 4.5 *Sirt1* mice. (a,b) Representative image of *Sirt1*$^{f/f}$.(c,d) Representative image of *Sirt1*$^{d/d}$ Group#1. (e,f) Representative image of *Sirt1*$^{d/d}$ Group#2. '*' indicate the implanted embryo. The magnification of each picture are (a,c,e) x200, (b,d,f) x400. B. Representative image of Picrosirius red staining in GD 5.5 *Sirt1* mice. (a,b) Representative image of *Sirt1*$^{f/f}$.(c,d) Representative image of *Sirt1*$^{d/d}$ Group#1. (e,f) Representative image of *Sirt1*$^{d/d}$ Group#2. '*' indicate the implanted embryo. The magnification of each picture are (a,c,e) x200, (b,d,f) x400.


A
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
COL1
a
b
c
d
e
f
200μm
100μm


B
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
COL1
a
c
e
b
d
f
200μm
200μm
200μm
100μm
100μm
100μm

C


Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
COL4
a
b
c
d
e
f
200μm
100μm

D

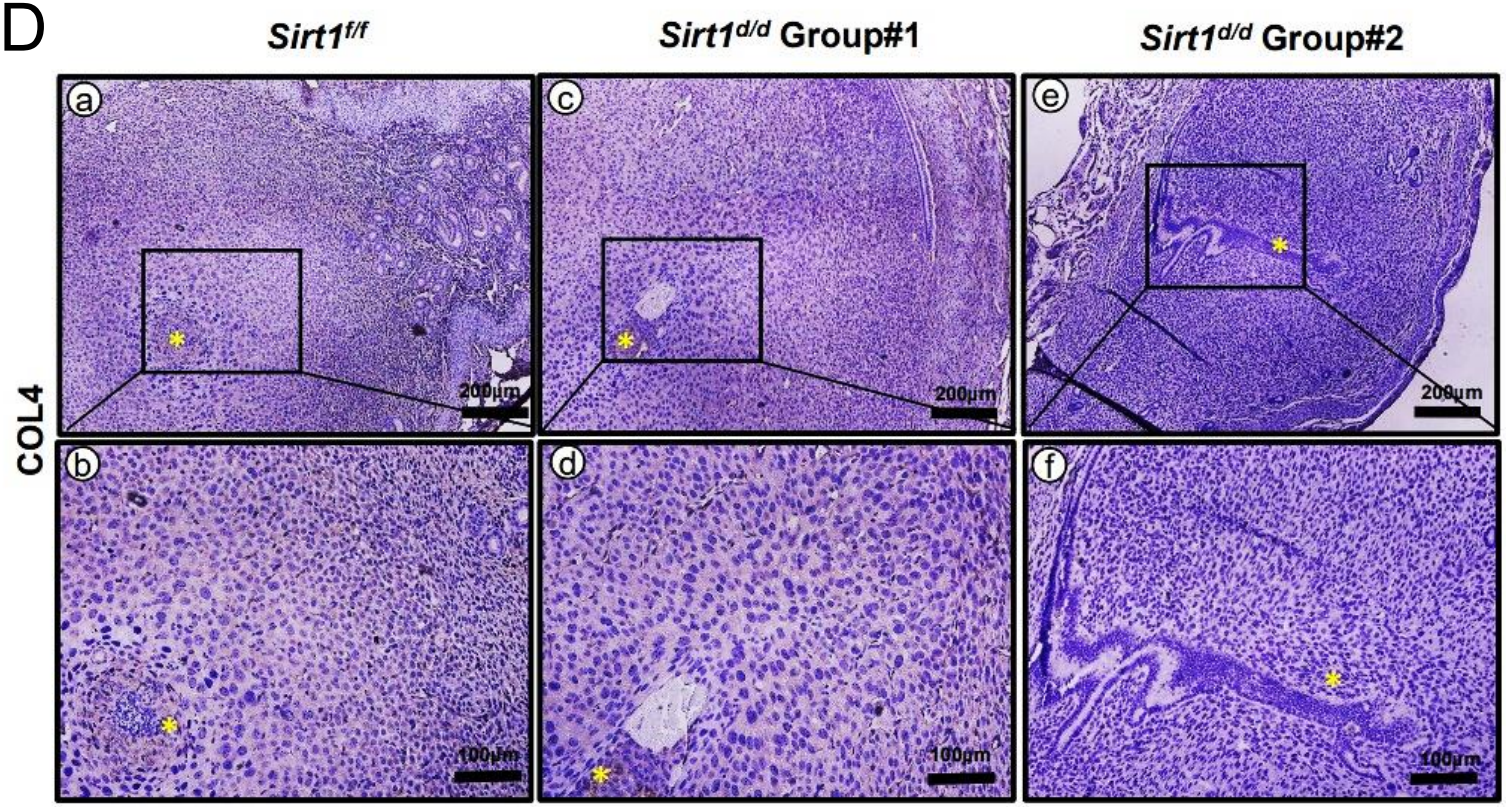

Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
COL4
a
b
c
d
e
f
200μm
100μm

**Figure 11. Abnormal distribution of COL1 and COL4 in *Sirt1$^{d/d}$***

A. Representative image of Immunohistochemistry of COL1 in GD 4.5 *Sirt1* mice. (a,b) Representative image of *Sirt1$^{f/f}$*. (c,d) Representative image of *Sirt1$^{d/d}$* Group#1. (e,f) Representative image of *Sirt1$^{d/d}$* Group#2. '*' indicate the implanted embryo. B. Representative image of Immunohistochemistry of COL1 in GD 5.5 *Sirt1* mice. (a,b) Representative image of *Sirt1$^{f/f}$*. (c,d) Representative image of *Sirt1$^{d/d}$* Group#1. (e,f) Representative image of *Sirt1$^{d/d}$* Group#2. '*' indicate the implanted embryo. The magnification of each picture are (a,c,e) x40, (b,d,f) x200. C. Representative image of Immunohistochemistry of COL4 in GD 4.5 *Sirt1* mice. (a,b) Representative image of *Sirt1$^{f/f}$*. (c,d) Representative image of *Sirt1$^{d/d}$* Group#1. (e,f) Representative image of *Sirt1$^{d/d}$* Group#2. '*' indicate the implanted embryo. D. Representative image of Immunohistochemistry of COL4 in GD 5.5 *Sirt1* mice. (a,b) Representative image of *Sirt1$^{f/f}$*. (c,d) Representative image of *Sirt1$^{d/d}$* Group#1. (e,f) Representative image of *Sirt1$^{d/d}$* Group#2. '*' indicate the implanted embryo. The magnification of each picture are (a,c,e) x40, (b,d,f) x200.

