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Research Article

iCre recombinase expressed in the anti-Müllerian hormone receptor 2 gene causes global genetic modification in the mouse†

Mackenzie J. Dickson, Artiom Gruzdev and Francesco J. DeMayo*

Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, NIH, Durham, NC, USA

***Correspondence:** Francesco J DeMayo, Reproductive and Developmental Biology Laboratory, National Institute of Environmental Health Sciences, NIH, Durham, NC, USA. E-mail: francesco.demayo@nih.gov

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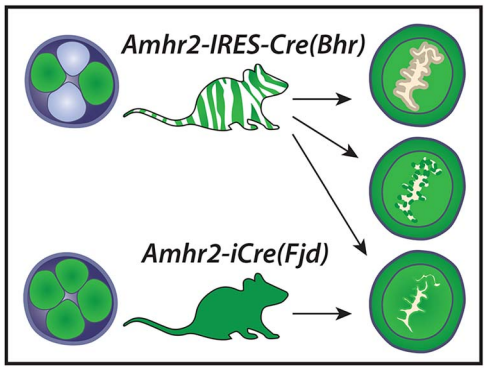
Abstract

Genetically engineered mice are widely used to study the impact of altered gene expression in vivo. Within the reproductive tract, the Amhr2-IRES-Cre(Bhr) mouse model is used to ablate genes in ovarian granulosa and uterine stromal cells. There are reports of Amhr2-IRES-Cre(Bhr) inducing recombination in non-target tissues. We hypothesized the inefficiency or off-target Cre action in Amhr2-IRES-Cre(Bhr) mice is due to lack of recombination in every cell that expresses *Amhr2*. To investigate, we created a new targeted knock-in mouse model, Amhr2-iCre(Fjd), by inserting a codon-optimized improved Cre (iCre) into exon 1 of the *Amhr2* gene. *Amhr2-iCre(Fjd)*/+ males were mated with females that contain a lox-stop-lox cassette in the *Sun1* gene so when DNA recombination occurs, SUN1-sfGFP fusion protein is expressed in a peri-nuclear pattern. In adult *Amhr2-iCre(Fjd)*/+ $Sun1^{LsL/+}$ mice, Amhr2-iCre(Fjd)-mediated genetic recombination was apparent in uterine epithelial, stromal, and myometrial cells, while *Amhr2-IRES-Cre(Bhr)*/+ $Sun1^{LsL/+}$ females demonstrated inter-mouse variability of Amhr2-IRES-Cre(Bhr) activity in uterine cells. Fluorescence was observed in Amhr2-iCre(Fjd)-positive mice at post-natal Day 1, indicating global genetic recombination, while fluorescence of individual Amhr2-IRES-Cre(Bhr)-positive pups varied. To determine the developmental stage that genetic recombination first occurs, $Sun1^{LsL/LsL}$ females were super-ovulated and mated with *Amhr2-IRES-Cre(Bhr)*/+ or $Amhr2^{(iCre/+)Fjd}$ males, then putative zygotes were collected and cultured. In the four-cell embryo, Amhr2-iCre(Fjd) and Amhr2-IRES-Cre(Bhr) activities were apparent in 100% and 25–100% of cells, respectively. In conclusion, Amhr2-IRES-Cre(Bhr) or Amhr2-iCre(Fjd) driven by the *Amhr2* promoter is active in the early embryo and can lead to global genetic modification, rendering this transgenic mouse model ineffective.

Summary Sentence

A new mouse model was created by inserting iCre recombinase in the *Amhr2* locus, to improve a previous genetic strain of mice. However, Amhr2-iCre causes DNA recombination in the preimplantation embryo, leading to total body genetic modification.

Graphical Abstract



Genetic recombination in the Amhr2-IRES-Cre(Bhr)/+ mouse has mosaic characteristics leading to variation in the genetic recombination in the whole mouse or apparent "leakiness" of Cre activity. The use of iCre to make an Amhr2-iCre(Fjd) mouse causes a more robust and consistent genetic recombination in all cells in the early embryo, leading to a global modification.

Keywords: genetics, transgenic mice, global genetic modification

Introduction

Since the introduction of genetic recombination strategies and transgenic mouse models in the past 50 years, research on genetic regulation of the female reproductive tract has dramatically increased. A major advancement has been the widespread use of the Cre-LoxP system. The Cre-loxP system is a powerful tool that can be employed to conditionally express a transgene or to ablate a gene of interest in a specific cell type to study the role of that gene or to trace the lineage of a cell that is derived from cells expressing developmental factors. Due to the rise in sequencing information and genetic editing, scientists have designed conditional knock-out mice where the Cre recombinase is under the control of a specific promotor, thus only causing genetic recombination in cells

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that express the Cre recombinase. One locus that has been used to target cell-specific gene in the female reproductive axis is the anti-Müllerian hormone receptor (Amhr2) locus in mouse.

In mammals, the initial development of the reproductive tract during embryogenesis originates from the mesoderm, one of three embryonic tissue layers that arise during the process of gastrulation. The primitive reproductive tract consists of undifferentiated gonads, and both paramesonephric, also known as Müllerian, and mesonephric, also known as Wolffian, ducts. Sex differentiation is dependent on the presence of the SRY gene on the Y chromosome, which leads to the development of the fetal testes [1, 2]. In males, anti-Müllerian hormone (AMH) is secreted from fetal testicular cells, which induces the regression of Mullerian ducts through AMH receptor 2 (AMHR2). In females, there is no Y chromosome or SRY gene present, therefore AMH is not secreted, and the Müllerian ducts remain, ultimately giving rise to the uterus and female reproductive tract. Amhr2 is expressed in the ovaries, oviduct, and uterus of the female reproductive tract. Due to the reproductive tract expression of *Amhr2*, an *Amhr2*-Cre mouse model was created by the insertion of a cassette containing an internal ribosome entry site (IRES)-Cre followed by a cassette containing neo flanked by FRT sites into exon 5 of the *Amhr2* gene [3]. Initial characterization demonstrated that expression of a functional Cre recombinase was present in the Müllerian ducts at embryonic day 11.5 and that Cre was also highly expressed in the gonads by embryonic day 12.5 [3]. Over the past 20 years, this Amhr2-IRES-Cre(Bhr) mouse model, *Amhr2*$^{tm3(cre)Bhr}$ (MGI 3042214), has been used for over 150 publications, especially to ablate genes in the reproductive tract including ovary, uterus, and testes [4].

