Different Cre systems induce differential microRNA landscapes and abnormalities in the female reproductive tracts of Dgcr8 conditional knockout mice

Yeon Sun Kim1 | Seung Chel Yang1 | Mira Park1 | Youngsok Choi2 | Francesco J. DeMayo3 | John P. Lydon4 | Hye-Ryun Kim1 | Hyunjung Jade Lim5 | Haengseok Song1

1Department of Biomedical Science, CHA University, Seongnam, Korea
2Department of Stem Cell and Regenerative Biotechnology, Konkuk University, Seoul, Korea
3Department of Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA
4Department of Molecular and Cellular Biology and Center for Reproductive Medicine, Baylor College of Medicine, Houston, TX, USA
5Department of Veterinary Medicine, School of Veterinary Medicine, Konkuk University, Seoul, Korea

Correspondence
Haengseok Song, Department of Biomedical Science, CHA University, Seongnam, Gyeonggi, Korea.
Email: hssong@cha.ac.kr
Hyunjung Jade Lim, Department of Veterinary Medicine, Konkuk University, Seoul, Korea.
Email: hlim@konkuk.ac.kr

Present address
Yeon Sun Kim, Division of reproductive sciences, Department of Pediatrics, Cincinnati Children’s Hospital, OH, USA

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Abstract

Objectives: The female reproductive tract comprises several different cell types. Using three representative Cre systems, we comparatively analysed the phenotypes of Dgcr8 conditional knockout (cKO) mice to understand the function of Dgcr8, involved in canonical microRNA biogenesis, in the female reproductive tract.

Materials and Methods: Dgcr8f/f mice were crossed with Ltfcre/+ , Amhr2cre/+ or PRcre/+ mice to produce mice deficient in Dgcr8 in epithelial (Dgcr8ed/ed), mesenchymal (Dgcr8md/md) and all the compartments (Dgcr8td/td) in the female reproductive tract. Reproductive phenotypes were evaluated in Dgcr8 cKO mice. Uteri and/or oviducts were used for small RNA-seq, mRNA-seq, real-time RT-PCR, and/or morphologic and histological analyses.

Result: Dgcr8ed/ed mice did not exhibit any distinct defects, whereas Dgcr8md/md mice showed sub-fertility and oviductal smooth muscle deformities. Dgcr8td/td mice were infertile due to anovulation and acute inflammation in the female reproductive tract and suffered from an atrophic uterus with myometrial defects. The microRNAs and mRNAs related to immune modulation and/or smooth muscle growth were systemically altered in the Dgcr8td/td uterus. Expression profiles of dysregulated microRNAs and mRNAs in the Dgcr8td/td uterus were different from those in other genotypes in a Cre-dependent manner.

Conclusions: Dgcr8 deficiency with different Cre systems induces overlapping but distinct phenotypes as well as the profiles of microRNAs and their target mRNAs in the female reproductive tract, suggesting the importance of selecting the appropriate Cre driver to investigate the genes of interest.


1 | INTRODUCTION

MicroRNAs (miRNAs) are evolutionarily conserved small non-coding RNAs that function in RNA silencing and post-transcriptional regulation of gene expression. miRNAs also regulate various cellular pathways necessary for the development and proper functions of organs, such as the ovary and uterus, in the female reproductive tract.\(^{1,2}\)

DGC8 is an RNA-binding protein that works with DROSHA to produce precursor miRNA in the nucleus, while DICER generates mature miRNAs and endogenous small interfering RNAs in the cytoplasm.\(^{3}\)

To study the function of miRNAs, especially canonical miRNAs in the female reproductive tract, we generated Dgcr8\(^{−/−}\), progesterone receptor \((PR)^{fr/+}\) \((Dgcr8^{fr/fr})\) mice and reported that canonical miRNAs are essential for uterine morphogenesis and physiology, including natural immune modulation.\(^{4}\) PR\(^{fr/+}\) (PR-Cre) mice have been mostly used to study uterine biology during pregnancy and various diseases. However, PR-Cre inactivates genes not only in the female reproductive tract but also in other progesterone-responsive organs, including the ovary, pituitary gland and mammary gland.\(^{5}\)

In the uterus, PR is spatiotemporally expressed in all the major uterine compartments: myometrium, stroma, and epithelium. Furthermore, PR is also expressed in immune cells, such as natural killer (NK) cells, macrophages, dendritic cells and T cells,\(^{6}\) suggesting that PR-Cre may affect various immune cells as well as all the major uterine cells in a spatiotemporal manner.