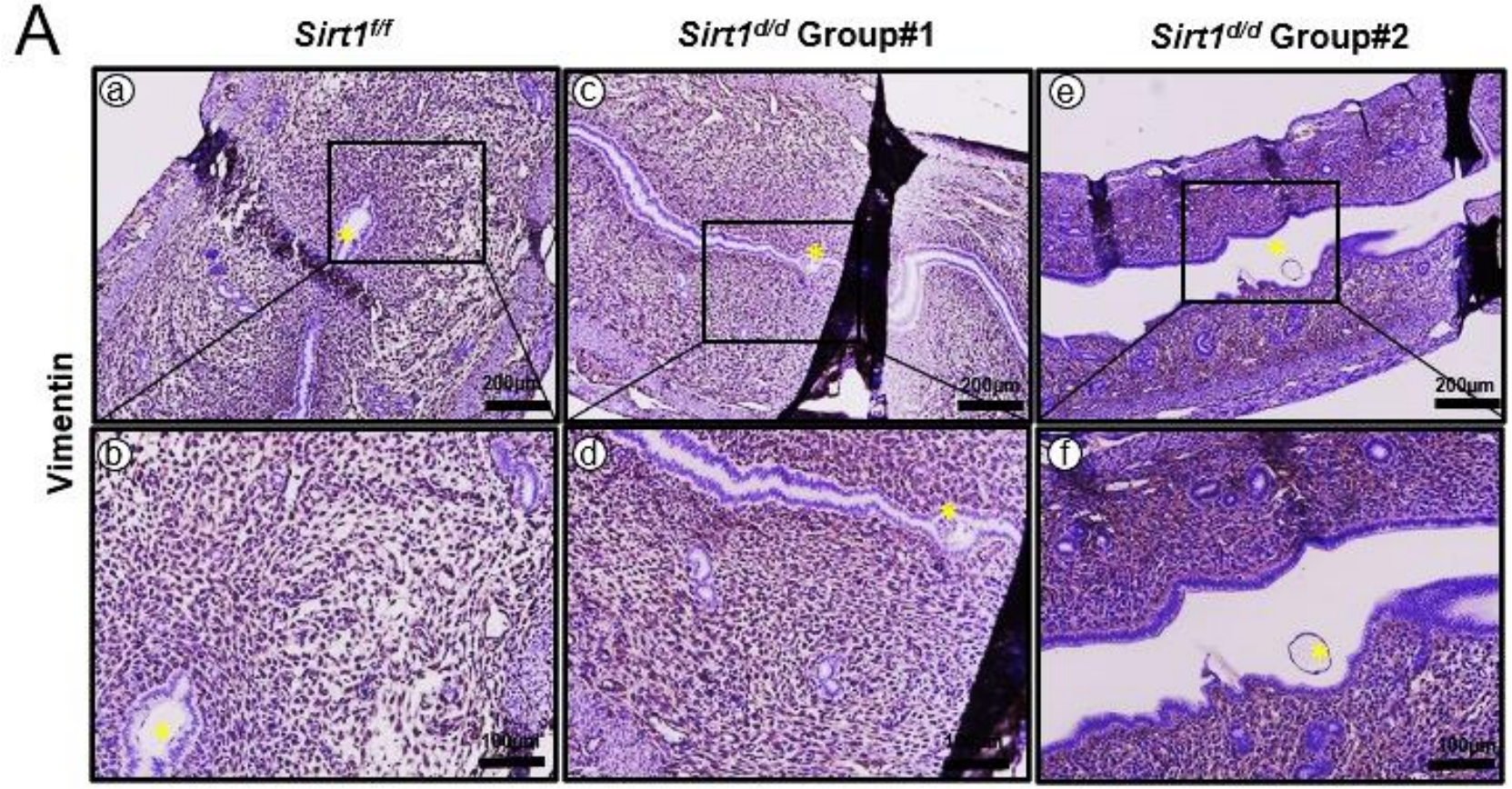
## Distribution of Vimentin, Elastin and Integrin β1

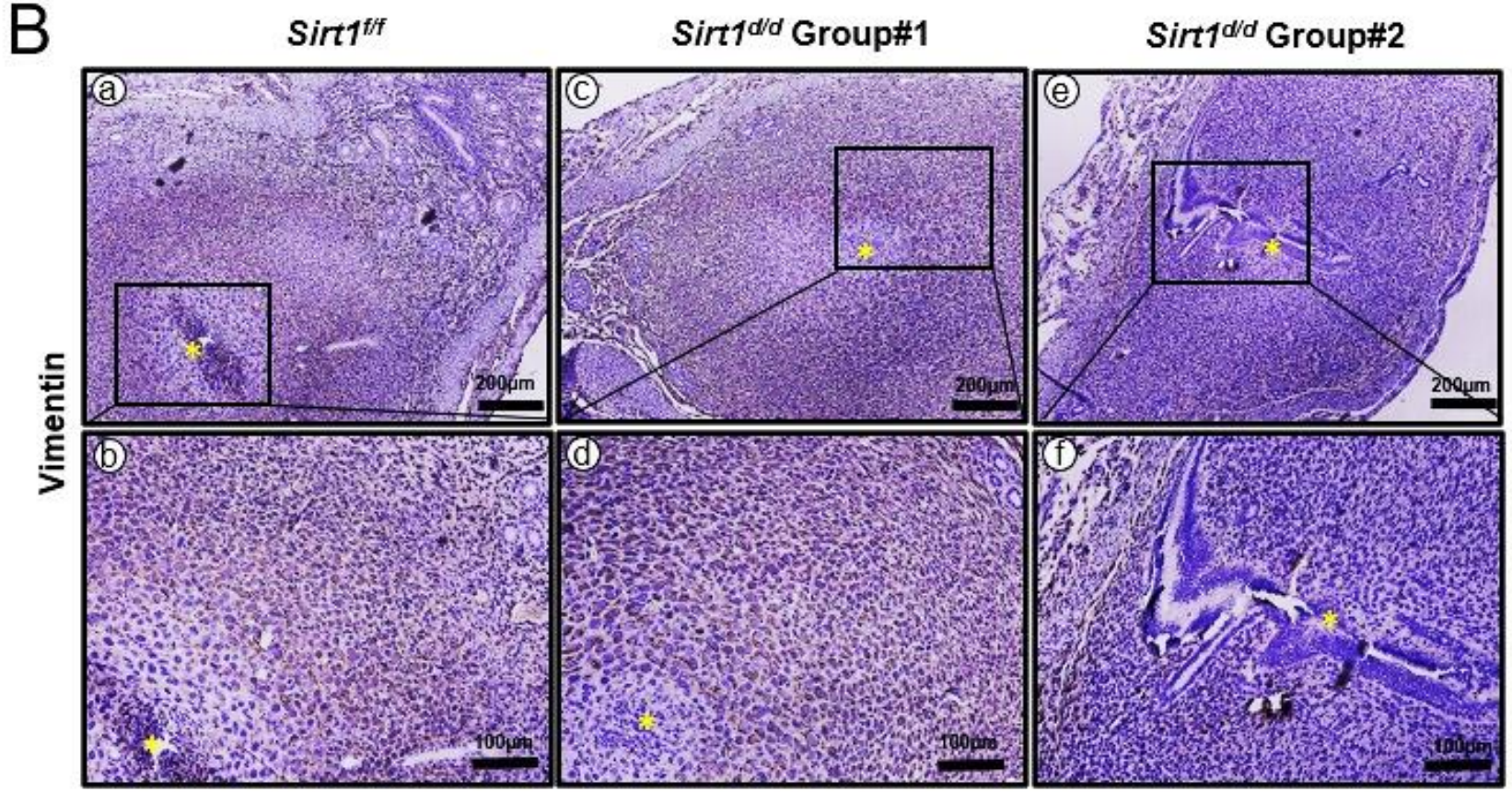
Vimentin is the type III filament protein that is ubiquitously expressed in mesenchymal tissue (Desouki et al., 2014). In early studies, vimentin has an important role to make up the cytoskeleton for normal cell shape, integrity also interaction between epithelial-mesenchymal interaction (Eriksson et al., 2009). Also, vimentin is reported as a marker for differentiating endometrial cells (Oh et al., 2013). Another ECM protein elastin is known as a critical molecule for maintaining the normal biological process (Wagenseil et al., 2010). Previous study identified the cardiovascular abnormalities, also elastin ablated mice ($Eln^{-/-}$) show lethal when neonatal stage (Li et al., 1998). therefore, we expect the vimentin and elastin have potential regulator of decidualization in $Sirt1^{d/d}$. However, there is no dramatic defected expression of these proteins at GD 4.5 and 5.5 of $Sirt1^{d/d}$.

