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Original Article

Adenovirus-mediated gene delivery restores fertility in congenitally infertile female mice

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Abstract. Oogenesis depends on close interactions between oocytes and granulosa cells. Abnormal signaling between these cell types can result in infertility. However, attempts to manipulate oocyte-granulosa cell interactions have had limited success, likely due to the blood-follicle barrier (BFB), which prevents the penetration of exogenous materials into ovarian follicles. Here, we used adenoviruses (AVs) to manipulate the oocyte-granulosa cell interactions. AVs penetrated the BFB and transduced granulosa cells through ovarian microinjection. Although AVs caused transient inflammation, they did not impair fertility in wild-type mice. Introduction of *Kitl*-expressing AVs into congenitally infertile $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mutant mouse ovaries, which contained only primordial follicles because of a lack of *Kitl* expression, restored fertility through natural mating. The offspring showed no evidence of AV integration and exhibited normal genomic imprinting patterns for imprinted genes. These results demonstrate the usefulness of AVs for manipulating oogenesis and suggest the possibility of gene therapies for human female infertility.
Key words: Adenovirus, Gene therapy, Kitl, Ovary

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Oogenesis is a long and complex process that depends on both intra- and extra-ovarian factors [1, 2]. At the time of birth, all oocytes are arrested at prophase I of the first meiotic division as primary oocytes in primordial follicles. Oocytes are surrounded by a layer of somatic cells called granulosa cells (GCs). A small number of primordial follicles are continuously recruited for fertilization. Recruitment of primordial follicles depends on the secretion of follicle stimulating hormone (FSH). Increases in circulating FSH levels during each reproductive cycle induce development of a cohort of follicles. GCs surrounding the oocyte increase in size and number, and begin to secrete estrogens. While most oocytes degenerate during oogenesis, stimulation by luteinizing hormone (LH) initiates ovulation of mature oocytes for subsequent fertilization.

Among the molecules involved in oogenesis, the interaction between the KIT tyrosine kinase receptor on oocytes and its ligand KITL on GCs has been extensively studied [3]. Mice with mutations in either of these molecules often exhibit impaired fertility, as the KITL-KIT interaction promotes both oocyte growth and survival. KITL is expressed in GCs in soluble (KITL1) or membrane-bound (KITL2) forms. Growth and differentiation factor 9 (GDF9) expression in oocytes inhibits the expression of both *Kitl1* and *Kitl2*. In contrast, bone morphogenetic protein 15 (BMP15), another molecule expressed in oocytes, promotes the expression of both *Kitl1* and *Kitl2* in monolayers of GCs [3]. Neutralizing antibody against KIT administered *in vivo* inhibited the onset of primordial follicle development, primary follicle growth, follicular fluid formation, and preovulatory follicle development [4]. These findings demonstrate the critical role of KITL-KIT signaling in oogenesis.

The development of a gene delivery method would be valuable for dissecting the complex molecular interactions between oocytes and GCs. However, the delivery of exogenous molecules into ovarian follicles is limited by the blood-follicle barrier (BFB), which consists of vascular endothelium, sub-endothelial basement membrane, thecal interstitium, follicular basement membrane, and membrane granulosa [5, 6]. BFB restricts access of molecules > 500 kDa into ovarian follicles [6]. Therefore, BFB likely prevents gene transduction into the oocyte-granulosa complex. However, we recently found that adeno-associated viruses (AAVs) can rescue the fertility of $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mutant mice with defective *Kitl* expression [7]. The ovaries of $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice completely lack *Kitl* expression, and oogenesis is arrested at the primordial follicle stage [8]. Microinjection of AAVs expressing the *Kitl* gene into their ovaries initiated oogenesis and allowed the production of offspring through natural mating. DNA analysis of the offspring revealed no evidence of germline integration of the transgene.

Although these results provide evidence that AAVs can penetrate the BFB and deliver a transgene to GCs, there are several critical drawbacks. AAVs have a very limited cargo capacity (4.7 kb) for transgenes [9]. Moreover, transgene expression occurs more slowly than with other viral vectors (3–21 days *in vivo*) [10]. Thus, although AAVs are considered the most promising gene therapy vectors, these drawbacks restrict their application. Among other gene therapy vectors, adenoviruses (AVs) appear to be good candidates for gene therapy in the ovaries, as they usually do not integrate into the host genome, which is the most serious side effect for germline cells. However, there are at least two problems associated with AV-mediated gene delivery. The first is inflammation, which often occurs in target tissue following AV-mediated gene delivery [11]. The death of one patient

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treated for ornithine-transcarbamylase deficiency using AV-mediated gene delivery raised safety concerns surrounding the application of AVs in humans, and resulted in a sharp decline in studies over the next decade [11]. The second problem is the duration of transgene expression [12]. The immunogenicity of AVs ordinarily limits the duration of their effect to a period of 2 weeks. AVs have several advantages over AAVs. Because AVs are larger than AAVs (90–100 nm), they can carry transgenes up to 30 kb in size [11, 13]. Moreover, the onset of expression can occur as early as 1–2 h after infection [14]. Therefore, AVs have several advantages that may overcome their shortcomings.

The purpose of this study was to examine the potential of AVs in gene therapy to treat congenital female infertility. Although our previous study showed the utility of AAVs in ovarian gene therapy, we sought to determine the utility of AVs because they have additional benefits not found in AAVs (i.e., increased cargo size and rapid onset of gene expression), which will increase the choice of strategies when AAV-based gene therapy cannot be applied. For example, AAVs may not be useful when the target gene is large, or when small promoters in AAVs are too weak or cannot recapitulate physiological gene expression. *Kitl*-expressing AVs were used to examine the utility of AVs in $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice. The successful production of offspring lacking viral vector integration suggests that AVs can serve as a useful vehicle for ovarian gene therapy.