In addition to PR-Cre, other Cre mice with unique purposes are currently available for conditionally inactivating gene(s) of interest in the female reproductive tract, especially in the uterus. Anti-mullerian hormone receptor type 2 \((Amhr2)\)-Cre mice are mainly used to target genes in stromal and myometrial compartments of the uterus and oviduct as well as of the ovary.\(^{10}\)

Temporally, Cre action starts from midgestational embryo development (embryonic day 12.5) under the control of the Amhr2 promoter in Amhr2-Cre mice. Recently, other Cre mice, such as lactoferrin \((Ltf)\)-iCre, small proline-rich protein 2f \((Sprf2f)\)-Cre, and Wnt family member 7a \((Wnt7a)\)-Cre, were generated to target genes in the epithelial compartment. The spatiotemporal actions of each Cre on the uterine epithelium are unique. Although Wnt7a-Cre is expressed throughout the epithelium of the prenatal Müllerian tract,\(^{11}\) Ltf and Sprf2f are not expressed in the immature mouse uterus, but robustly expressed in the uterine epithelium of adult mice.\(^{12,13}\) Ltf and Sprf2f, well-known oestrogen-responsive genes, are expressed not only in the uterine but also in the oviductal epithelium after puberty.\(^{13}\)

Collectively, the spatiotemporal modes of each Cre system may provide diverse reproductive phenotypes. Thus, insights into the mode(s) of actions of multiple Cre systems are required to precisely delineate the functions of genes of interest in a cell type-specific manner in the female reproductive tract.

2 | MATERIALS AND METHODS

2.1 | Animals and genotyping of Dgcr8 cKO mice

All mice used in this study were maintained in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC170174) of CHA University. Dgcr8\(^{fr/+}\) mice were initially generated and provided by Dr Elaine Fuchs.\(^{11}\) PR-Cre, Amhr2-Cre and Ltf-iCre mice were generously provided by Dr Francesco DeMayo,\(^{5}\) Dr Richard Behringer\(^{10}\) and Dr Sudhansu K. Dey,\(^{12}\) respectively.

Genotyping PCR was performed using specific primers (Table S1) and genomic DNA extracts from mouse tail biopsies.

2.2 | Fertility analysis and preimplantation embryo culture

Dgcr8\(^{fr/fr}\) and Dgcr8\(^{fr/fr}\) mice have an aneuploid or achiasmate meiotic configuration,\(^{14,15}\) and Dgcr8\(^{fr/fr}\) mice were administrated intraperitoneal (IP) injections of 5 IU PMSG (Sigma-Aldrich, St. Louis, MO, USA) for 48 hours, followed by IP injections of 5 IU hCG (Sigma-Aldrich). Dgcr8\(^{fr/fr}\) mice were then bred with wild-type fertile males, and pregnancy was evaluated by the presence of a vaginal plug the next morning. The other Dgcr8 cKO mice were naturally mated. The 2-cell embryos and/or fragmented oocytes were flushed from the oviducts on day 2 of pregnancy (Day 2).

2.3 | RNA extraction, reverse transcription-PCR (RT-PCR) and real-time RT-PCR

Total RNA was extracted from the mouse uterus using TRizol Reagent (Invitrogen Life Technologies, San Diego, CA, USA) according to the manufacturer’s protocols. First-strand cDNA was synthesized from 1 μg of total RNA using M-MLV reverse transcriptase (Promega, Madison, WI, USA) and RNasin Ribonuclease Inhibitor (Promega). For quantification of expression levels, real-time RT-PCR was performed using IQ\™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) on a BIO-RAD iCycler as previously described.\(^{4}\) The synthesized cDNA was utilized for PCR and real-time RT-PCR with specific primers (Table S1).

2.4 | Tissue collection and histological analysis

Female reproductive organs were dissected and fixed in 4% paraformaldehyde (PFA) for histology or snap-frozen for RNA and/or protein preparation. Tissues were embedded in paraplast (Leica Biosystems, St. Louis LLC, Diemen, the Netherlands). Sections were cut at 5 μm and stained with haematoxylin and eosin (H&E) (Sigma-Aldrich), and observed by light microscopy.

2.5 | Immunofluorescence

Sections were subjected to antigen retrieval in 10 mM sodium citrate buffer (pH 6.0) for 20 min. Non-specific staining was blocked using protein block serum (Dako, Carpinteria, CA, USA). Sections were then incubated with primary smooth muscle actin (α-SMA) (Abcam, Cambridge, UK, 1:100) and acetylated tubulin (Sigma-Aldrich, 1:200), E-cadherin (Cell Signaling, Danvers, MA, USA, 1:200), Desmin (Santa
KIM et al. performed following the protocol from the HB miR Multi Assay Kit (Heim Biotek, Gyeonggi-do, Korea). cDNA was converted to cDNA, and real-time RT-PCR (50 ng of cDNA) was considered statistically significant for more than 3 groups.

PRO-3-iodide (Invitrogen) or DAPI (Thermo, Waltham, MA, USA). performed at 48 hours post-transfection. Renilla luciferase and firefly luciferase activities were analysed using the Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer’s instructions. The firefly luciferase activities were normalized using Renilla luciferase activity.