However, despite extensive use in recent decades, there have been reports of the Cre recombination activity "being leaky" and causing recombination in other tissues or of total body recombination [5, 6]. In fact, for DNA recombination to occur in this Amhr2-IRES-Cre(Bhr) mouse model, the *Amhr2* promoter must function and splice normally as well as the IRES must work. Due to the conflicting results, we hypothesize the inefficiency or off target activity of the Amhr2-IRES-Cre(Bhr) mouse model is due to (1) lack of Cre-induced recombination in every cell that expresses *Amhr2* or (2) due to inconsistent *Amhr2* expression in various cell types. To investigate these possibilities, we created a new simpler targeted knock-in mouse model, Amhr2-iCre(Fjd), by inserting a codon-optimized improved Cre (iCre) into exon 1 of the *Amhr2* gene. Our objective was to make a new mouse model by inserting iCre in the *Amhr2* locus to decrease leakiness reported from the old mouse model and to make a more robust genetic disruption, specifically in uterine stromal cells.

Materials and methods

All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and animal protocols approved by the Animal Care and Use Committee (ACUC) at the National Institute of Environmental Health Sciences.

Generation of Amhr2-iCre(Fjd) mice

The Amhr2-iCre(Fjd) locus was generated by replacing the genomic sequence immediately downstream of the start codon in exon 1 with the codon improved iCre recombinase open reading frame (ORF) and Sv40 polyadenylation sequence (Figure 1). The repair donor plasmid was generated in the pUC19 backbone by conventional molecular biology techniques and consists of a 409 bp 5′ homology (chr15:102,353,509-102,353,917 GRCm39), codon-improved iCre recombinase ORF, SV40 polyadenylation signal, and a 400 bp 3′ homology arm (chr15:102,354,000-102,354,399 GRCm39). In the final Amhr2-iCre(Fjd) locus structure, 82 bp of endogenous sequence (chr15:102,353,918-102,353,999 GRCm39) was replaced by the iCre-pA genetic payload (1205 bp). As designed, Amhr2-iCre(Fjd) allele is likely an Amhr2 loss-of-function allele due to genetic disruption of endogenous exon 1. The resulting allele should express iCre recombinase under the control of the endogenous *Amhr2* promoter in place of AMHR2 protein.

Gene targeting was done in B6129F1 embryonic stem (ES) cells (G4; 129S6/SvEvTac x C57BL/6Ncr). ES cells were transfected with a 6:1 molar ratio of donor plasmid and Cas9-Puro/sgRNA (TAAGAGTCTACAGAGCGGGGCGG*N GG[PAM]*) delivery plasmid (pSpCas9(BB)-2A-Puro (PX459) V2.0), a gift from Feng Zhang (Addgene plasmid # 62988, [7].

After transfection, the cells were exposed to 48-h of puromycin selection (0.9 μg/mL) followed by standard clonal expansion/screening. Clones were screened with 5′ and 3′ screens external to the homology arms and genetic payload/WT zygosity screen to identify Amhr2-iCre/WT clones. Screening primers: Amhr2-iCre 5'Scr (Fwd: ATGCTACAGATCTGAGTTCTCCAT, Rev: CTTCCTGACTTCATCAGAGGTGG), Amhr2-iCre 3'Scr (Fwd: ATTGCCGAAATTGCCAGAATCA, Rev: ACTTGGTCATTCACCTTGCATCT), and Amhr2 WT Scr (Fwd: ATGCTACAGATCTGAGTTCTCCAT, Rev: GTGTACTTGGTCATTCACCTTGC). Polymerase chain reaction (PCR) amplicons from targeted clones were fully sequenced to confirm proper insertion of the iCre-pA construct. For the chimeric founder generation, targeted ES cells were microinjected into e3.5 albino B6J blastocyst (JAX stock #000058) isolated from naturally mated females. Injected blastocysts were then non-surgically transferred to pseudo-pregnant recipient SWISS mice. Germline male chimera founders were mated to C57BL/6 J females to establish the Amhr2-iCre(Fjd) mouse line. The Amhr2-iCre(Fjd) allele was re-sequenced in the F1 chimera offspring as described above in the original ES cell targeting. The line was maintained by crossed to C57BL/6 J wild-type mice. The mouse colony was subsequently genotyped at Transnetyx using primer/probe assays; Amhr2 WT (Fwd: GGCTTTGGACACTGCTTCCT, Rev: CCGCCCCGCTCTGTA, Probe: CAGCACAAGGTAAGAGTC) and Amhr2-iCre (Fwd: CAGCTGGCATCCTTTTGCA, Rev: CTTCATCAGAGGTGGCATCCA, Probe: CCAACCTGCTGACTGT). Long-range locus-specific PCR as described above in original screening was used to confirm that the Transnetyx primer/probe genotyping assay results were consistent in identifying the targeted and wild-type alleles. For the first 12 litters of heterozygous x C57BL/6 J WT, average litter size was 7.75 mice at weaning per litter, with a 40:53 male to female ratio, and 44:49 WT to Cre + ratio. Based on Mendelian results and consistency between long-range PCR and Transnetyx primer probe assay, there is no indication that a parallel random integration of the targeting vector