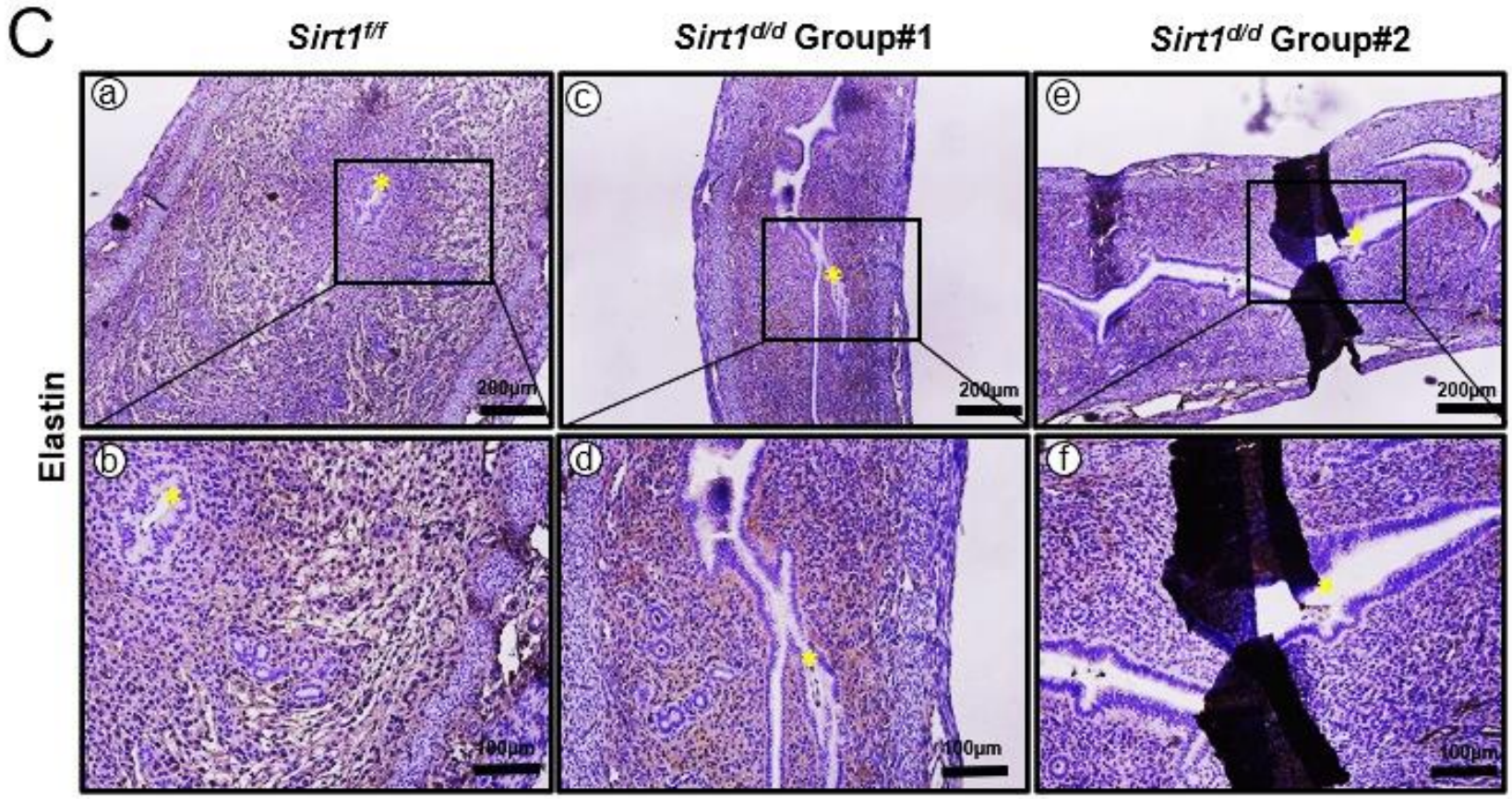
In GD 4.5 $Sirt1^{f/f}$ and $Sirt1^{d/d}$ Group #1 shows decreased endometrial Integrin β1 at blastocyst attached site. However, defected implantation site of $Sirt1^{d/d}$ Group #2 shows strong expression of Integrin β1 (Fig. 13A). In primary decidual zone of $Sirt1^{f/f}$ and $Sirt1^{d/d}$ Group #1 at GD 5.5, Integrin β1 was very weak. But there is no positive staining in $Sirt1^{d/d}$ Group #2 (Fig. 13B).

**Figure 12. No difference of Vimentin, Elastin between *Sirt1$^{f/f}$*, *Sirt1$^{d/d}$***

A. Representative image of Immunohistochemistry of Vimentin in GD 4.5 *Sirt1$^{f/f}$* mice. (a,b) Representative image of *Sirt1$^{d/d}$* Group#1 (c,d), Representative image of *Sirt1$^{d/d}$* Group#2 (e,f). '*' indicate the implanted embryo. B. Representative image of Immunohistochemistry of Vimentin in GD 5.5 *Sirt1$^{f/f}$* mice. (a,b) Representative image of *Sirt1$^{d/d}$* Group#1 (c,d), Representative image of *Sirt1$^{d/d}$* Group#2 (e,f). C. Representative image of Immunohistochemistry of Elastin in GD 4.5 *Sirt1$^{f/f}$* mice. (a,b) Representative image of *Sirt1$^{d/d}$* Group#1 (c,d), Representative image of *Sirt1$^{d/d}$* Group#2 (e,f). D. Representative image of Immunohistochemistry of Elastin in GD 5.5 *Sirt1$^{f/f}$* mice. (a,b) Representative image of *Sirt1$^{d/d}$* Group#1 (c,d), Representative image of *Sirt1$^{d/d}$* Group#2 (e,f). The magnification of each picture are (a,c,e) x100, (b,d,f) x200


A
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Vimentin
a
b
c
d
e
f
200μm
100μm


B
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Vimentin
a
c
e
b
d
f
200μm
200μm
200μm
100μm
100μm
100μm


C
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Elastin
a
b
c
d
e
f
200μm
100μm
200μm
100μm
200μm
100μm