Materials and Methods

Animals and microinjection procedure

For *in vivo* examination of AV expression, we used 4- to 6-week-old C57BL/6 (B6) mice. We also used 4- to 8-week-old *R26R-Eyfp* mice (a gift from Dr. Franklin Constantini, Columbia University Medical Center) [15]. For fertility restoration experiments, 4- to 8-week-old B6.Cg-Kitl<Sl-t>/Rbrc mice with a mixed B6, DBA/2 background were used (RIKEN BRC, Ibaraki, Japan) [8]. The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols.

For the ovarian microinjections, a ventral-lateral flank incision was made to insert a glass needle. After extracting the ovaries with fine forceps, the glass needle was inserted under the tunica albuginea. Filling was confirmed by adding a small amount of Trypan blue (Invitrogen, Carlsbad, CA, USA). Approximately 2 µl of viral particles was microinjected into the ovarian stroma.

Virus production

AxCAN-*Cre*, AxCAN-*LacZ*, and AxCAN-*Kitl* (RIKEN BRC) were used to transfect 293 cells. Viral particles were purified from 293 cells using CsCl centrifugation [16]. For AAVs, we used pAAV9-CAG-*Kitl* and produced viral particles through transient transfection, as described previously [17]. The virus titers were adjusted to 2×10^8/ml.

Measurement of hormones

To evaluate hormone levels, we collected peripheral blood and conducted a competitive-based enzyme-linked immunosorbent assay (ELISA) using an estrogen ELISA kit (R&D Systems, Minneapolis, MN, USA), a mouse FSH ELISA kit, and an LH ELISA Kit (both from MyBiosource, San Diego, CA, USA). Absorbance at 450 nm was measured using a SpectraMax ABS device (Molecular Devices, San Jose, CA, USA). Data are expressed as pg of estrogen per ml of serum and ng of FSH or LH per ml of serum.

Analysis of ovaries

The ovaries were fixed in 4% paraformaldehyde for 2 h, followed by embedding in paraffin or Tissue-Tek OCT compound for cryosectioning. For the analysis of *R26R-Eyfp* mice, ovaries were examined under ultraviolet light. Cryosections were immunostained by treating samples with 0.1% Triton-X in phosphate-buffered saline (PBS). After immersion in blocking buffer (0.1% Tween 20, 1% bovine serum albumin, and 1% donkey or goat serum in PBS) for > 1 h, samples were stained with the indicated primary antibodies at 4°C overnight. After three washes with 0.1% Tween 20 in PBS, the samples were stained with a secondary antibody. The antibodies used in this study are listed in Supplementary Table 1.

DNA analysis

Genomic DNA was isolated from the offspring using a standard phenol/chloroform extraction procedure, followed by ethanol precipitation. Polymerase chain reaction (PCR) was performed using specific primers (Supplementary Table 2). To detect viral DNA through southern blotting, we digested 20 µg of DNA with the indicated restriction enzymes and separated the fragments by electrophoresis on a 1.0% agarose gel. The DNA was transferred onto a nylon membrane (Hybond-N+; Amersham Biosciences, Buckinghamshire, UK). Hybridization was performed as previously described [16]. Genomic DNA was hybridized with a pAdex1cw cosmid containing the entire Adex1cw genome. For PCR analysis, a 309 bp region of pAdex1cw was amplified using specific primers (Supplementary Table 2).

Combined bisulfite restriction analysis (COBRA)

Genomic DNA was collected and treated with sodium bisulfite, which deaminates unmethylated cytosines to uracils, but does not affect 5-methylated cytosines. This DNA was used as a template for amplification of differentially methylated regions (DMRs) using specific primers (Supplementary Table 2). The PCR product was digested with the indicated restriction enzymes that recognize DNA sequences containing CpG in the original unconverted DNA.

Bisulfite sequencing

DNA methylation analysis was performed as described previously [7]. Bisulfite-treated DNA was amplified by PCR using specific primer sets and the products were purified using AMPure XP (Beckman Coulter, Brea, CA, USA) according to the manufacturer's protocol. Subsequently, the purified PCR products were amplified using nested PCR primers conjugated with Ion PGM Barcode X (comprising 13 bases each) and the barcode adapter GAT and then sequenced. Sequence data were processed in FASTA format files, and DNA methylation analysis was performed using QUMA, a quantification tool for methylation analysis. The PCR primers used are listed in Supplementary Table 2.

Statistical analyses

Significant differences between means for individual comparisons were determined using Student's *t*-test.

Results

AV-mediated gene delivery into the ovary

We previously failed to observe any signal by AV-mediated gene delivery into ovarian follicles. In that study, we microinjected AVs expressing enhanced green fluorescent protein (EGFP) into the ovaries of wild-type mice and examined EGFP expression by immunostaining

[7]. However, we were unable to observe apparent signals in the cells within the ovarian follicles. Therefore, we conclude that the AVs could not penetrate the BFB. However, it is possible that the signals were not strong enough for detection by immunofluorescence. Alternatively, cells expressing EGFP might have disappeared before the time of analysis because of apoptosis or transgene silencing. Therefore, in the present study we used *R26R-Eyfp* mice [15]. These mice contained a *loxP*-flanked STOP sequence followed by the *Eyfp* gene, which was inserted into the Gt(ROSA)26Sor locus (Fig. 1A). These mice allow for more sensitive detection of gene expression in a variety of tissues, including the testes [18]. Even if originally infected cells are lost, their progeny may contribute to enhanced yellow fluorescent protein (EYFP) expression. We produced *Cre*-expressing AV (AxCAN-*Cre*) and monitored the expression of EYFP after microinjection into the ovaries of *R26R-Eyfp* mice.