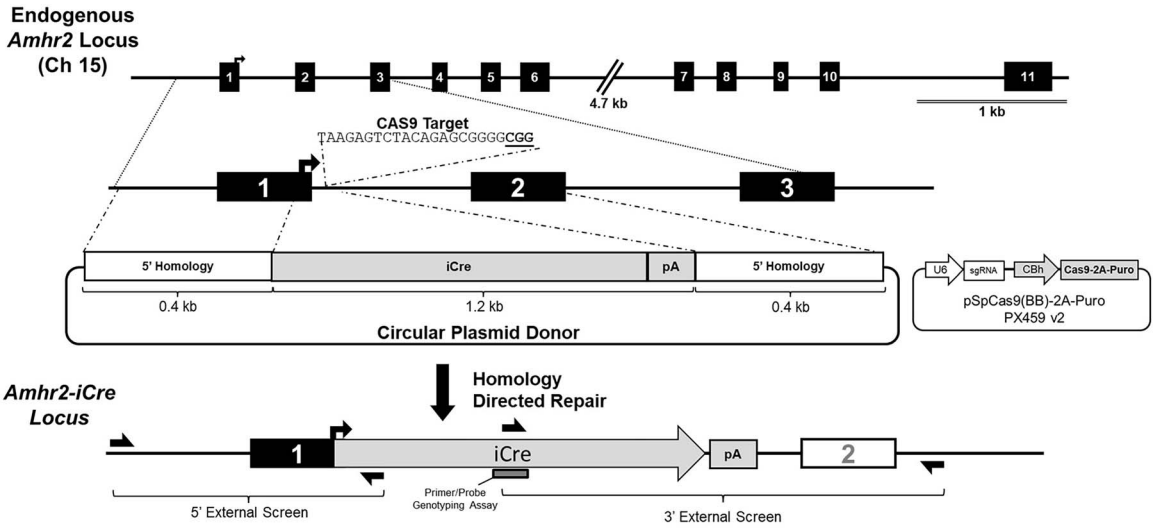

Figure 1. Targeting strategy to make the *Amhr2-iCre(Fjd) allele*. CRISPR/Cas9-mediated targeting strategy to generate the Amhr2-iCre(Fjd) allele. The iCre ORF and polyA signal were inserted immediately downstream of the start codon in exon 1. Locus-specific PCR screens and Transnetyx primer/probe genotyping assays are indicated in the final allele.

occurred elsewhere in the genome. These mice are available upon request.

Verification of iCre recombinase activity

Adult Amhr2-iCre(Fjd) or Amhr2-IRES-Cre(Bhr) male mice were crossed with CAG-Sun1sfGFP ($Sun1^{LsL/LsL}$) females [8] that were purchased from Jackson Laboratory to generate Amhr2-iCre(Fjd)/+ $Sun1^{LsL/+}$ and Amhr2-IRES-Cre(Bhr)/+ $Sun1^{LsL/+}$ mice. Adult mice between 7 and 12 weeks of age were euthanized for analysis of Amhr2-iCre(Fjd) or Amhr2-IRES-Cre(Bhr) activity in the uterus ($n = 6$) and other tissues. Breeding pairs were set up of *Amhr2-iCre(Fjd)*/+ or *Amhr2-IRES-Cre(Bhr)*/+ males crossed with $Sun1^{LsL/LsL}$ females. Breeding pairs were monitored and the day after a litter was born, pups were euthanized at post-natal day 1 for analysis of Amhr2-iCre(Fjd) or Amhr2-IRES-Cre(Bhr) activity. Adult mice were euthanized by CO_2 inhalation followed by cervical dislocation, and post-natal day 1 pups were euthanized by decapitation. Tissues were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 h at 4°C.

Superovulation and embryo culture

Adult female $Sun1^{LsL/+}$ mice were injected intraperitoneally with 5 IU pregnant mare serum gonadotropin, followed by 5 IU human chorionic gonadotropin (hCG) 48 h later. After hCG injection, female mice were housed with adult Amhr2-iCre(Fjd)/+ or Amhr2-IRES-Cre(Bhr)/+ or CD1 male mice overnight. The next morning, female mice were removed and euthanized via CO_2 inhalation and cervical dislocation. To collect putative zygotes, oviducts were excised, and opened with dissecting forceps into wash medium (Embryo Max® M2; Millipore MR-015-D). Putative zygotes were then transferred into hyaluronidase for dissociation of cumulus cells. Following a 5 min incubation at room temperature in hyaluronidase (Embryo Max® M2 with hyaluronidase; Millipore MR-051-F), putative zygotes were washed three times with wash medium, and twice in embryo culture medium (Embryo Max® KSOM; Millipore MR-106-D), then placed in embryo culture medium, overlaid with mineral oil (Fisher Scientific O121-1). The volume of embryo culture media was dependent on the number of putative zygotes present (1 μl/embryo). Putative zygotes were cultured at 37°C in 5% CO_2 and 5% O_2 for 4 days.