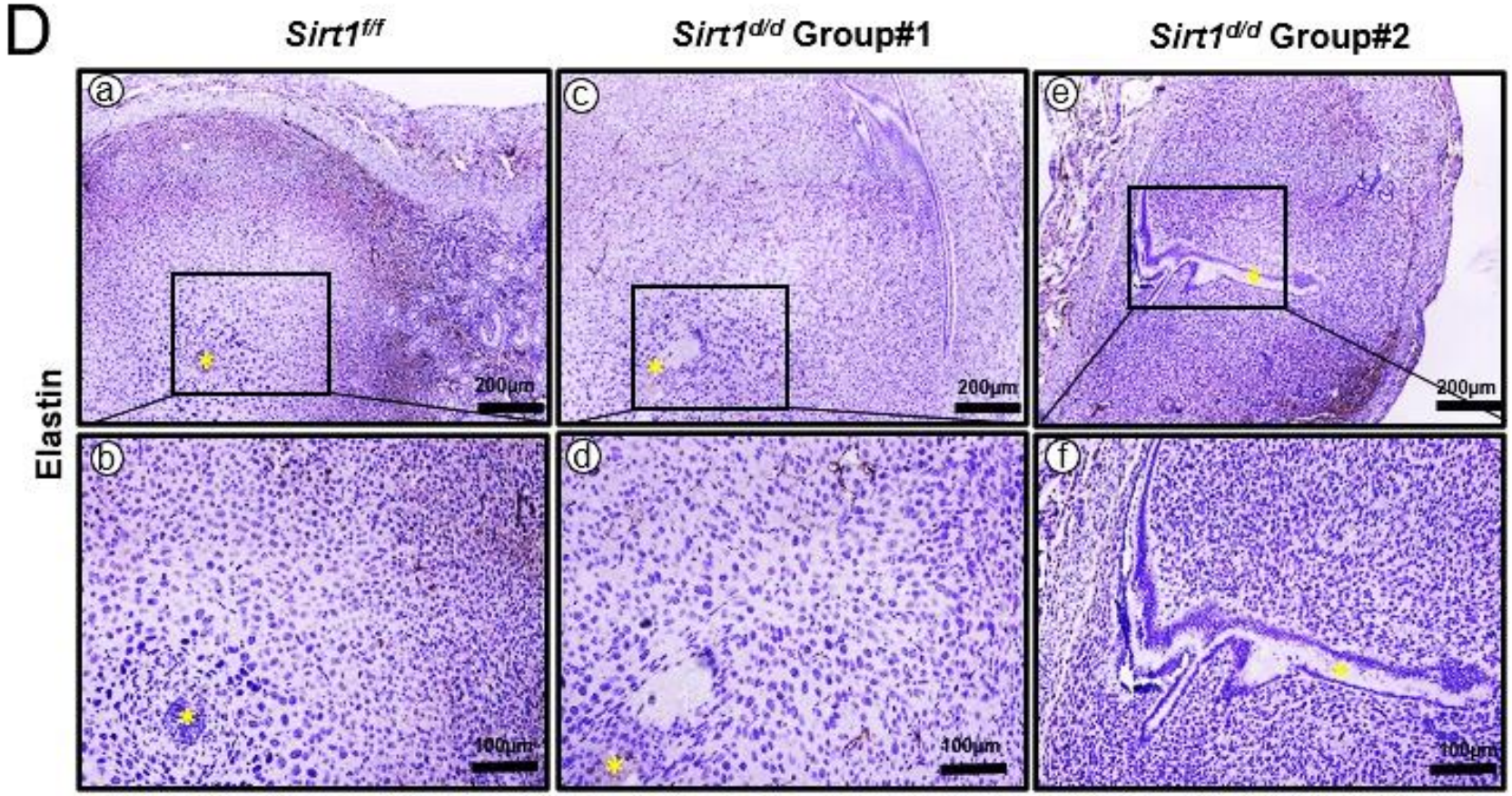
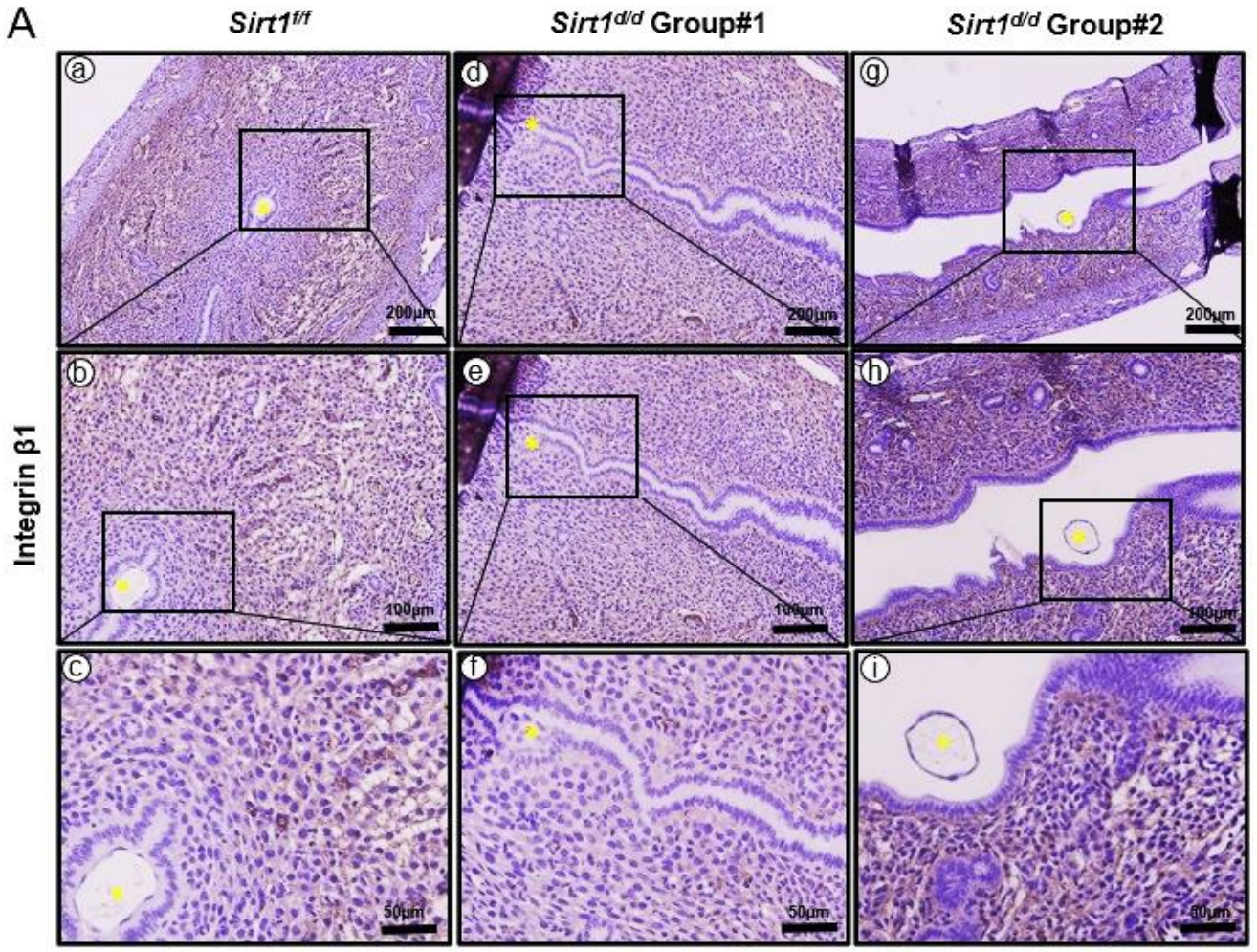
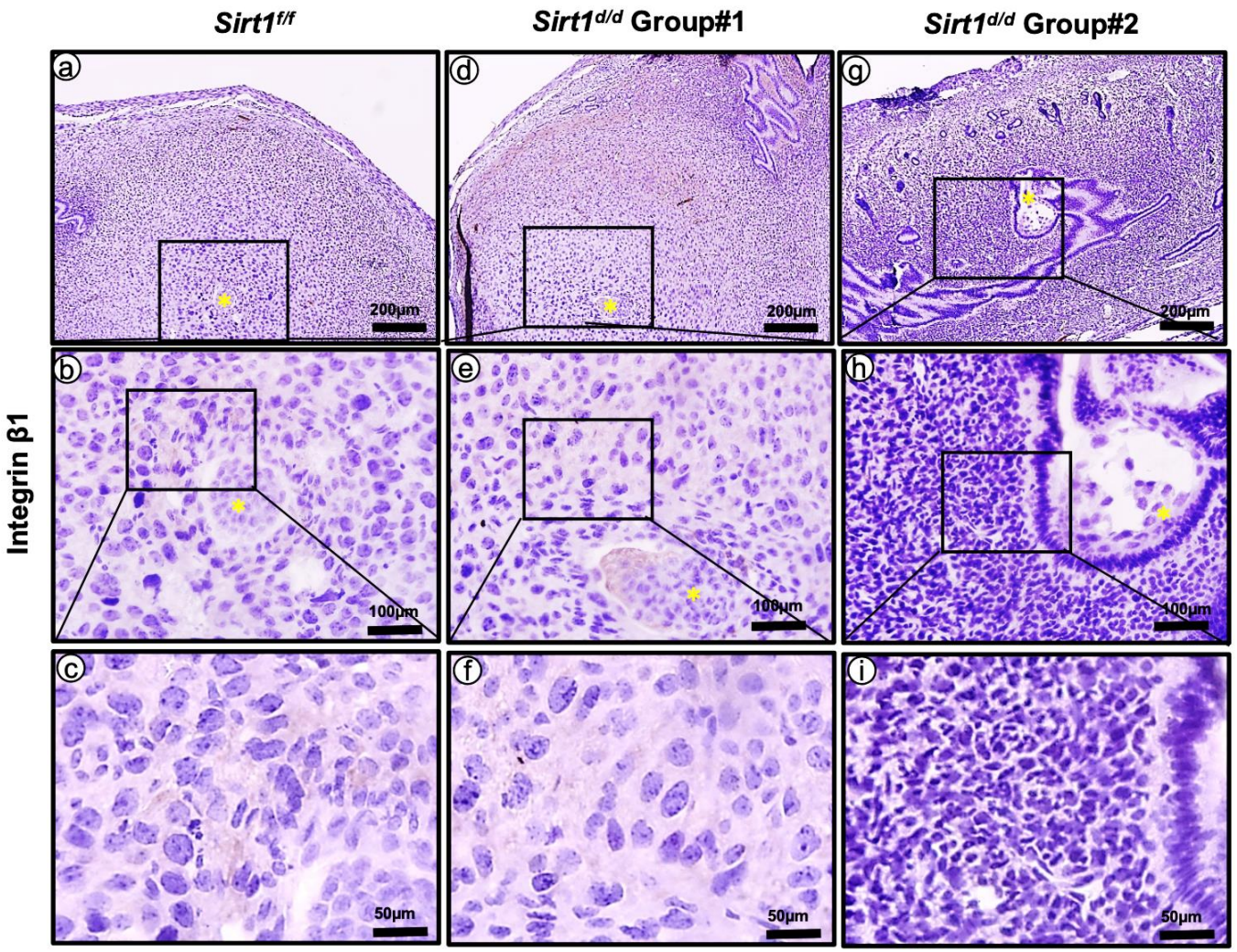