2.6 | Library preparation for small RNA sequencing (RNA-seq) and miRNA expression analysis

For control and test RNAs, the construction of the library was performed using the NEBNext Multiplex Small RNA Library Prep kit (New England BioLabs, Inc, USA) according to the manufacturer’s instructions. Briefly, for library construction, total RNA from each sample was used 1 μg to ligate the adapters, and then, cDNA was synthesized using reverse transcriptase with adaptor-specific primers. To quantify the miRNA expression levels, total RNA (1 μg) was converted to cDNA, and real-time RT-PCR (50 ng of cDNA) was performed following the protocol from the HB miR Multi Assay Kit (Heim Biotek, Gyeonggi-do, Korea).

2.7 | mRNA-Seq and data analyses

Uteri were dissected and snap-frozen. Quant-3’ mRNA-seq was initially performed using total uterine RNA pooled by genotype (n = 2-3 pools per genotype; 3 mice per pool; Ebiogen, Seoul, Korea). High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc, USA). Gene classification was based on searches performed using GSEA software (Gene Set Enrichment Analysis\(^\text{15}\)) and QuickGO (https://www.ebi.ac.uk/QuickGO/), and the target genes of miRNAs were identified using miRmap (miRmap score ≥ 90; https://mirmap.ezlab.org/).

2.8 | Cell culture, transfection and luciferase assay

293T cells were grown in high glucose DMEM supplemented with 10% FBS, penicillin at 37°C, and 5% CO\(_2\). 293T cells were transiently transfected using Lipofectamine 3000 (Invitrogen Life Technologies). Transfection of each 3’ UTR into a pmirGLO basic luciferase reporter vector (Promega; 300 ng) and each mimic miRNA (Bioneer, Daejeon, Korea; 25 pg) was performed in a 24-well plate. Luciferase assay was performed at 48 hours post-transfection. Renilla luciferase and firefly luciferase activities were analysed using the Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer’s instructions. The firefly luciferase activities were normalized using Renilla luciferase activity.

2.9 | Statistical analysis

All values represent the mean ± standard deviation. Statistical analyses were performed using the unpaired Student’s t tests and P < 0.05 was considered statistically significant for more than 3 groups.

3 | RESULTS

3.1 | Multiple Cre systems effectively delete Dgcr8 in the female reproductive tract

The uterine cell type-specific actions of the three representative Cre systems used in this study were summarized based on previous reports (Figure 1A). To understand the temporal activity of Cre drivers in the female reproductive tract, the expression profiles of Amhr2, Pgr and Ltf were first examined during postnatal days (PND), oestrous cycle and early pregnancy (Figure 1B-D and Figure S1). RT-PCR results showed that Amhr2 is highly expressed in the developing uterus at PND 0, but maintained at undetectable levels during the oestrous cycle and early pregnancy. Pgr expression was very low at PND 0 and 3 but increased after PND 7 in the uterus. Ltf mRNAs were detected in the mouse uterus at PND 28 (4 weeks of age). During the oestrous cycle, Pgr and Ltf showed stage-specific expression patterns. During early pregnancy, Ltf expression was low on Days 1 and 2, followed by substantial increases on Days 3-5.

To validate the actions of each Cre system in the female reproductive tract of Dgcr8 cKO mice, Dgcr8\(^{\text{f/f}}\) mice were crossed with Ltf-\(^{-i}\)Cre, Amhr2-\(^{-C}\)re and PR-Cre mice to produce Dgcr8\(^{\text{td/td}}\) (epithelium-specific), Dgcr8\(^{\text{ed/ed}}\) (mesenchyme-specific) and Dgcr8\(^{\text{dd/dd}}\) (all the major uterine cell types) mice, respectively. Cre-mediated deletion of exon 3 of the Dgcr8 allele produced a 262 bp PCR product (white arrowhead), whereas a floxed allele resulted in a 1085 bp product (black arrowhead) (Figure 1E). Consistent with a previous report\(^{12,16}\) that Ltf is expressed in the epithelium of the oviduct and uterus, and in some immune cells, PCR results using genomic DNA of tissues from Dgcr8\(^{\text{ed/ed}}\) mice showed a deletion of exon 3 in the oviduct and uterus, but not in other tissues. Dgcr8\(^{\text{ed/ed}}\) mice showed deletion of Dgcr8 only in the ovary, oviduct and uterus, among all the tested tissues. PR-Cre activity was detected not only in the female reproductive tract but also in the pituitary of Dgcr8\(^{\text{td/td}}\) mice.