Tissue collection and processing, and immunofluorescence

Following fixation, tissues were transferred to 70% ethanol for at least 24 h, followed by dehydration and paraffin embedding. Embedded tissues were sectioned at 5 μm thickness and mounted on glass slides. Staining for SUN1-sfGFP was performed as previously described [9]. Briefly, slides were rehydrated, then antigen retrieval was performed by microwaving slides in Antigen Unmasking Solution (Vector H-3300) for 3.5 min at 70% power, followed by 12 min at 20% power. After slides were cooled to room temperature, slides were washed with PBS, followed by PBS containing 0.2% Triton-X, then washed again with PBS. Slides were blocked for 1 h at room temperature in PBS containing 5% normal donkey serum, and incubated in primary antibody for GFP (1:500, Abcam, ab5450), overnight at 4°C. The following day, slides were washed thrice in PBS, and incubated in secondary antibody (1:500, Donkey anti-Goat IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, ThermoFisher A-11055) for 1 h at room temperature. Slides were washed thrice in PBS and mounting media containing DAPI (Vectashield H-1200) was applied.

Microscopy and imaging

Images of fixed tissue immunofluorescence and live embryos were captured using a ZEISS LSM 780 UV confocal microscope at ×20 magnification. Endogenous GFP expression was captured in embryos and a dye (1 μg/mL Hoechst 33342, ThermoFisher) was added to embryo culture as a nuclear marker. Brightfield and GFP whole mount images of fresh tissue or post-natal day 1 were collected on a Leica M165 FC stereo microscope using a Leica DFC310 FX camera. Image processing was performed in ZEN 2.3SP1.

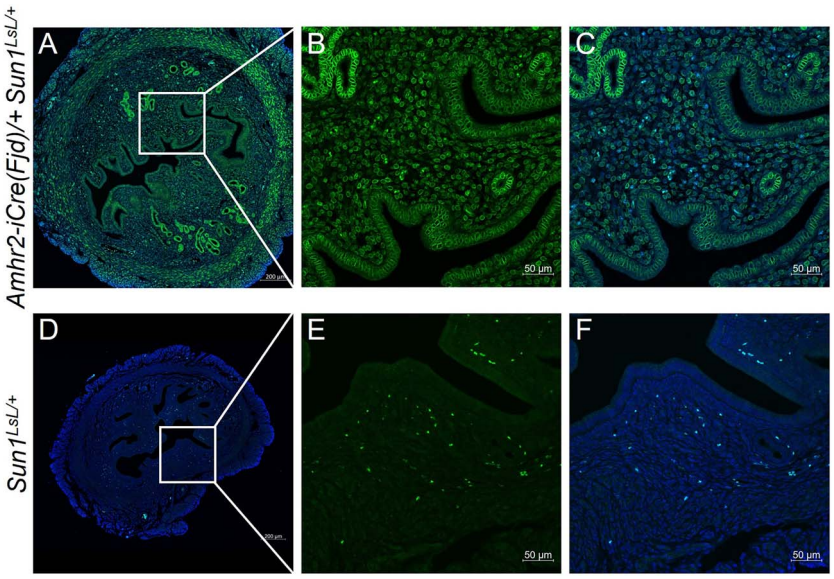

Figure 2. Amhr2-iCre(Fjd) *activity in the adult uterus.* Adult female mice ($n = 6$) aged 7 to 12 weeks were euthanized and the uterus was assessed for Amhr2-iCre(Fjd) activity and SUN1-sfGFP fusion protein expression. A cross-section of the whole uterus from an *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ mouse depicts SUN1-sfGFP expression throughout the tissue (*A*). Close-ups of the uterus show SUN1-sfGFP expression indicative of Amhr2-iCre(Fjd) activity in epithelial, stromal, and myometrial cells (*B, C*). Wild-type (Cre negative) mice did not express GFP in the uterus (*D–F*). Blue indicates nuclear staining and green is GFP.

Results

Amhr2-iCre(Fjd) mouse strain generation

To create a new Amhr2-iCre(Fjd) mouse strain, CRISPR/Cas9-based genome editing was employed to insert the codon-improved Cre recombinase ORF (iCre) into the *Amhr2* locus. The iCre ORF was placed directly after the endogenous start site in exon 1 (Figure 1), and the resulting allele should express iCre recombinase under the control of the endogenous *Amhr2* promoter in place of AMHR2 protein. Both male and female heterozygous Amhr2-iCre(Fjd)/+ mice were visually indistinguishable from wild-type litter mates.

Amhr2-iCre(Fjd) activity in the adult mouse

Cross-sections of the uterus from *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ adult mice ($n = 6$) were processed, sectioned, and analyzed for Amhr2-iCre(Fjd) activity. Amhr2-iCre(Fjd) activity was observed by the expression of the SUN1-sfGFP fusion proteins, creating peri-nuclear GFP stain. In the adult uterus, Amhr2-iCre(Fjd) activity was present in uterus of *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ mice (Figure 2A), while there was no SUN1-sfGFP fusion proteins or peri-nuclear GFP signal in the iCre negative, *Amhr2*$^{+/+}$ *Sun1*$^{LsL/+}$, mice (Figure 2D). Higher magnification of the uterus revealed iCre activity in the epithelial, stromal, and myometrial cells of the *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ uterus (Figure 2B, C) but not in the iCre negative, *Amhr2*$^{+/+}$ *Sun1*$^{LsL/+}$ adult uterus (Figure 2E, F). Apparent variation in the fluorescent intensity could be due to differential expression of sfGFP due to specific cell-cycle stages or due to immunofluorescence antibody staining. Due to the pan-uterine Amhr2-iCre(Fjd) activity and genetic recombination, we assessed other organs of the adult *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ mice and found fluorescence in multiple organs, including the testes, lung, spleen, and female reproductive tract (Supplemental Figure 1).