D
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Elastin
a
b
c
d
e
f
200μm
100μm

**Figure 13. Altered expression of Integrin β1 between *Sirt1$^{f/f}$*, *Sirt1$^{d/d}$***

A. Representative image of Immunohistochemistry of Integrin β1 in GD 4.5 *Sirt1$^{f/f}$* mice. (a,b,c) Representative image of *Sirt1$^{d/d}$* Group#1 (d,e,f), Representative image of *Sirt1$^{d/d}$* Group#2 (g,h,i). '*' indicate the implanted embryo. B. Representative image of Immunohistochemistry of Integrin β1 in GD 5.5 *Sirt1$^{f/f}$* mice. (a,b,c) Representative image of *Sirt1$^{d/d}$* Group#1 (d,e,f), Representative image of *Sirt1$^{d/d}$* Group#2 (g,h,i). The magnification of each picture are (a,d,g) x100, (b,e,h) x200, (c,f,i) x400


A
Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Integrin β1
a
b
c
d
e
f
g
h
i
200μm
100μm
50μm
200μm
100μm
50μm
200μm
100μm
50μm


Sirt1f/f
Sirt1d/d Group#1
Sirt1d/d Group#2
Integrin β1
a
b
c
d
e
f
g
h
i
200μm
100μm
50μm
200μm
100μm
50μm
200μm
100μm
50μm

## Discussion

The effect of *Sirt1* deletion in the uterus on fertility *Pgr-cre* driver to determine the effect of *Sirt1* deletion in the mouse uterus on fertility was examed. A 6-month fertility trial showed that female *Sirt1*$^{d/d}$ mice were subfertile. Because *Pgr* is also expressed in ovarian granulosa cells, we needed to rule out a potential ovarian defect caused by *Sirt1* deletion. The average number of blastocysts, and serum P4 and E2 levels in *Sirt1*$^{d/d}$ mice at GD3.5 were not different than control mice. PGR is not expressed in the oocyte, and *Sirt1*$^{d/d}$ mice showed normal ovarian function. Together, *Sirt1*$^{d/d}$ mice exhibit normal ovarian function, and the subfertility of *Sirt1*$^{d/d}$ females is due to a uterine defect.

Previous research identified that SIRT1 was overexpressed in eutopic endometrium from women with endometriosis (Teasley et al., 2020). In women, this SIRT1 overexpression was not dependent on the menstrual cycle stage; rather, overexpression was consistent at all stages (Huang et al., 2015). Therefore, SIRT1 is a potential target of endometriosis treatment. On the other hand, one study showed that SIRT1 was reduced in damaged human endometrial stromal cells, which may be the cause of repeated implantation failures (Engin-Ustun et al., 2017). Therefore, it appears that a properly regulated amount of SIRT1 is important for normal uterine processes such as decidualization.

The invasion of the blastocyst to the luminal epithelium triggers the process of decidualization that includes the differentiation of the fibroblastic stromal cells into morphologically different decidual cells (Rytkonen et al., 2019). These

differentiated cells have a special biosynthetic and secretory propertie (Murata et al., 2021). In the normal implantation period, the stromal decidual cell marker COX2 (Huang et al., 2015) induces prostaglandin, an important molecule for angiogenesis (Arai et al., 2015). However, COX2 was not sufficiently induced in *Sirt1d/d* implantation sites. In *Sirt1*$^{d/d}$ Group #1, expression of COX2 was not limited to the stromal cells but also extended to epithelial cells and was not specific to the invasion site. On the other hand, COX2 was almost negative in *Sirt1*$^{d/d}$ Group #2. Therefore, endogenous decidualization was disrupted at the molecular level by loss of SIRT1.