3.2 | Dgcr8 cKO mice crossed with different Cre systems show a distinct spectrum of fertility

To compare the fertility of Dgcr8 cKO female mice with different Cre systems, they were mated with mature fertile males for 8-10 weeks (Figure 1F). Dgcr8\(^{\text{dd/dd}}\) female mice never produced any litter, as we previously reported.\(^{5}\) However, Dgcr8\(^{\text{ed/ed}}\) mice were sub-fertile and Dgcr8\(^{\text{dd/dd}}\) mice were normal with respect to the number of pups produced. By monitoring oestrous cycles with daily vaginal smears over a 2-week period, we observed that Dgcr8\(^{\text{td/td}}\) female mice were anestrus, whereas Dgcr8\(^{\text{ed/ed}}\) and Dgcr8\(^{\text{dd/dd}}\) mice exhibited regular 4-5 day oestrous cyclicity similar to that of Dgcr8\(^{\text{f/f}}\) control mice (Figure 1G).
Conditional deletion of Dgcr8 in female reproductive tracts by various uterine Cre systems and fertility tests in Dgcr8 cKO mice. A, A schematic diagram summarizing the expression of various Cre systems. GE, Glandular epithelium; LE, Luminal epithelium; S, Stroma; M, Myometrium; [M], Mesometrium; [AM], Anti-mesometrium; Im, Immune cell. (B-D) RT-PCR analyses of Amhr2, Pgr, Ltf and Dgcr8 were performed using total RNA extracted from the mouse uterus at various postnatal days (PND), during the oestrous cycle, and early pregnancy (n = 5 per group). P, Proestrus; E, Oestrus; M, Metestrus; D, Dioestrus; IS, Implantation site; Inter IS, Inter-implantation site. E, Representative images of PCR results with the genomic DNA of various tissues. Black and white arrowheads indicate PCR products for inclusion (1085 bp) and deletion (262 bp) of Dgcr8 exon 3, respectively. T, Tail; B, Brain; P, Pituitary gland; O, Ovary; Ov, Oviduct; U, Uterus. F, Litter size in Dgcr8f/f and Dgcr8 cKO mice. Numbers in bars indicate the number of mice examined in each group. Unpaired Student’s t test, *P < 0.05, **P < 0.01. G, Representative graphs to demonstrate changes in epithelial cells/total cells (%) obtained by a vaginal smear method for two weeks (n = 4 per genotype).
3.3 | Dgcr8 deficiency in the oviduct affects quality of ovulated oocytes followed by fertilization in Dgcr8\textsuperscript{md/md} mice

To explore the underlying causes of sub-fertility in Dgcr8\textsuperscript{md/md} mice, we examined in detail the reproductive phenotypes during early pregnancy. Since Dgcr8\textsuperscript{td/td} mice are anovulatory due to pituitary defects, the numbers and fertilization rates of ovulated metaphase II (MII) oocytes were examined only in Dgcr8\textsuperscript{md/md} and Dgcr8\textsuperscript{ed/ed} mice. The number of ovulated MII oocytes was not statistically different between genotypes, although there was a moderate reduction in Dgcr8\textsuperscript{ed/ed} and Dgcr8\textsuperscript{md/md} mice (Figure 2A). However, the fertilization rate was significantly reduced in oocytes from Dgcr8\textsuperscript{md/md} mice (Figure 2B). When 2-cell embryos harvested from the Dgcr8\textsuperscript{md/md} oviduct were cultured in vitro, they developed to the blastocyst stage similar to that of Dgcr8\textsuperscript{f/f} and Dgcr8\textsuperscript{ed/ed} mice (Figure 2C). We then investigated the quality and quantity of the oocyte right after ovulation at post-hCG 16 hours. As shown in Figure 2D, the quantity and quality of oocytes were similar to those of Dgcr8\textsuperscript{f/f} mice immediately after ovulation. This is consistent with the fact that zona pellucida remnants and degenerated oocytes were often observed from the oviducts of Dgcr8\textsuperscript{md/md} mice on Day 2 (Figure 2E-F). Furthermore, histological observation of the ovaries of Dgcr8\textsuperscript{md/md} mice indicated that ovulation was not affected in these mice (Figure 2G).

3.4 | Oviduct development was affected in Dgcr8\textsuperscript{md/md} but not in Dgcr8\textsuperscript{td/td} and Dgcr8\textsuperscript{ed/ed} mice

We next examined whether Dgcr8\textsuperscript{md/md} mice showed any morphological abnormalities in the oviduct. The length of the oviduct in Dgcr8\textsuperscript{md/md} mice was shorter than that in other Dgcr8 cKO mice.