When the mice were sacrificed 1 week after microinjection, a strong EYFP signal was detected (Fig. 1B). Histological sections of the ovaries revealed EYFP signals in the cells within the ovarian follicles. Double immunostaining of *R26R-Eyfp* mouse ovaries with ovarian cell markers indicated that the EYFP signal was present in extrafollicular HSD3B$^+$ theca cells and intrafollicular AMH$^+$ GCs (Figs. 1C, D). However, no signal was observed in oocytes. These results suggest that AVs can penetrate the BFB and infect GCs without transduction of oocytes.

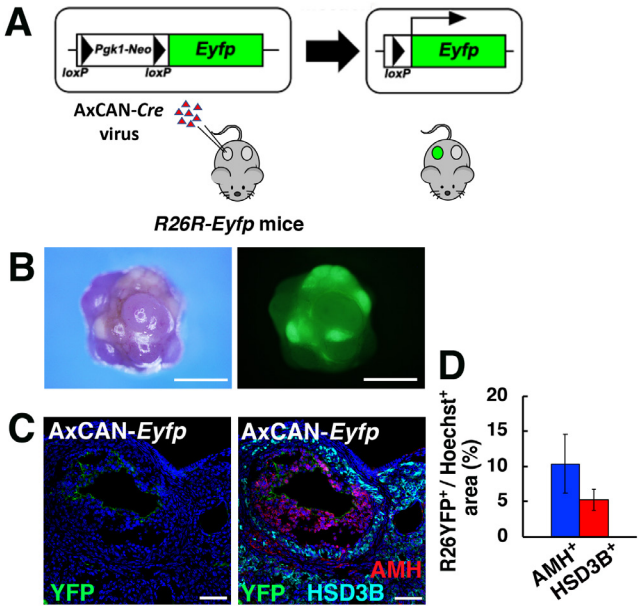

Fig. 1. AV-mediated gene delivery into GCs. (A) Experimental diagram. *R26R-Eyfp* mice were microinjected with AxCAN-*Cre*. CRE-mediated recombination removed the *neo* cassette, and EYFP expression was initiated under control of the *ROSA26* promoter. (B) Macroscopic appearance of *R26R-Eyfp* ovary 7 days after AxCAN-*Cre* injection. (C) Immunostaining of *R26R-Eyfp* ovary. (D) Quantification of cells expressing EYFP (n = 12 for AMH; n = 18 for HSD3B). Bar = 1 mm (B), 50 μm (C). Counterstain, Hoechst 33342 (C).

Comparison of AVs and AAVs for ovarian transduction

Because the results presented in the preceding section demonstrate successful infection of ovarian follicles by AVs, we compared the effects of AVs and AAVs. For this experiment, we used AVs that express *Kitl* (AxCAN-*Kitl*) [19]. In our previous study of this virus for male infertility treatment, we were able to restore spermatogenesis in congenitally infertile *Kitl*^{Sl}/*Kitl*$^{Sl\text{-}d}$ mutant mice, which contained only undifferentiated spermatogonia due to a lack of membrane-bound *Kitl* [20]. Although this vector was useful for restoration of spermatogenesis, we observed infiltration of lymphocytes into the seminiferous tubules, and it was impossible to obtain offspring through natural mating. This result suggested that transgene expression may not be sustained long enough to generate a sufficient amount of sperm [19]. Nevertheless, we tested whether exogenous *Kitl* overexpression stimulates oogenesis.

To examine the differences between AVs and AAVs, we microinjected AVs and AAVs into the same mice (AVs into right ovaries and AAVs into left ovaries). Both viruses were used at the same concentrations. Ovaries recovered 7 and 14 days after microinjection and were examined for signs of inflammation. We found no differences in the macroscopic appearance between ovaries treated with AVs or AAVs at either time point. However, histological analysis revealed that AxCAN-*Kitl* caused inflammation. Although lymphocyte invasion was not evident 7 days after infection, many lymphocytes were found after 14 days (Figs. 2A–C). Immunostaining of the ovaries confirmed the presence of both CD4$^+$ and CD8$^+$ lymphocytes in the ovaries 2 weeks after injection (Figs. 2B, C). These lymphocytes were found mostly in the interstitial areas, and no invasion into the follicles was apparent. In contrast, no lymphocyte infiltration was observed in ovaries after AAV transduction. These results suggested that AV transduction induces inflammation, confirming our previous observations in the testes [19].

As these results suggest that AAVs are superior to AVs, we examined the number of apoptotic cells using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (Supplementary Fig. 1). Although the number of TUNEL$^+$ cells was slightly higher for AAVs among both AMH$^+$ GCs and HSD3B$^+$ theca cells, the differences were not statistically significant. We found no apparent effect on oocytes despite inflammation, suggesting that ovarian inflammation does not cause oocyte depletion. These results suggest that AVs cause inflammation and apoptosis in ovarian somatic cells and have effects comparable to those of AAVs.

Impact of Kitl overexpression on reproductive performance

Since oocytes could survive after AxCAN-*Kitl* expression, it is possible that the animals would retain fertility despite the observed inflammatory reactions. Based on this possibility, we examined the impact of AxCAN-*Kitl* overexpression on reproductive performance in wild-type mice. In this study, we microinjected AxCAN-*Kitl* into the ovaries of mature female mice. AxCAN-*LacZ* at the same viral concentration was used as a control. These females were housed with wild-type males for at least 7 days after microinjection, and the number of offspring and timing of birth were recorded.