It is well-known that ovarian hormones are critical for decidualization, especially P4 signaling through PGR, which is necessary to maintain the decidual tissue (Rytkonen et al., 2019). There are two different ways to activate the PGR: (1) ligand binding dependent (Arai et al., 2015) and (2) ligand binding independent (Arai et al., 2015). The amount of ovarian progesterone was similar in the GD 3.5 *Sirt1*$^{f/f}$ and *Sirt1*$^{d/d}$, so it can be assumed that the decrease in PGR was not due to a decrease in the ligand. Therefore, our results suggest that changes in PGR-related decidual signaling in stromal cells lead to abnormal implantation phenotypes in *Sirt1*$^{d/d}$ mice .

FOXO1 is one of the proteins that interact most tightly with PGR (Arai et al., 2015), and FOXO1 and PGR were established as important to inducing in vitro decidualization (Rytkonen et al., 2019). Our results showed that SIRT1 contributes to the activation of the FOXO1-PGR axis, and ablation of SIRT1 decreases decidual cell proliferation. Together, these data suggest that SIRT1 contributes to

the regulation of the FOXO1-PGR axis at implantation. At the beginning of the implantation period, GD 4.5, the withdrawal of PGR in epithelial cells and the rapid accumulation of FOXO1 in the endometrial epithelium is necessary to start the epithelial degradation necessary for embryo implantation (Baker et al., 2015). However, in *Sirt1*$^{d/d}$ mice, PGR was still localized to the nucleus in the epithelial region at this stage, and FOXO1 failed to translocate to the nuclei (Arai et al., 2015). Also, *Sirt1*$^{d/d}$ Group #2 implantation regions showed an intact FOXO1 positive epithelium compared with no luminal epithelial cells in control and *Sirt1*$^{d/d}$ Group #1 implantation regions. We thus speculate that the subfertility of *Sirt1*$^{d/d}$ females is due to biological adaptation of individual PGR signaling mechanisms at specific endometrial regions. Our fertility experiment revealed that *Sirt1*$^{d/d}$ mice exhibit subfertility instead of infertility, and delayed embryo implantation could have a ripple effect on reducing litter sizes. Ovariectomy before embryo implantation results in blastocyst dormancy and delayed implantation in the mouse (Shin et al., 2017). These conditions are maintained by continued P4 treatment but can be terminated with an injection of E2 leading to blastocyst activation and subsequent implantation. The window for successful implantation defines as a limited time span when the activated stage of the blastocyst is superimposed on the receptive state of the uterus (Shin et al., 2017). Although the serum P4 level is normal in *Sirt1*$^{d/d}$ mice, Group #2 of *Sirt1*$^{d/d}$ mice showed delayed progression of implantation. The expression of SIRT1 is weak in uterine epithelium and stroma until GD 4.5 (Figure 1), and the effect of Sirt1 knock-out on artificial decidualization response is only a partially defect. These results suggest that Sirt1 ablation in uterine cells using *Pgr-*

*cre* model could result in a compensation or redundancy effect of other SIRT1 family proteins.

*Sirt1*$^{d/d}$ mice had only 2.40 ± 0.60 litters/mouse compared with 5.40 ± 0.30 litters/mouse from control mice. This result suggests that the mating latency from cohabitation to mating for *Sirt1*$^{d/d}$ mice is remarkably longer than control mice. We also found the varying phenotypes in *Sirt1*$^{d/d}$ mice of GD 4.5 and GD 5.5 that appear to represent failures of embryo attachment and decidualization process at two different stages. Although this study could not determine the source of these variations in *Sirt1*$^{d/d}$ mice, it could possibly have resulted from functional redundancy or compensation of other family members, variables related to SIRT1 deficiency, or slightly different timing from mating event to sample collection between mice. There are also possible pregnancy losses that led to reduced number of litters per mouse. Therefore, further study would be needed to investigate pathophysiological effect of SIRT1 loss during pregnancy. Decidualization is stromal cell proliferation and differentiation into specialized type of cells (decidual cells) with polyploidy (Huang et al., 2015) The decidual cell polyploidy is characterized by the formation of large mono or bi-nucleated cells, a characteristic of nuclear endoreduplication, consisting of DNA with four, eight, and even higher multiples of the haploid complement (Kandoth et al., 2013). Although control mice revealed abundant decidual cell morphology in the stimulated horn, decidual cells were decreased in the stimulated horn of *Sirt1*$^{d/d}$ mice. Although we did not provide experimental results on the molecular effects of SIRT1 deficiency on our artificial decidual experiment, the phenotypes of *Sirt1*$^{d/d}$ mice at GD4.5 and

GD5.5 suggest a critical function of SIRT1 in decidualization. Therefore, further study would be needed to determine the molecular mechanisms of SIRT1 in decidualization using artificial decidual experiments as well as in vitro decidualization of primary stromal cells. In conclusion, SIRT1 has a role in implantation and decidualization.

The ECM components are multifunctional molecular complexes formed by proteins that are produced by the cells, including endometrial decidualized cells. Also, there is no mobility in the extracellular space (Ramirez and Rifkin, 2003). The structure of ECM proteins includes multiple domains, to acquire the ability to interact with other molecules in ECM which are cell-surface reporters, and a variety of growth factors (Berrier and Yamada, 2007).

Integrins are one of the cell surface receptors and are connected by cytoplasmic tail structure to the cytoskeleton. Also, it has a connection with the signaling complex that regulates the gene expression, finally could affect to cell behavior (Hynes, 2009). In addition, communication between growth factors and ECM proteins may control the growth factor function in intra, extra cellular region. Also, these molecules are presented to cognate receptors or even storage and regulated releasing by the perform of ECM-remodeling proteins (Naba et al., 2012). Finally, ECM could involve all cellular processes more than structure role, including cell proliferation, differentiation, survival, and death (Hynes and Naba, 2012).

Previous data shows abnormal expression of COX2 in *Sirt1* ablated uterus. So, Its expected that there is defect in ECM not just decidualized stromal cells.

Cummings and their research group identify the different disctribution of Collagen Subtype I and III in aged *Sirt1*$^{d/d}$, shows decreased collagen structure. In addition, IHC in GD 4.5, 5.5 *Sirt1*$^{d/d}$ result suggests that loss of Collagen Subtype IV, which has relation to the implantation site. Altogether, *Sirt1* can involve decidual structure and maintain the extracellular matrix (Figure. 14). In summary, *Sirt1* deletion in endometrium shows disrupted decidualization and dysregulating PGR/FOXO1 siganling. Failure of decidualization is one cause leading the subfertility (Figure. 14). Interestingly, communication pattern of decidualization was changed by *Sirt1* disruptions. Base on this, SIRT1 is important for decidualization.