Amhr2-iCre(Fjd) activity in neonatal mice

Due to multiple tissues indicating Amhr2-iCre(Fjd) activity in the adult, neonatal pups ($n = 24$; 3 litters) were collected at post-natal day 1 and analyzed for Amhr2-iCre(Fjd) activity and SUN1-sfGFP expression. Pups were removed from the dam within 24 h of littering and imaged on a fluorescent dissection stereo microscope. Pups that were *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ ($n = 13$) showed fluorescence throughout their bodies (Figure 3A–C), whereas iCre negative, *Amhr2*$^{+/+}$ *Sun1*$^{LsL/+}$, pups ($n = 11$) did not fluoresce (Figure 3D). Organs, including the heart and testes, dissected from the post-natal day 1 pups exhibited fluorescence, indicating Amhr2-iCre(Fjd) activity, while organs from iCre negative, or wild-type (WT), *Amhr2*$^{+/+}$ *Sun1*$^{LsL/+}$, pups were not fluorescent (Supplemental Figure 2).

Amhr2-IRES-Cre(Bhr) activity in neonatal and adult mice

Previous reports have described the Amhr2-IRES-Cre(Bhr) mouse model to be leaky and have reported recombination in other tissues, such as the lung or the brain [5]. Cross-sections of uterus from adult *Amhr2-IRES-Cre(Bhr)/+ Sun1*$^{LsL/+}$ mice ($n = 6$) showed variability in the Amhr2-IRES-Cre(Bhr) activity (Figure 4). In some mice, there was little to no Amhr2-IRES-Cre(Bhr) activity in the luminal epithelial cells (Figure 4A), while in other mice there was substantial Amhr2-IRES-Cre(Bhr) activity present (Figure 4D). Upon closer investigation, Amhr2-IRES-Cre(Bhr) activity was consistently present in the stromal cells and glandular epithelium but variable in the luminal epithelial cells (Figure 4B, E).

To determine the origin of the variability in Amhr2-IRES-Cre(Bhr) activity in the adult uterus, neonatal pups were collected ($n = 28$; 3 litters) and analyzed for fluorescence indicative of Amhr2-IRES-Cre(Bhr) activity. Based on appearance, the pups were classified as wild-type ($n = 15$), or Cre positive ($n = 13$). Unexpectedly, there was not consistency between

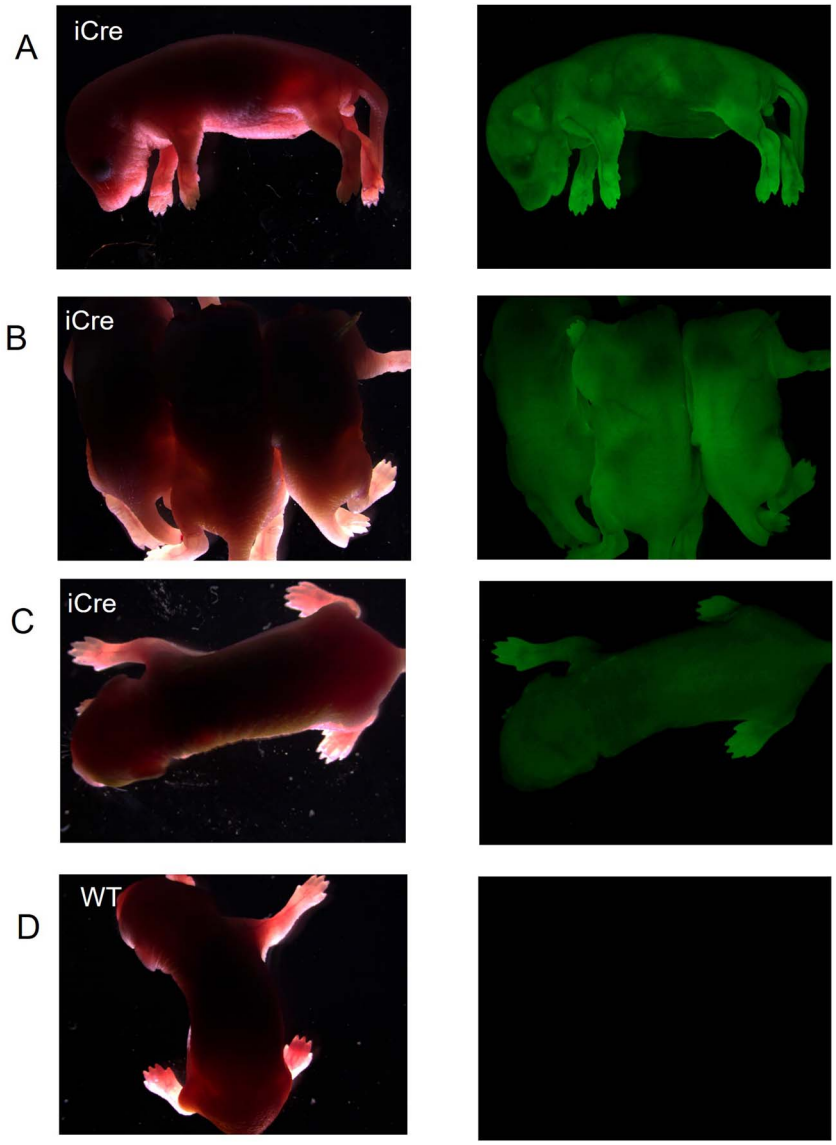

Figure 3. Amhr2-iCre(Fjd) *activity in neonatal pups.* Pups from *Amhr2-iCre(Fjd)/+* mice crossed with *Sun1*$^{LsL/LsL}$ mice were assessed the day after being born (post-natal day 1; ($n = 24$; 3 litters). Pups that were *Amhr2-iCre(Fjd)/+* positive appeared fluorescent indicative of SUN1-sfGFP expression and Amhr2-iCre(Fjd) activity (*A*–*C*). Wild-type (WT; Cre negative) mice did not appear green (*D*).

visual assessment of genetic recombination and genotype. Based on genotypes from tail snips, there were 10 wild-type and 18 Cre positive pups. These data indicate that pups that were genetically positive for Amhr2-IRES-Cre(Bhr) varied in their fluorescence, and therefore genetic recombination (Figure 4G, H). Further, organs dissected from *Amhr2-IRES-Cre(Bhr)/+Sun1*$^{LsL/+}$ mice, including the heart, testes, and intestines, showed variability in their green fluorescence (Supplemental Figure 2).