Overall, four of the five females microinjected with AxCAN-*Kitl* were fertile, as were all five females microinjected with AxCAN-*LacZ* (Table 1). In *Kitl*-overexpressing females, the first offspring were born as early as 22 days after mating, with an average timing of 26.5 ± 3.9 days. In contrast, the first offspring were born 28 days after mating in mice with AxCAN-*LacZ* transduction, with an average of 60.8 ± 11.7 days. The difference in the timing of offspring production was statistically significant. For litter size, an average of 5.1 ± 0.9 offspring were born after AxCAN-*Kitl* transduction. Although AxCAN-*LacZ* transduction led to a larger number of offspring (6.1 ± 0.8), there was no statistical difference between the two groups. AxCAN-*Kitl* transduction did not deplete the oocyte pool, as all four fertile females bore additional litter. Together, these results show that AxCAN-*Kitl* transduction does not impair fertility in wild-type animals.

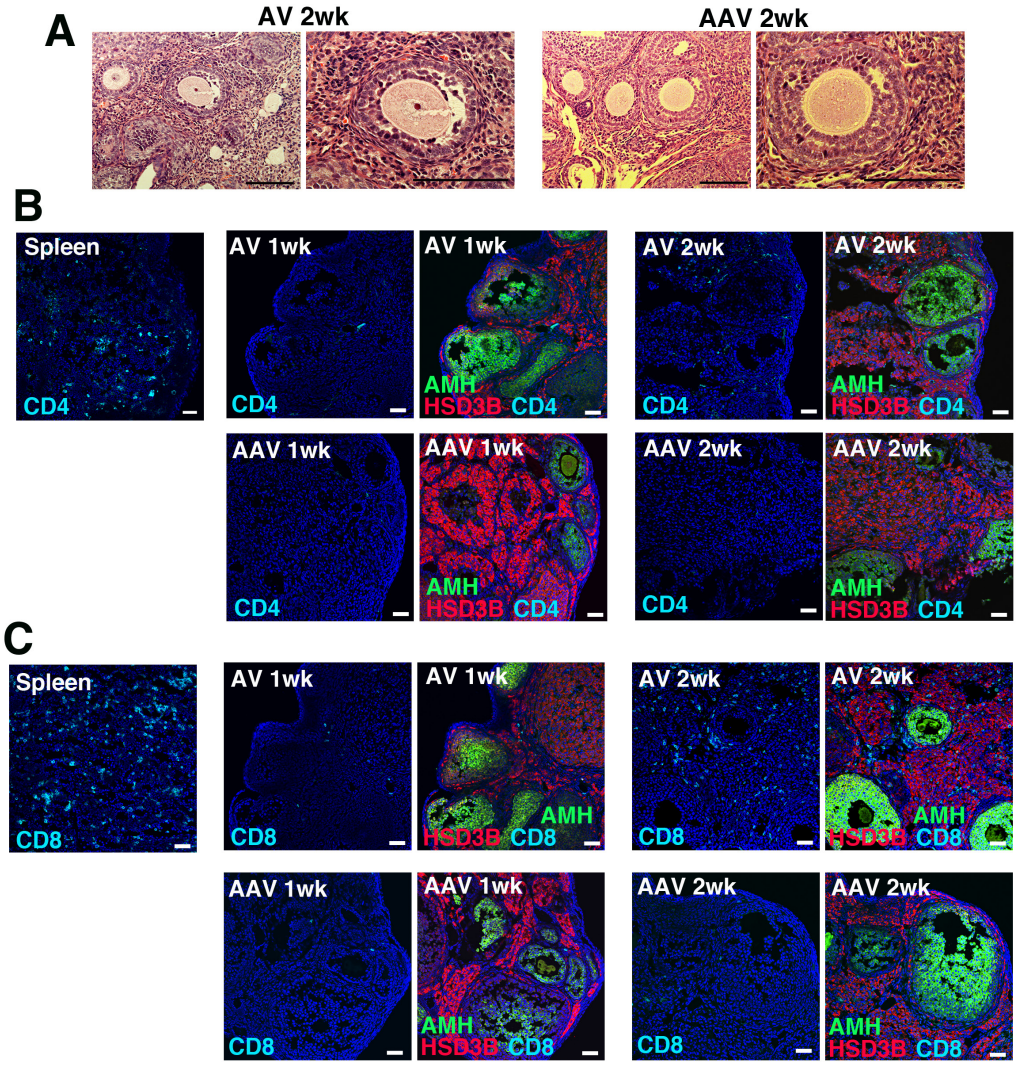

Fig. 2. Induction of inflammation by AVs. (A) Histological appearance of ovaries transduced with AxCAN-*Kitl* or AAV9-*Kitl* 2 weeks after microinjection. (B, C) Immunostaining of ovaries with anti-CD4 (B) or CD8 (C) antibodies 1 or 2 weeks after microinjection. Spleen was used as a positive control. Bar = 100 µm (A), 50 µm (B, C). Stain, H & E (A), Counterstain, Hoechst 33342 (B, C).