**FIGURE 2** The comparison of ovulation and embryo development rate in Dgcr8 cKO mice on Day 2. (A-C) 8-week-old Dgcr8\textsuperscript{f/f} and Dgcr8 cKO mice were mated with wild-type fertile males. Ovulation and embryo development are comparable between Dgcr8\textsuperscript{f/f} and cell type-specific Dgcr8 cKO mice (n = 4-6 per genotype). D, The number of ovulated eggs via superovulation in Dgcr8\textsuperscript{f/f} and Dgcr8\textsuperscript{md/md} mice (n = 12-15 per genotype). E, The percentage of degenerated oocytes in the oviducts of Dgcr8\textsuperscript{md/md} mice on Day 2. F, Representative images of 2-cell embryos collected from the oviducts of Dgcr8\textsuperscript{f/f} and Dgcr8 cKO mice. Scale bar: 100 µm. G, Histological analyses of the ovaries of Dgcr8\textsuperscript{f/f} and Dgcr8 cKO mice collected on Day 2. * indicates corpus luteum, scale bar: 200 µm. Unpaired Student’s t test, *P < 0.05, **P < 0.01
at 4 (pubertal) and 9 weeks (mature adult) of ages (Figure 3A-B). Furthermore, the smooth muscle thickness of the isthmus in the oviduct was significantly reduced in both 4- and 9-week-old Dgcr8<sup>td/td</sup> mice, as observed using α-SMA immunostaining (Figure 3C-D). However, oviducts of all the genotypes, including Dgcr8<sup>md/md</sup> mice, were histologically indistinguishable from those of Dgcr8<sup>+/+</sup> mice and showed normal oviductal organization (Figure 3E). Furthermore, immunofluorescence staining for acetylated Tubulin was performed to examine whether the oviducts of Dgcr8 cKO mice had cilia defects in the epithelial compartment (Figure 3F). The results were similar between oviducts of all the genotypes of Dgcr8 cKO mice, suggesting that the distribution and function of oviductal cilia of Dgcr8 cKO mice were different from those of Dgcr8<sup>+/+</sup> mice.

### 3.5 | Deletion of Dgcr8 affects the uterine architecture with Cre-specific spectrum

To examine whether Dgcr8 deficiency affects uterine development, we investigated the uteri of all the Dgcr8 cKO mice at 4 and 9 weeks of ages. The gross morphology and uterine weight to body weight ratio of 4-week-old Dgcr8<sup>td/td</sup> mice were different from all the other genotypes. However, at 9 weeks of age, these defects were observed not only in Dgcr8<sup>td/td</sup> mice, but also in Dgcr8<sup>md/md</sup> mice (Figure 4A-B). Interestingly, a severely atrophic myometrium was observed only in adult Dgcr8<sup>td/td</sup> mice (Figure 4C-D). In general, gross histology and real-time RT-PCR analysis and/or immunostaining of cell type-specific markers showed that Dgcr8<sup>td/td</sup> and Dgcr8<sup>md/md</sup> uteri were similar to those of Dgcr8<sup>+/+</sup> mice (Figure 4E-F), whereas Dgcr8<sup>td/td</sup> uteri had severe abnormalities, such as reduced myometrial thickness, and reduced stromal and epithelial area (Figure 4G-H).

### 3.6 | miRNAs that control immune responses and negatively regulate smooth muscle cell proliferation were systemically upregulated in the uteri of Dgcr8 cKO mice

We then performed small RNA-seq and mRNA-seq for uteri of 4-week-old Dgcr8<sup>td/td</sup> mice to elucidate the molecular mechanisms underlying the severe uterine phenotypes (Figure 5). Uteri from 4-week-old Dgcr8<sup>td/td</sup> mice were chosen because they showed the onset of multiple uterine defects, and PR was also expressed in all the major uterine cell types at this stage. In mRNA-seq data, 573 and 424 genes with 1.5-fold cut-off values were upregulated and downregulated in Dgcr8<sup>td/td</sup> mice, respectively (Figure S2 and Table S2). GSEA analyses showed the systemic upregulation of the gene sets associated with 'immune response', including leucocyte proliferation, migration, chemotaxis and blood vessel dilation (Figure 5A-C). In addition, gene sets associated with 'negative regulation of smooth muscle cell proliferation and development' were upregulated (Figure 5A-C). These results are consistent with the phenotypes in Dgcr8<sup>td/td</sup> mice, such as acute inflammatory infiltration of immune cells in the female reproductive tract and severe atrophy in uterine smooth muscle (Figures 4 and 6). In contrast, gene sets involved in ribosome biogenesis, RNA methylation (RNA stability), and negative regulation of T cell-mediated immunity were mainly downregulated in Dgcr8<sup>td/td</sup> mice (Figure 5B-D). Small RNA-seq data showed that 1035 out of the 1976 mouse miRNAs were detected in the uterus. However, only 32 and 27 miRNAs were down- and upregulated, respectively, with a 1.5-fold cut-off value in Dgcr8<sup>td/td</sup> mice (Figure S3 and Table S3-S4).