Amhr2-iCre(Fjd) and Amhr2-IRES-Cre(Bhr) activity in the early embryo

Due to the variation seen in the Amhr2-IRES-Cre(Bhr) mouse model and the consistency seen in the Amhr2-iCre(Fjd) mouse model at post-natal day 1, we performed embryo culture to determine how early Amhr2-IRES-Cre(Bhr) or iCre activity occurred. *Sun1*$^{LsL/LsL}$ mice were super-ovulated and mated with either *Amhr2-iCre(Fjd)/+* or *Amhr2-IRES-Cre(Bhr)/+* males and putative zygotes were collected and placed in culture at 0.5 days post coitus. *Amhr2* expression has been reported as early as the two-cell embryo [6, 10], and we found Amhr2-iCre(Fjd) activity was present in some two-cell embryos, as indicated by the expression of peri-nuclear SUN1-sfGFP fusion protein. Consistently, there was SUN1-sfGFP fusion protein expression in the four- to eight-cell embryo in embryos from *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ and *Amhr2-IRES-Cre(Bhr)/+ Sun1*$^{LsL/+}$ breeders (Figure 5). Embryos from *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ consistently had SUN1-sfGFP expression in all cells of the early four- to eight-cell embryo, whereas embryos from *Amhr2-IRES-Cre(Bhr)/+ Sun1*$^{LsL/+}$ breeders expressed SUN1-sfGFP proteins in a portion of cells in some embryos and all cells in other embryos (Figure 5). Blastocysts from *Amhr2-iCre(Fjd)/+ Sun1*$^{LsL/+}$ and *Amhr2-IRES-Cre(Bhr)/+ Sun1*$^{LsL/+}$ breeders expressed SUN1-sfGFP in both the trophectoderm and inner cell mass. Expression of SUN1-sfGFP is evidence of genetic recombination in the early embryo which will give

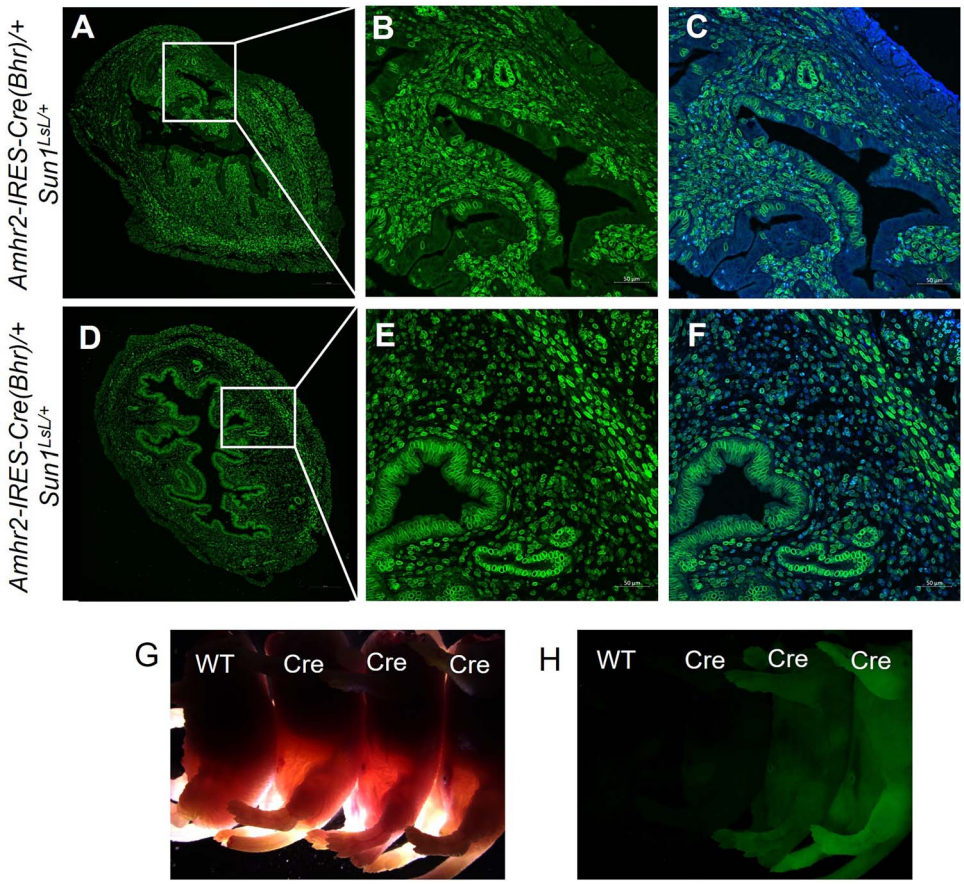

Figure 4. Amhr2-IRES-Cre(Bhr) activity is variable in adult tissues and neonatal pups. Adult female mice ($n = 6$) aged 7–12 weeks were euthanized and the uterus was assessed for Amhr2-IRES-Cre(Bhr) activity and SUN1-sfGFP expression. Uteri from *Amhr2-IRES-Cre(Bhr)/+ Sun1*$^{LsL/+}$ mice had variation in the expression of SUN1-sfGFP fusion protein with some mice lacking epithelial recombination (*A–C*) and some mice having strong recombination in epithelial, stromal, and myometrial cells (*D–F*). Blue indicates nuclear staining and green is GFP. To assess Amhr2-IRES-Cre(Bhr) activity in neonatal mice, pups ($n = 28$; 3 litters) were collected at post-natal day 1 from *Amhr2-IRES-Cre(Bhr)/+* male mice mated with *Sun1*$^{LsLLsL+}$ females. There was wide variation of fluorescence in *Amhr2-IRES-Cre(Bhr)/+* pups (*G*). Wild-type (Cre negative) mice did not appear green (*H*).

rise to global modification in the mouse. Embryos from *CD1 Sun1*$^{LsL/+}$ breeding pairs did not have SUN1-sfGFP expression (Supplemental Figure 3).