Table 1. Progeny from mice injected with AVs

Exp	Type of animals	No. of animals	Virus	Fertile animals (%)	Days to first progeny	Period of analysis (day)	Number of litters (total)	Litter size
#1	B6	5	AxCAN-*Kitl*	4 (80)	22	89–93	2.5 ± 0.3 (10)	5.1 ± 0.9
	B6	5	AxCAN-*LacZ*	5 (100)	28	92–93	2.0 ± 0.5 (10)	6.1 ± 0.8
#2	$Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$	5	AxCAN-*Kitl*	1 (20)	63	180	1 (1)	2

The titers of the virus were adjusted to 2×10^8/ml. $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ females were mated with $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ male to confirm the genotype of the offspring.

Histological analysis of ovaries after AxCAN-Kitl transduction

To evaluate the impact of AxCAN-*Kitl* transduction on oogenesis, we collected ovaries from both types of mice and performed histological analysis 3 months after microinjection. Because we observed lymphocyte infiltration 2 weeks after AV injection, we expected that AV transduction would induce fibrosis in the long term. However, no fibrosis or lymphocyte infiltration was observed at this stage. Instead, we observed large clusters of extrafollicular cells (Fig. 3A). Because these cell clusters were found in control ovaries that received AxCAN-*LacZ*, it is likely that they resulted from AV infection rather than *Kitl* overexpression. Although *Kitl* overexpression can cause tumors [21], no evidence of tumor formation was found in AxCAN-*Kitl*-transduced ovaries.

When we conducted immunostaining to confirm the cell type, the cell clusters were reactive with anti-HSD3B antibody (Fig. 3B), suggesting that they were derived from thecal cells. However, because these cells expressed low levels of MKI67, they did not actively proliferate at the time of analysis. In contrast, MKI67 signals

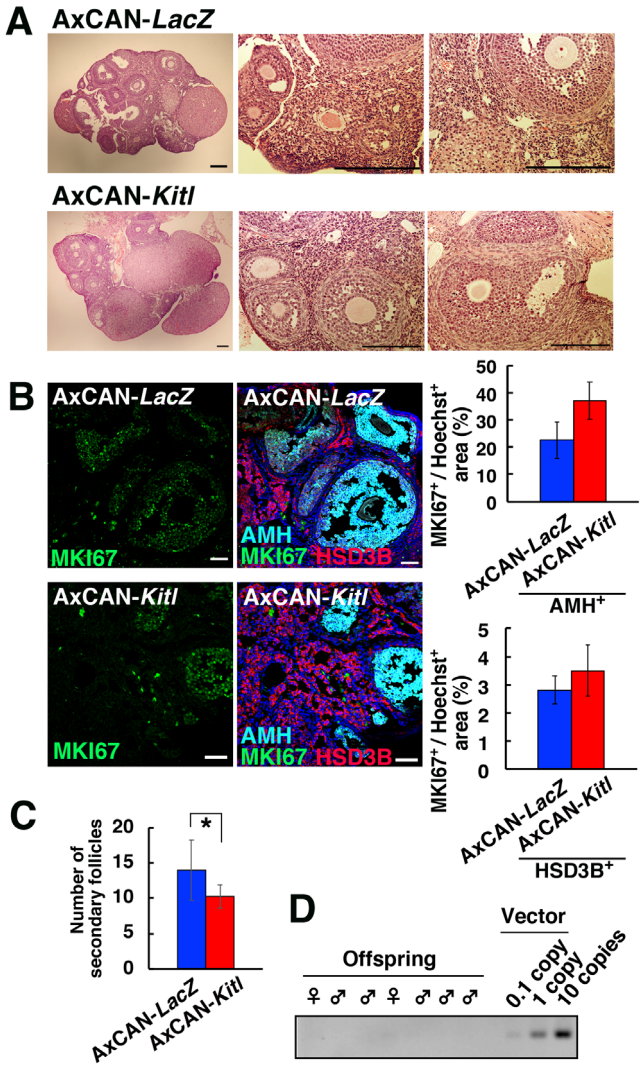

Fig. 3. Impact of AxCAN-*Kitl* expression on oogenesis in wild-type mice. (A) Histological section of ovaries transduced with AxCAN-*Kitl* or AxCAN-*LacZ* 3 months after microinjection. Clusters of theca cells are evident. (B) Immunostaining of ovaries using ovarian somatic cell markers (n = 16–21 for AMH; n = 8–9 for HSD3B). (C) Number of secondary follicles (n = 4). (D) PCR analysis of transgene integration. Bar = 200 μm (A), 50 μm (B). Stain, H & E (A). Counterstain, Hoechst 33342 (B). Asterisk indicates statistical difference ($P < 0.05$).

were stronger in AMH^+ GCs in both ovarian types. However, no significant difference was observed between the two vectors. Likewise, TUNEL staining showed that there was no significant enhancement of apoptosis due to AxCAN-*Kitl* transduction (Supplementary Fig. 2).

Despite the development of theca cell clusters, histological analysis showed normally growing oocytes in the control ovary that received the AxCAN-*LacZ* injection (Fig. 3A). Oogenesis also appeared normal in ovaries that received AxCAN-*Kitl* injections with many antral follicles. However, quantitative examination of oogenesis revealed that the number of secondary follicles was slightly, but significantly, reduced after AxCAN-*Kitl* transduction (Fig. 3C). This finding raised the possibility that *Kitl* overexpression might have influenced oocyte differentiation. To exclude the possibility of germline integration of the transgene, we examined the presence of adenovirus DNA by PCR. We collected tail DNA from all offspring and performed PCR using a virus-specific primer set. None of the offspring from AxCAN-*Kitl*- or AxCAN-*LacZ*-transduced ovaries showed evidence of germline integration (Fig. 3D), suggesting that the reduced number of secondary follicles might have been an indirect effect of *Kitl* expression in surrounding cells.