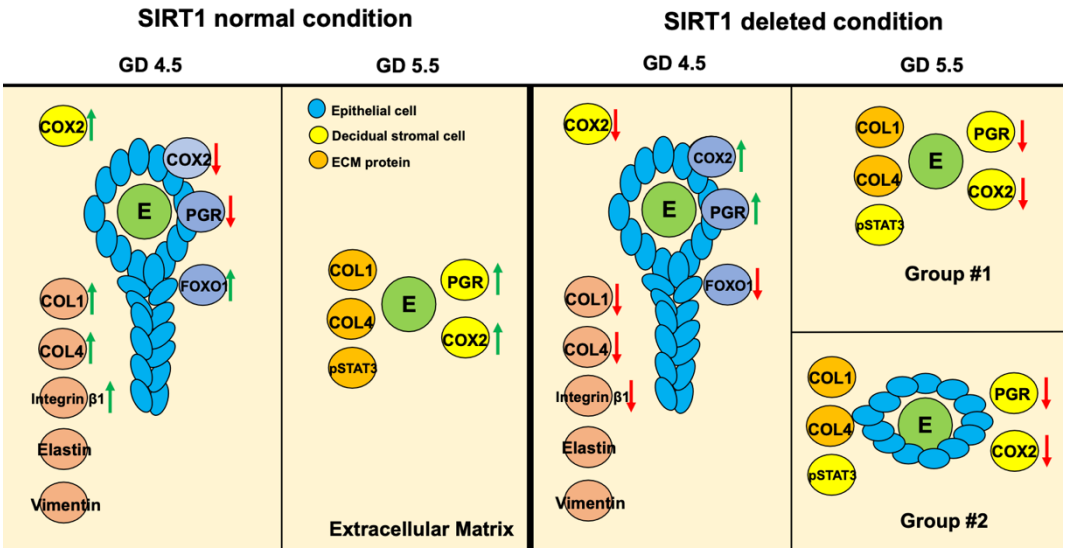



**Figure 14. SIRT1 contribute to maintain the normal decidual process**.

During implantation period in mouse, SIRT1 has the role in luminal epithelial cell, decidual stromal cell, and extra cellular region. Reduction of implantation relative marker show the negative regulation of SIRT1. Also, Group #2 *Sirt1*$^{d/d}$ mice has delayed implantation. And Sirt1 selectively affect to ECM molecules. Altogether, SIRT1 contribute to maintain the normal decidualization and implantation.

# 논문개요

배아의 착상은 배아와 자궁내막 사이에서 일어나는 복잡한 과정이다. 착상시기 동안 자궁내막은 조직학적, 기능적 변화를 겪는다. 세포 외 표지들은 미세 환경을 유지하는 핵심 물질이다. 또한 배아 착상과 배반포의 발달을 지지한다. 탈락막화된 조직은 수용성을 가지고 침입하는 영양세포를 엄격하게 통제한다. Sirtuin 단백질(SIRT) 계열은 신경변성, 암 성장, 노화와 관련된 결함, 그리고 비만을 포함한 생리적 및 다양한 병리학적 과정에 관여하는 SIRT1~7이다. Sirtuin 계열의 가장 중요한 구성원 중 하나인 SIRT1은 지난 몇 년간 특히 암생물학에서 연구되었다.

SIRT1의 기능은 종양의 성장에 억제제 또는 촉진제 역할을 할 수 있기 때문에 여전히 논란이 있다. 또한 SIRT1은 여성 생식계에서 흥미로운 표적 유전자였다. 이전의 연구들의 결과에서 보여 준 바와 같이 인간과 개코원숭이의 자궁내막증에서 유의미한 SIRT1 상향조절을 확인했기 때문이다.
이러한 결과는 적절한 SIRT1 조절이 건강한 여성 생식계에 중요하다는 것을 시사한다. 그러나 자궁에서 SIRT1의 기능은 직접적으로 연구되지 않았다. 여기서 면역조직화학 분석을 사용한 자궁 특이적 Sirt1은 생쥐에서 임신 초기 내내 SIRT1 발현을 보였으며, 결정화 세포에서 GD4.5와 GD5.5에서 가장 강하게 발현되는 것으로 나타났다.

6개월 간의 임신 시험 결과, *Sirt1*$^{d/d}$ 암컷은 난임이며, 새끼를 낳는 횟

수가 현저히 감소하였다(n=5, *p<0.05). GD 3.5에서 *Sirt1*$^{d/d}$ 자궁에서 수집된 배아의 수는 대조군과 차이가 없었으며 Progesterone 과 Estrogen의 수준 또한 대조군과 유의적인 차이가 없었다. 따라서 *Sirt1*$^{d/d}$ 은 정상적인 난소 기능을 나타냈다. 그러나 *Sirt1*$^{d/d}$ 생쥐에서는 GD5.5 ($p < 0.05$)의 착상부위 수가 대조군에 비해 유의하게 감소했다.

착상 결손의 원인을 보다 면밀하게 평가하기 위해 GD4.5의 자궁에서 조직학적 특성과 marker 유전자 발현을 분석하였다. *Sirt1*$^{d/d}$ 의 착상 부위는 대조군에 비해 자궁내강 폐쇄가 정상적으로 일어난 Group #1(14/20, 70%)과 비특이적인 Cyclooxygenase 2(COX2) 발현인 Group #26(6/20, 30%)의 두 그룹으로 나눌 수 있었다.

대조군 생쥐의 상피에서 Fork heads box protein O1(FOXO1)은 핵에 정상적으로 국한되었으며, PGR은 기질세포에 제한되었다. 그러나 *Sirt1*$^{d/d}$ Group #1 의 내강 상피세포에서 세포 핵 내 FOXO1 발현이 유의미하게 감소(세포의 20%)하였고, 세포질 FOXO1 발현이 증가(세포의 80%, p<0.05)하였다. 반정량 H-Scoring 결과는 *Sirt1*$^{d/d}$ Group #1, #2의 상피 PGR이 대조군에 비해 유의하게 증가함을 보여주었다(p<0.001). 또한 *Sirt1*$^{d/d}$ Group #2에서 핵 FOXO1 발현을 볼 수 있었으며 정상적인 대조군 상피세포에서는 PGR의 발현이 거의 없지만 *Sirt1*$^{d/d}$ Group #2의 상피세포에서는 PGR 발현이 강하게 일어난다. GD5.5 시기의 *Sirt1*$^{d/d}$ 배아착상 영역에서는 두 가지 패턴의 E-cadherin 염색을 보였다. Group #1은 배아를 둘러싼 E-cadherin에 대한 양성 정도가 대조군과 유사

했으며. Group #2에서 E-cadherin 양성 상피가 배아를 둘러 싼 형태로 남아있었다.

기질세포의 PGR과 COX2는 Group #1 과 Group #2 *Sirt1*$^{d/d}$ 배아 영역 모두에서 대조군($p<0.001$)에 비해 유의하게 감소했으며, 이는 불완전한 탈락막화를 나타낸다. FOXO1은 자연 조건에서 GD 5.5 착상부위 주변에서 발현되지 않으며, *Sirt1*$^{d/d}$ Group #1의 결과는 대조군과 유사했다.

그러나 Group #2 착상부위 에서는 상피 세포의 핵에 FOXO1이 양성을 나타냈는데, 이 결과는 Group #2에서 분자 신호 전달이 지연되었음을 나타내고 있다. 인공적으로 유도된 탈피검사에서 *Sirt1*$^{d/d}$ 암컷은 자궁과 체중비($p<0.05$)가 유의하게 감소한 것을 근거로 탈락막화 5일째에 결함을 보였다. 이 발견은 자궁내막 상피의 수용성이 이상이 있음을 나타낸다.