Discussion

The power of expressing Cre recombinase under the control of cell- or compartmental-specific genes allows scientists to better investigate the role of cell-to-cell communication in a specific organ. The uterus consists of several cellular compartments: the luminal epithelium, the glandular epithelium, the stroma, the myometrium, and vascular cells. Cre mouse models that are used to study the female reproductive tract include the progesterone receptor (*Pgr*) and lactoferrin (*Ltf*) Cre mouse models. PgrCre mice are a useful tool to genetically manipulate a gene of interest in the major uterine cell types (epithelial, stromal, myometrial), and LtfCre mice are useful for genetic recombination in epithelial cells [11, 12]. Previously, Amhr2-IRES-Cre(Bhr) was considered specific to the mesenchymal cells, including stromal and myometrial cells within the uterus [13], despite reports of recombination in other cells, including the uterine epithelium [14]. These reports of leakiness or patchiness have been attributed to the limitations of the expression of the Cre recombinase. Here we demonstrate that insertion of a Cre recombinase modified to improve expression, iCre, into the *Amhr2* gene increased the robustness of genetic recombination in cells expressing *Amhr2*. The difference between the new Amhr2-iCre(Fjd) and the original Amhr2-IRES-Cre(Bhr) mice recombination activity is not likely due to variable activity of the Amhr2 promoter between the two Cre strains since both are knock-in alleles using the same promoter. The mouse-to-mouse variability observed within each strain may be partially explained by variability/leakiness of the endogenous Amhr2 promoter. However, the Amhr2-IRES-Cre(Bhr) allele relies on an IRES element for CRE protein translation from the Amhr2-Cre fusion transcript generated from the Amhr2 promoter, while the Amhr2-iCre(Fjd) allele relies on CRE translational start very similar to the endogenous AMHR2 protein. Additionally, the Amhr2-iCre(Fjd) alleles use an improved Cre (iCre) ORF. Cre recombinase is originally derived from bacteriophages, however the iCre ORF is optimized for mammalian expression instead of prokaryotic, which leads to increased levels of CRE activity and likely increases efficiency of genetic recombination of loxP flanked sequences [15]. This increase in genetic recombination revealed iCre-induced genetic recombination under the control of the *Amhr2* gene can occur in the early embryo, leading to global modification in the mouse.