Rescue of infertility in $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice

Because experiments using wild-type ovaries showed that AxCAN-*Kitl* transduction does not impair fertility, we next tried to restore fertility in congenitally infertile $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice [8]. The size of the ovaries was significantly reduced in these mice (Fig. 4A). Histological analysis of the ovaries showed that they contained only primordial follicles (Fig. 4B). Owing to the lack of growing oocytes, none of the more than 100 female mutant mice housed with wild-type males produced offspring. Although the exact nature of this mutation remains to be determined, we recently found that $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ female mice completely lacked *Kitl* expression in their ovaries [7]. Therefore, the mutation responsible for the $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ phenotype likely disrupts the regulatory mechanism, thereby reducing the levels of *Kitl* mRNA in the ovary. In contrast, $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ male mice exhibit normal spermatogenesis and are fertile, despite two point mutations in the *Kitl* gene [7]. Analysis of the peripheral blood of these mice revealed elevated FSH and LH levels [7]. In contrast, the estrogen levels were significantly reduced. Therefore, these mice can serve as models for primary gonadal failure in humans.

We microinjected AxCAN-*Kitl* into the ovaries of $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice and examined the impact of *Kitl* overexpression. Histological analysis of the ovaries at 1 month after microinjection showed evidence of resumed oogenesis (Fig. 4B). Although only primordial follicles were found in the uninjected ovaries, a small number of oocytes grew in secondary follicles. No evidence of lymphocyte infiltration or tumor formation was observed. No abnormal theca cell proliferation was observed in the ovaries. As these results suggested that AxCAN-*Kitl* overexpression can stimulate oogenesis, we microinjected AxCAN-*Kitl* into the ovaries of five mature female mice (Table 1). One week after the microinjection, the mice were housed with $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ male mice to produce offspring. Although four mice remained infertile, one female bore two female offspring 63 days after AxCAN-*Kitl* microinjection, demonstrating restoration of normal fertility (Fig. 4C). Despite successful offspring production, analysis of the peripheral blood of AxCAN-*Kitl*-transduced mice showed no apparent changes in the levels of estrogen, FSH, or LH (Fig. 4D).

DNA analysis of offspring

To test whether AV transduction resulted in integration of the transgene, we collected DNA from the offspring produced by treated mice and examined their transgene integration patterns. Using an AV-specific probe, we first performed a southern blot analysis and found no evidence of transgene integration (Fig. 5A). We then performed more sensitive PCR analysis, which confirmed the lack of transgene integration (Fig. 5B).

Because genomic imprinting in the female germline is initiated after birth [22], it is possible that forced expression of *Kitl* causes abnormal imprinting. To test this possibility, COBRA was performed on the DNA of the mature offspring. Control DNA from germline stem (GS) cell cultures showed complete androgenetic DNA methylation patterns. Although the DMR of *H19* was heavily methylated, no methylation was apparent in the DMR of *Igf2r*. In contrast, the offspring of $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice exhibited somatic cell methylation patterns, and both the *H19* and *Igf2r* DMRs were partially digested with methylation-specific restriction enzymes, similar to those found

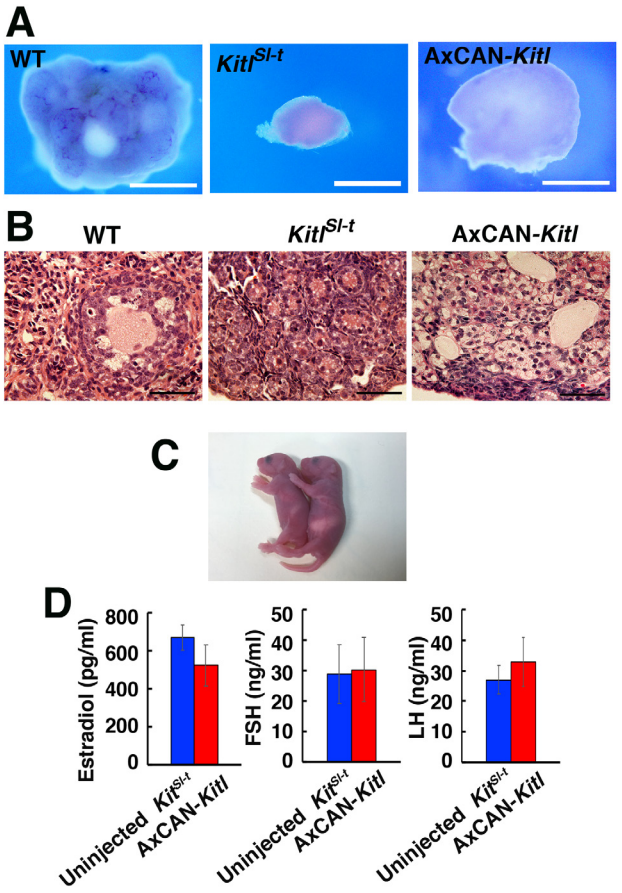

Fig. 4. Rescue of congenital infertility in *Kitl*$^{Sl\text{-}t}$/*Kitl*$^{Sl\text{-}t}$ mice through transduction of AxCAN-*Kitl*. (A, B) Macroscopic (A) and histological (B) appearance of *Kitl*$^{Sl\text{-}t}$/*Kitl*$^{Sl\text{-}t}$ ovary after AxCAN-*Kitl* transduction. (C) Offspring born after AxCAN-*Kitl* transduction. (D) Quantification of estrogen, FSH, and LH levels (n = 4–6 for estrogen and FSH; n = 4–5 for LH). Bar = 1 mm (A), 50 μm (B). Stain, H & E (B).