### 3.7 | Dgcr8 cKO mice provide distinct and overlapped target profiles in a Cre-dependent manner

To identify the direct target miRNAs of differentially expressed miRNAs (DEmiRNAs) in the uteri of Dgcr8<sup>td/td</sup> mice, we obtained a dataset of potential target genes of DEMiRNAs in Dgcr8<sup>td/td</sup> mice using miRmap and compared this dataset with mRNA-seq data. We found that 208 upregulated and 132 downregulated genes were overlapped between both datasets (Figure 6A). Since miR-149-5p, miR-29c-3p and miR-446b-3p miRNAs are representative of miRNAs with a Cre-specific unique expression in the uterus, these miRNAs and their target miRNAs associated with 'immune response' and/or 'negative regulation of smooth muscle cell proliferation and development' were further evaluated (Figure 6B). When luciferase constructs that included the 3' UTR of Cxcl12, Agtr2, Itga9 or Tspan2 mRNAs were co-transfected with miRNA mimics, the luciferase activity was significantly reduced (Figure 6C). This suggests that the target miRNAs that control the immune response are directly regulated by the miRNAs. We further examined and compared the expression profiles of these miRNAs and their target genes between Dgcr8 cKO mice.
with different Cre systems. miR-29c-3p was reduced in all the cKO genotypes and miR-466b-3p was differentially regulated in a Cre-specific manner, whereas miR-149-5p was significantly reduced only in Dgcr8<sup>td/td</sup> mice (Figure 6D). In general, the unique expression profiles of these miRNAs were inversely correlated with their target mRNAs in the uterus (Figure 6E,F). For example, among the miR-149-5p targets, Fbxo32, Npr3, and Tpm1 were specifically upregulated only in the Dgcr8<sup>td/td</sup> uterus, where miR-149-5p was significantly
reduced. Cxcl12 was upregulated not only in Dgcr8<sup>td/td</sup> but also in the Dgcr8<sup>md/md</sup> uteri. Furthermore, the expression patterns of Itga9 and Agtr2, targets of miR-29c-3p, were inversely upregulated in all the three Dgcr8 cKO mice. Wisp1, a target of miR-29c-3p and miR-466b-3p, were inversely correlated with their regulator miRNAs. The upregulation of Npr3 and Tpm1 as well as downregulation of their regulator miR-149-5p only in Dgcr8<sup>td/td</sup> uterus (Figure 6E) were consistent with the fact that smooth muscle defects were observed only in the Dgcr8<sup>td/td</sup> uterus (Figure 4D). Interestingly, whereas miRNAs and their target mRNAs associated with immune response were differentially dysregulated in Dgcr8 cKO mice in a Cre-dependent manner (Figure 6F), the percentage of CD45-positive immune cells were significantly increased only in Dgcr8<sup>td/td</sup> mice at 4 weeks of age (Figure 6G,H). Interestingly, the acute immune infiltration into the organs in the female reproductive tract was persistently observed in the Dgcr8<sup>td/td</sup> uterus, but not in other genotypes (Figure S4).
DISCUSSION

The uterus is a complex organ that consists of three major tissue compartments: myometrium, stroma and epithelium, with dynamic changes in various immune cells during the reproductive cycle. In this aspect, PR is a good Cre driver given its expression in all the major uterine cell types, and thus, PR-Cre mice have been exploited for gene deletion studies in all the major uterine compartments.\(^5,18\) Recently, we also demonstrated that canonical miRNAs are essential for uterine physiology and fertility using Dgcr8\(^{td/td}\) mice.\(^6\) However, PR-Cre mice may display compound phenotypes in multiple cell types. Thus, comparative analyses of all the three Dgcr8 cKO mice in this study will improve the understanding of the spatiotemporal action of each Cre system in the female reproductive tract. When Dgcr8 is deleted by Amhr2-Cre, defects in the oviduct and uterus were observed before and after puberty, respectively (Figures 3 and 4). These results suggest that phenotypes are expressed much later than the onset of Cre expression and/or follow a tissue-specific expression in Dgcr8\(^{td/td}\) mice. Unlike Dgcr8\(^{td/td}\) mice that suffer from infertility and uterine deformities, Dgcr8\(^{ed/ed}\) and Dgcr8\(^{md/md}\) mice showed regular oestrous cycles, showed normal architecture, and produced pups, suggesting that the deletion of Dgcr8 in epithelial and mesenchymal cells does not significantly deteriorate fertility (Figure 1). However, considering that both PR and Amhr2 are expressed in the stroma and myometrium in the female reproductive tract,\(^\text{19}\) it is noteworthy that Dgcr8\(^{ed/ed}\), but not Dgcr8\(^{md/md}\) mice exhibited acute inflammation in all the tissues of the female reproductive tract (Figure 6 and Figure S4). These results suggest that immune cells deficient in canonical miRNAs may affect uterine development and physiology. In fact, PR is present in a variety of cell types, including immune cells, such as NK cells,\(^6\) macrophages,\(^7\) dendritic cells\(^8\) and T cells,\(^9\) and non-immune cells, such as neuronal cells. However, it was also reported that Ltf is expressed in some immune cells, especially neutrophils and macrophages.\(^12,16\) Thus, deletion of Dgcr8 using immune-cell-specific Cre, such as macrophage-specific LyzM-Cre,\(^20\) may provide clues to decipher these puzzled phenotypes that are observed in Dgcr8\(^{ed/ed}\) mice but not in others.