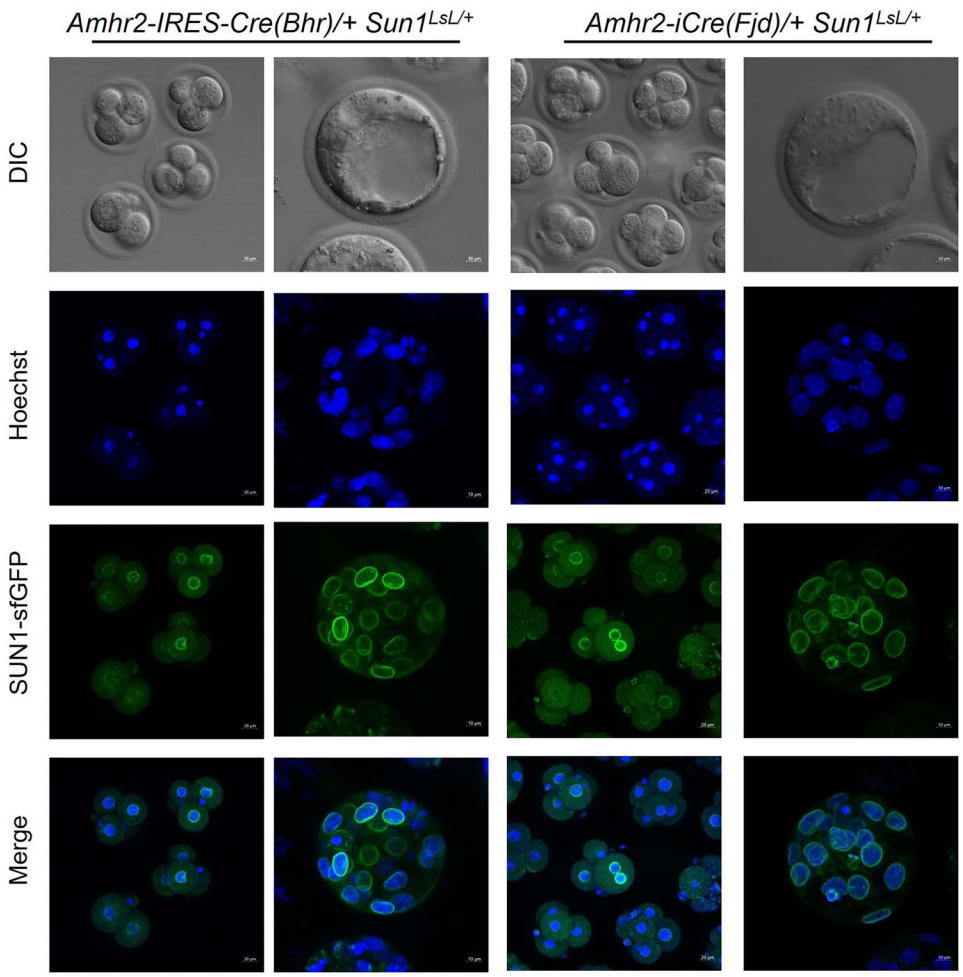

Figure 5. Embryo culture depicts Amhr2-iCre(Fjd) *and Amhr2-IRES-Cre(Bhr) activity in the early embryo.* Female *Sun1*$^{LsL/Lsl}$ mice were super-ovulated and mated with either *Amhr2-IRES-Cre(Bhr)/+* or *Amhr2-iCre(Fjd)/+* male mice. The morning after mating, female mice were euthanized, and putative zygotes were recovered and placed in culture. SUN1-sfGFP expression is apparent as early as the two- to four-cell stage in some embryos, indicating Amhr2-IRES-Cre(Bhr) and Amhr2-iCre(Fjd) activity. By the blastocyst stage, the majority of cells are SUN1-sfGFP positive, indicating Amhr2-IRES-Cre(Bhr) or Amhr2-iCre(Fjd) recombination has occurred. Genetic recombination this early in development demonstrates that Amhr2-IRES-Cre(Bhr) and Amhr2-iCre(Fjd) are not specific to the reproductive tract. Blue is a nuclear stain (Hoechst 33342), and green is the endogenous expression of sfGFP.

Initial characterization of the Amhr2-IRES-Cre(Bhr) mouse model showed Cre recombination, indicated by beta-galactosidase and X-gal staining, as early as 11.5 days post coitus [3, 16]. However, these reports did not investigate earlier timepoints in embryonic development. More recently, there have been reports documenting the temporal dynamics of *Amhr2* expression. Gene expression of *Amhr2* has been documented as early as the two-cell embryo, followed by decreased *Amhr2* gene expression at the blastocyst stage, and increased *Amhr2* expression during embryonic sexual differentiation [6, 10, 17, 18]. Due to the temporal nature of *Amhr2* expression, genetic recombination could occur as early as the two-cell embryo. In fact, there has been some work implying Amhr2-IRES-Cre(Bhr) could be active in the early embryo. *Pten* is required for embryonic development in mice [19], and previous work found high rates of pregnancy failure in *Amhr2-IRES-Cre(Bhr)/+ Ptenf/f* mice [20]. Conversely, there are discrepancies, as another report using *Amhr2-IRES-Cre(Bhr)/+ Ptenf/f* mice did not report significant pregnancy failure [21]. Moreover, there have been reports of *Amhr2* being expressed in the brain and motor neurons [22, 23], and indeed, the expression overview in the Mouse Gene Expression Database depicts *Amhr2* expression in nearly every tissue in the body [24].

Specifically in the uterus, multiple research groups have used the Amhr2-IRES-Cre(Bhr) mouse model to investigate endometrial regeneration and the hypothesis that stromal cells transition to epithelial cells in the murine uterus [25, 26]. Using the Amhr2-IRES-Cre(Bhr) model with either lacZ or a lox-stop-lox EYFP mating strategy, previous work identified mosaicism of Amhr2-IRES-Cre(Bhr) genetic modification in the uterus of mice postpartum [25, 26]. The method of assessing β-galactosidase expression as indicative of Cre activity is commonly used. However, this method requires the insertion of a *lacZ* cassette into the gene of interest, followed by collection and clearing of tissues, and counterstaining with X-gal. This enzyme-based assay requires additional steps compared with a lox-stop-lox reporter mouse, where if Cre-mediated recombination occurs, expression of EYFP is initiated. It is important to note that each experiment was terminal for the individual mouse, so within a mouse, the Cre activity cannot be examined pre- and post-partum. These results must

be interpreted with caution or potentially repeated using different models to ensure the results are not an artifact of the transgenic mouse model.

Our data, in agreement with previously published work [6], demonstrate that Cre or iCre under the *Amhr2* promoter is active in the early embryo and can induce recombination in undesired cells thereby rendering the Amhr2-iCre(Fjd) mouse model being a global knock-out mouse and the Amhr2-IRES-Cre(Bhr) mouse model inconsistently being a global knock-out mouse. In this study, we utilized the CAG-Sun1sfGFP reporter mouse to test both Amhr2-iCre(Fjd) and Amhr2-IRES-Cre(Bhr) recombination efficiency. However, there is a possibility that the *Sun1* locus is easier to genetically edit compared with other genes. Therefore, it is possible there could be more tissue or cell specificity when using either Amhr2-iCre(Fjd) or Amhr2-IRES-Cre(Bhr) mouse depending on the targeted gene.

In conclusion, Amhr2-iCre(Fjd) mice have genetic recombination occurring in the early embryo, leading to a global modification. This enhancement of a previous mouse model, Amhr2-IRES-Cre(Bhr), explains why previous studies reported genetic recombination in other organs in addition to the original cell types considered altered by Amhr2-IRES-Cre(Bhr). Evidence herein has revealed the *Amhr2* promoter is not as specific as originally thought, and further solidifies the importance of evaluating global Cre activity and genetic recombination when new transgenic Cre mouse models are created. Scientists should consider this work and exercise caution when interpreting results from experiments that have utilized Amhr2-IRES-Cre(Bhr) mice. Further, investigators should be wary to use this mouse model for future studies.

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Supplementary material

Supplementary Material is available at *BIOLRE* online.

Conflict of interest

The authors have declared that no conflict of interest exists.

Author contributions

MJD collected samples, performed analysis, and wrote the manuscript. AG created the transgenic mice and revised the manuscript, and FJD designed the project and revised the manuscript.

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

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