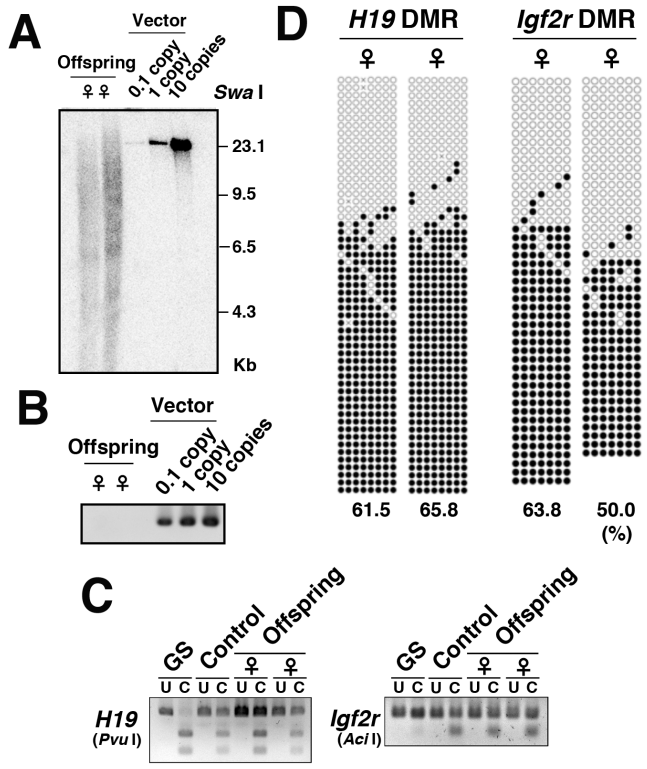

Fig. 5. DNA analysis of offspring born after AxCAN-*Kitl* transduction. (A, B) Southern blot (A) and PCR (B) analyses of F1 DNA samples from *Kitl*$^{Sl\text{-}t}$/*Kitl*$^{Sl\text{-}t}$ mutant mice. Controls represent genomic DNA spiked with viral DNA in amounts equivalent to indicated copies of viral DNA per diploid genome. (C) COBRA of DMRs in *H19* and *Igf2r* by COBRA. DNA of germline stem (GS) cells and normal mice were used as controls. (D) Bisulfite sequencing of DMRs in *H19* and *Igf2r*. Black ovals indicate methylated cytosine-guanine sites (CpGs), and white ovals indicate unmethylated CpGs.

in offspring born after natural mating (Fig. 5C). Bisulfite sequencing of the *H19* and *Igf2r* DMRs also confirmed the somatic cell type imprinting patterns (Fig. 5D). These results suggest that AV-mediated ovarian rescue does not induce abnormalities in offspring.

Discussion

Oogenesis involves numerous genes that require complex cellular and hormonal interactions [1, 2]. Therefore, it is not surprising that a large proportion of couples have impaired fertility. Currently, approximately 10% of women of reproductive age are unable to conceive or carry a pregnancy to term [23]. Owing to rapid advances in elucidating the mechanism of oogenesis in recent decades, the basic mechanism of oogenesis in mice is now well understood. However, few attempts have been made to restore fertility caused by defective oogenesis *in vivo*. The current study aimed to establish a strategy to rescue oogenesis in *Kitl*$^{Sl\text{-}t}$/*Kitl*$^{Sl\text{-}t}$ mice, which only have primordial follicles. These mice resemble the primary gonadal failure in humans, which accounts for approximately 1% of human infertility cases [24]. This defect is based on the deregulation of *Kitl* expression in GCs, which is likely the most intensively studied signal in the crosstalk between oocytes and GCs. Therefore, the analysis of this mouse model will provide a useful platform for the development of gene therapies for ovarian defects.

Our results using wild-type mice suggest that KITL overexpression did not deplete the oocyte pool after transduction. This was one of the greatest concerns in the current study because we expected that excessive KITL expression might trigger the activation of numerous oocytes or interfere with ovulation. However, we did not find such abnormalities, but instead noticed that they sired their offspring in relatively shorter periods after AV transduction. Considering that the size of the litters was comparable between control and KITL-expressing animals, these results suggest that KITL is not directly involved in determining the number of activated oocytes. Rather, it promotes the development of oocytes that are committed to activation. Although the number of animals in the current study may be too small to conclude such an effect, further investigation of this point by experiments on a larger scale will clarify the role of the KITL-KIT interaction in oocyte development. As the aim of this experiment was to confirm the normal fertility of animals with AVs, we applied AVs to infertile *Kitl*$^{Sl\text{-}t}$/*Kitl*$^{Sl\text{-}t}$ mice.

Our successful fertility restoration in *Kitl*$^{Sl\text{-}t}$/*Kitl*$^{Sl\text{-}t}$ mice suggests that AVs could be used to restore fertility in animals with ovarian defects. Because AVs cannot penetrate the blood-testis barrier [18], we initially thought that AVs are not useful for ovarian gene therapy. However, we found evidence of follicular infection with AVs in *R26R-Eyfp* mice. We do not currently know how AAVs (25 nm) or AVs (90 nm) can penetrate BFB, which serves as a molecular sieve

to prevent the diffusion of proteins > 20 nm in size [25–27]. One possibility for this is transcytosis. Transcytosis pathways are utilized by blood components and by viruses, such as human immunodeficiency virus and poliovirus [28]. AAVs have been shown to penetrate the epithelial barrier through transcytosis [29]. However, transcytosis of AVs across the blood-brain barrier appears to be inefficient or impossible [28]. Because the composition and nature of barrier function likely differ in the ovary, whether AVs employ transcytosis to penetrate the BFB remains to be determined.