Dgcr8 is required for the biogenesis of canonical miRNAs, whereas DICER is indispensable in the production of both canonical and non-canonical miRNAs. Thus, it is expected that Dicer and Dgcr8 cKO mice would show overlapping phenotypes in the female reproductive tract. In fact, Dgcr8\(^{md/md}\) mice showed similar but less severe abnormalities compared to Dicer\(^{md/md}\) mice. For example, Dgcr8\(^{md/md}\) and Dicer\(^{md/md}\) mice showed abnormally short oviducts, whereas Dgcr8\(^{ed/ed}\) and Dicer\(^{ed/ed}\) mice did not display morphologic deformities in the oviduct (Figure 7). While embryos were trapped in the oviduct that often harboured cysts during early pregnancy in Dicer\(^{md/md}\) mice,\(^21-23\) but not in Dgcr8\(^{md/md}\) mice (Figure 2), the increased

![Figure 5](https://example.com/figure5.png)

**Figure 5** GSEA analysis revealed upregulated and downregulated gene sets via mRNA seq in the uterus of Dgcr8\(^{ed/ed}\) at 4 weeks of age. (A, B) GSEA was performed to identify upregulated (A) and downregulated (B) gene ontology (GO) term in Dgcr8\(^{ed/ed}\) mice at 4 weeks of age. Gene sets with an FDR q-value < 0.25 (red dotted line) were considered significant. (C, D) GSEA enrichment plots and heatmaps of upregulated (C) and downregulated (D) GO gene sets from RNA-seq data. The normalized enrichment score (NES) and the corresponding FDR q-value are reported in each graph.

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FIGURE 6  Expression of potential target genes related to immune response and negative regulation of smooth muscle proliferation in the uteri of Dgcr8 cKO mice at 4 weeks of age. A, Venn diagram indicates overlapping between downregulated miRNA (a; 32, \( P < 0.05 \), 1.5-fold change) target genes (n = 7094) and upregulated genes (n = 573), and upregulated miRNA (b; 27, \( P < 0.05 \), 1.5-fold change) target genes (n = 6775) and downregulated genes (n = 424) in Dgcr8<sup>td/td</sup> mice at 4 weeks of age. The target genes of miRNA were searched using miRmap. B, Potential target genes for miRNAs related to immune response and negative regulation of smooth muscle proliferation in the uterus of Dgcr8<sup>td/td</sup> mice at 4 weeks of age. C, Luciferase reporter assay of Dgcr8 potential target miRNAs binding to 3’ UTR of putative target genes. (D-F) Real-time RT-PCR analyses of relative miRNAs and their targeted mRNA levels in the uterus of Dgcr8<sup>fl/fl</sup> and Dgcr8 cKO mice. (n = 4-8 per group). G, Immunofluorescence of CD45 in the uterine sections from Dgcr8<sup>fl/fl</sup> and Dgcr8 cKO mice. CD45 (immune cell marker) was visualized by green, and nuclei were stained with TO-PRO-3 (red), Scale bar: 20 \( \mu \)m. H, Percentage of CD45-positive cell /total number of cells counted in the uteri of Dgcr8<sup>fl/fl</sup> and Dgcr8 cKO mice at 4 weeks of age (n = 10-11 in each group). Unpaired Student’s t test, *\( P < 0.05 \), **\( P < 0.01 \)
number of degenerated oocytes in the oviduct of Dgcr8<sup>md/md</sup> mice could be associated with this phenotype. The uterus of Dicer<sup>md/md</sup> mice showed morphological and histological defects<sup>21-23</sup> whereas Dgcr8<sup>md/md</sup> mice had a normal uterine structure (Figure 4). In contrast, when PR-Cre was used, Dgcr8<sup>td/td</sup> and Dicer<sup>td/td</sup> mice showed similar uterine phenotypes, such as decreased number of glands and atrophic stroma.<sup>24</sup> In addition, considering that Dgcr8 is an essential factor for the biogenesis of canonical miRNAs, it was quite surprising that only 32 miRNAs with a 1.5-fold change were downregulated in the uteri of Dgcr8<sup>td/td</sup> mice (Figure 6). Intriguingly, only a handful of miRNAs were downregulated in the uteri of Dicer<sup>td/td</sup> mice as well.<sup>24</sup>