또한 Vimentin과 Elastin을 포함한 중요한 ECM 단백질은 표현형에 차이가 없다. 단, Integrin α1의 식은 GD 4.5 및 5.5에서 *Sirt1*$^{d/d}$ Group #2에서 이상을 나타낸다. 정상 조건에서 Integrin α1은 배아 부착 부위에서 검출되지 않았으나 Integrin α1은 GD 4.5에서 *Sirt1*$^{d/d}$ Group #2의 동일한 면적에서 발현을 유지한다. 또한 *Sirt1*$^{d/d}$ 의 자궁은 ECM의 구조적 결함이 발견되었다. GD 4.5 *Sirt1*$^{d/d}$ 자궁에서 Collagen에 대한 Picro Sirius Red 염색 결과는 ECM에서 콜라겐 다발이 축적됨을 보여준다. 또한 GD 5.5 *Sirt1*$^{d/d}$ Group #2는 모든 자궁 세포층에서 콜라겐이 극적으로 감소함을 보여준다. 결과는 SIRT1이 착상 기간 동안 정상적인 ECM 구조를 유지하는데 필요하다는 것을 시사한다. 전체적으로, 이러한

결과들은 SIRT1이 탈락막화에 중요하며 성공적인 착상을 위한 수용성 자궁내막을 준비하는 데 기여한다는 것을 알 수 있다.

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## 감사의 글

약 6년동안 박사과정을 지내면서 저는 종종 박사과정 진학 여부를 고민스러워 하는 친구들이랑 대화 할 기회가 있었습니다. 그 중에서도 가장 많이 듣는 질문은 이것인데, '언니 석사 한 김에 박사도 할까요?' 입니다. 저는 그때마다 같은 대답을 합니다. '석사 한 김에 박사를 한다는 말은 니가 만원짜리 한장들고 슬리퍼 바람에 편의점을 가는 중인데 나온김에 세계여행 갈까' 같은 거라고요. 언니는 그래서 슬리퍼바람에 세계여행 하느라 죽을 맛이고 집으로 돌아가자니 이미 많이 멀어져 버려서 울며 겨자먹기 식으로 계속 여행중이라고 웃었습니다. 박사 두번 하라고 하면 절대로 못 할거고 하고 싶지도 않습니다. 그렇지만 절대 후회하지 않을 선택이 였고 연구를 업으로 삼은 제 자신이 너무나도 좋습니다. 그리고 고생보다 더 큰 가치와 크기를 가늠하기 힘든 감사함을 느끼고 있습니다.

가장 먼저 30대 중반까지 뒷바라지하면서 기다려준 우리 가족들 엄마, 아빠, 와렁, 할머니 할아버지, 외가 식구들 언니 오빠 그리고 새로 가족이 된 형부 정말 감사합니다. 박사가 성공을 보장하는 시대는 이미 지났지만 우리 가족의 연정아가는 어떻게든 잘 될 테니 걱정마시고 모두 건강하세요. 제 인생의 뮤즈 우리 엄마 김봉희 선생님 저는 타고난 거의 모든 운을 엄마를 만나기 위해 썼다고 한다면 앞으로의 삶에 있을 매우 작고 작은 고난과 불운 정도는 아무것도 아니라고 생각합니다. 엄마 더 행복하고 즐겁게 살자.

바쁘신 와중에도 졸업논문에 고견을 보태시고 심사해 주신 상명대학교 이성호 교수님, 서울여자대학교 양현원 교수님, 바이오신약과 전민영 교수님께 진심으로 감사드립니다. 학부생 시절부터 박사까지 저를 가르치신 우리과 강혜순 교수님, 윤진호 교수님, 김상태 교수님, 최상철 교수님 그리고 교편을 옮기셔도 저에게 응원을 아끼지 않으시는 백영빈 교수님께 진심으로 감사의 인사를 드립니다. 또한 성신여대에서 봤던 모든 입학시험 (학부입학, 석사입학, 박사입학, 박사졸업) 을 심사하신 강창수 교수님 저의 중요한 순간마다 나타나셔서 저를 한걸음 나아가게 해주신 것 감사드립니다. 인사를 자주 못드려서 죄송할 뿐인, 저를 잊지 않으시고 안부 물어주신 서울대학교 의과대학 구승엽 교수님과 김윤영 연구교수님께도 진심으로 감사드립니다. 그리고! 항상 아빠 같은 한양대학교 의과대학 백두진 교수님께 깊은 사랑과 존경을 드립니다. 교수님을 만난 것은 박사생활 중 다시없을 행운 이였어요.

8년 동안 연구실에 함께 있었던 모든 선후배들께도 감사의 인사를 전합니다. 박사동지 박수현 박사님 감사합니다. 앞으로도 나와 함께 씩씩한 여성과학자가 되어줘. 학부생으로 연구실 생활 하는게 쉽지 않을텐데도 꼬박꼬박 자리 지켜가면서 실험하는 친구들 고맙고 장합니다. 그리고 가장 마지막에 함께한 솔아, 주은이, 태은이, 지선, 보영, 정빈이에게는 나이 많고 별난 언니 항상 잘 챙겨주고 많이 도와줘서 정말 고맙다는 말을 전합니다. 여러분

은 이제 다음 세대의 처음으로써 하고 싶은대로 소신을 지켜가면서 용기있고 아름답게 사세요. 연정언니가 언제나 응원하고 있습니다. 그리고 매일 보던 얼굴을 한달 두달마다 보아서 그게 안타까울 뿐인, 특별히 저와 같은 길을 선택한 사랑하는 후배, 전우, 동생, 친구인 양희선 선생님은 저와 종신계약을 맺은 것으로 하겠습니다 항상 고맙습니다.

정말 긴 학위과정 기간동안 그저 학교만 다닐뿐인 저를 옆에서 항상 응원해 주고 이해해준 몇 안되는 친구들에게도 감사의 인사를 전합니다. 정혜미님 박혜리 선생님 제가 정말 사랑합니다.

2021년은 길지 않은 미국 생활 이였지만 인생에서 가장 소중하고 갚진 1년이였습니다.갑자기 한국에서 뚝 떨어진 저를 돌봐주신 구은미 여사님께 진심으로 감사드립니다. 성기준 박사님, 재희씨 저랑 낯선 타지에서 친구도 되어주고 선배박사로써 많이 가르쳐줘서 정말 감사합니다.
그리고 그리고, 갑자기 미시간으로 날아든 저를 붙들어 주시고 몇 년을 걸려도 못 다할 가르침을 주신 Michigan State University에 정재욱 교수님, 김태훈 교수님 진심으로 감사드립니다. 논문표지에 한국인으로 추정되는 영어로 쓰인 존함을 보면서 저는 뵐 기회가 평생 없을 것만 같았던 연예인 같은 교수님이랑 무려 같은 연구실에 있었다는 사실이 아직도 믿을 수 없습니다. 두분께서는 항상 저의 슈퍼스타 이십니다. 항상 건강하시고 다시한번 감사드립니다.

마지막으로 감사와 존경으로는 부족한 우리 선생님, 앞으로 저의 삶에 조금이라도 성취가 있다면 그것은 전부 교수님의 덕입니다. 평생 제 앞에서 걸어주시고 가끔 돌아봐 주세요. 저는 옛날부터 지금까지 석사이건 박사이건 그리고 그 어디에 있어도 그냥 교수님의 연정이 일 뿐입니다. 보시기에 마음에 드는 아이가 아니여서 언제나 죄송했습니다. 그래도 사랑해요 선생님 싫어도 받으셔야 합니다. 항상 아프지말고 건강하세요

그 밖에 말씀 못드린 모든 분께 다시한번 감사의 말씀을 드리며

2022년 7월 황연정