The birth of offspring after AV transduction confirms our previous observation that oogenesis can resume with appropriate stimulation even in mature infertile female mice [7]. The remarkable flexibility of the oocyte-GC interaction is reminiscent of our previous research on the testes. Adult Sertoli cells were found to support spermatogenesis in undifferentiated spermatogonia that had never been exposed to membrane-bound *Kitl* during postnatal testicular development. To date, three types of viruses –AAVs, AVs, and lentiviruses– have been used for fertility restoration in male animals [18, 19, 30]. However, the efficiency of this method in males may be insufficient because no offspring are produced through natural mating. All offspring were born by collecting sperm from testes for microinsemination. This low efficiency is likely due to the relatively small amount of sperm that could mature through simple *Kitl* overexpression. In this sense, the present results in female mice are striking, as they produce offspring through natural mating. This finding is even more surprising considering that $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice may have additional defects in the oviduct, as neural crest cells, which also depend on KIT, colonize the oviduct and serve as pacemaker cells [31].

Fertility restoration by AVs was unexpected because of the previous failure to restore fertility in *Fshr* knockout mice via AV transduction [32]. Because the types of mutations and vectors used in the study are different, a direct comparison between the two studies is not easy. However, the constitutive CMV5 promoter used in a previous study was speculated to have been downregulated to support the increased expression of *Lhr* in the later phase of oogenesis. Although the use of the authentic human *Fshr* promoter may prevent such problems, the reconstruction of the complex inverse regulatory loop between *Fshr* and *Lhr* in the growing follicle that orchestrates successful maturation and ovulation is a daunting task. Although we were able to rescue fertility in the present study by simply overexpressing *Kitl,* these results suggest that the development of vectors reflecting endogenous gene expression patterns may be a more appropriate method, depending on the nature of the infertility. Therefore, AVs may be preferable to AAVs because of their larger cargo size, which would allow a larger promoter to be incorporated into the virus.

One important concern in AV-mediated gene therapy is inflammation, which is a major problem with this vector [11]. In our previous study on male mice [19], we used AVs to treat spermatogenic defects because the testis is an immune-privileged part of the body [33]. Therefore, we hypothesized that the suppression of immunity may modulate inflammation caused by AVs. Although AV infection attracted lymphocytes after microinjection in the seminiferous tubules of the testis, spermatogenic recovery was not inhibited, and it was possible to obtain sperm for offspring production. Because the ovary is also considered an immune-privileged site [34], we expected that AV infection might also be attenuated in the ovary relative to other organs. As expected, infiltration of lymphocytes was observed but did not persist in the long term, and the ovaries were functionally normal in the production of offspring. Therefore, AVs did not compromise fertility despite the apparent induction of inflammation.

Given these results, the most important concern for human application is germline integration of the transgene. Fortunately, offspring born after gene therapy did not carry the transgene, indicating no integration into the germline. As the life cycle of wild-type AV is extrachromosomal [11], AV vectors are considered non-integrating vectors. However, it was previously observed that injection of AVs can result in chromosomal integration *in vivo* [35]. In a previous study, 0.0001–1% of cells showed evidence of integration when the frequency was estimated *in vitro* [36]. *In vivo* integration has also been reported for AAVs [37]. Similar to AVs, most AAVs exist in an extrachromosomal state. AAV integration can occur at non-homologous locations that are sites of DNA damage, or at specific sites via homologous recombination [38]. Therefore, further studies are required to examine the safety of gene therapy in fertility treatment.

We observed impairment of the hormonal response despite successful offspring production. $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice have elevated levels of FSH and LH, resembling primary gonadal failure in humans [7]. Hormonal regulation is important not only for ovulation, but also for oviduct transport in embryos. Since pacemaker cells that drive such transport also depend on KIT signaling [31], it is possible that $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice have an abnormal oviduct. However, the birth of offspring suggests that an appropriate hormonal response and gamete/embryo transport occurred in $Kitl^{Sl\text{-}t}/Kitl^{Sl\text{-}t}$ mice. Although we do not yet know the mechanisms underlying these observations, we speculate that AV transduction transiently restores the hormonal response and allows for normal ovulation and offspring production. Because oogenesis is accompanied by GC proliferation, and AVs cannot integrate into the host genome, it is likely that *Kitl*-expressing GCs cannot maintain transgene expression at a constant level throughout oogenesis. It is possible that AVs may gradually become depleted in infected cells after several rounds of cell division, resulting in an impaired hormonal response. However, further research is required to examine these mechanisms.

Our results on AV-mediated gene delivery raise the possibility that gene therapy may be applied to human female infertility in the future. Through extensive analysis of the genes involved in fertility, the number of candidate genes for female infertility is increasing rapidly [23]. Our strategy based on AV- or AAV-based gene delivery into ovaries may be applicable to some of these genes. Because AVs can be applied locally to the ovarian stroma, they are safer than systemic injections, which often cause severe side effects in other organs. Our successful rescue of infertility by AVs increases the range of target diseases because they have a large cargo size, which allows the incorporation of large promoters to achieve sufficient or physiological gene expression levels. The identification of appropriate target genes is necessary at this stage of research, as it investigates the risk of transgene integration through large-scale experiments with other animal models. Such endeavors may eventually overcome fertility problems that cannot be treated using conventional assisted reproduction technologies.

Conflicts of interests: The authors declare no conflicts of interest associated with this manuscript.

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