PR-Cre, Amhr2-Cre and Ltf-iCre mice have been used to understand the functions of other genes important for uterine biology. For example, each Cre mouse was individually used to examine the function of Pten, a well-known tumour suppressor gene, in tumorigenesis of endometrial cancer.<sup>25-28</sup> Pten<sup>td/td</sup> mice showed rapid development of endometrial cancer with full penetration, whereas Pten<sup>md/md</sup> mice failed to initiate tumorigenesis. As Amhr2 is not expressed in the epithelial compartment of the uterus, it is reasonable that Pten deletion in the stroma and myometrium could not provoke endometrial cancer.<sup>25,26</sup> However, Pten<sup>td/td</sup> mice developed atypical epithelial hyperplasia but did not develop endometrial cancer,<sup>27</sup> suggesting that Pten signalling in the stroma restrains epithelial cell transformation from hyperplasia to carcinoma. Deletion of Tsc1, a direct inhibitor of mTORC1, in the female reproductive tract sterilized both Tsc1<sup>td/td</sup> and Tsc1<sup>md/md</sup> female mice, resulting from oviductal hyperplasia, retention of embryos in the oviduct, and implantation failure.<sup>19</sup> However, embryo development was disrupted in Tsc1<sup>td/td</sup>, but not in Tsc1<sup>md/md</sup> mice.<sup>19</sup> Collectively, these reports, as well as this study, suggest that selection of the Cre system leads to a differential spectrum of phenotypes in tissues with multiple cell types in the female reproductive tract.

Previously, we demonstrated that DGCR8-dependent canonical microRNAs are essential for uterine development and physiological processes such as proper immune modulation, reproductive cycle and steroid hormone responsiveness in mice.<sup>6</sup> Especially, we observed that an excessive influx of immune cells occurs in the ovary, oviduct and uterus of Dgcr8<sup>td/td</sup> mice on Day 2.<sup>4</sup> In addition, percentage of CD45-positive cells were increased in the uterus of 4-week-old Dgcr8<sup>td/td</sup> mice (Figure 6G, H). These are consistent with the results of small RNA- and mRNA-seq, which revealed that miRNAs and their potential target mRNAs involved in immune responses were dysregulated in the uteri of Dgcr8<sup>td/td</sup> mice. However, acute inflammation in Dgcr8<sup>td/td</sup> mice was not observed in Dgcr8<sup>md/md</sup> and Dgcr8<sup>ed/ed</sup> mice (Figure 6 and Figure S4), suggesting that the profiles of dysregulated miRNAs could depend on the Cre system. Although many miRNAs are known to regulate immune responses, such as Toll-like receptor (TLR) signalling,<sup>29</sup> the functions of these miRNAs in the uterus are poorly understood. Luciferase assays validated that miRNAs directly inhibit Cxcl12, Itga9, Agrt2 and Tspan2 (Figure 6C). Itga9 plays a very important role in neutrophil migration,<sup>30</sup> and Cxcl12 induces cell migration via the CXCR4/CXCR7 complex.<sup>31-33</sup> TSPAN2 induces M2 polarization in microglia, and AGTR2 has vasodilating and blood pressure-reducing effects.<sup>34,35</sup> Another unique phenotype observed in Dgcr8<sup>td/td</sup> uteri was that the inner circular smooth muscle became atrophic at the adult stage (Figure 4). The role of miRNAs in the proliferation of uterine smooth muscle cells is unknown, but Npr3 and Tpm1, potential target genes of miR-149-5p and miR-29c-3p, are known to negatively regulate smooth muscle cell proliferation. NPR3 increases...
endothelial cell proliferation and inhibits vascular smooth muscle growth via ERK 1/2 phosphorylation. In addition, TPM1 inhibits vascular smooth muscle proliferation and migration progression via the HIF-1α/miR-21/TPM1 pathway. Although the expression of miR-21 was not reduced in Dgcr8td/td mice, it is thought that the expression could be sufficiently regulated by other miRNAs. Thus, it is assumed that the downregulation of miRNAs maintains the increased levels of their target mRNAs, subsequently inducing leucocyte migration and differentiation, blood vessel dilation, and/or suppression of smooth muscle proliferation. Collectively, differential phenotypes and landscapes of miRNAs and mRNAs in Dgcr8 cKO mice with different Cre systems suggest that selection of Cre is critical to understanding the function of the gene of interest in the female reproductive tract in a spatiotemporal manner.

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CONFLICT OF INTEREST
The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS
YS Kim, SC Yang, HJ Lim and H Song conceived and designed the experiments. YS Kim, SC Yang, M Park and HR Kim carried out the experiments. YS Kim, SC Yang, M Park, Y Choi, FJ DeMayo, JP Lydon, HJ Lim and H Song analysed the data. YS Kim, SC Yang, HJ Lim and H Song wrote the manuscript. All authors agreed to be responsible for the content of the work.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID
Seung Chel Yang https://orcid.org/0000-0003-2027-0393

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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