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Generation and characterization of an Endothelin-2 iCre mouse

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Abstract

A novel transgenic mouse line that expresses codon-improved Cre recombinase (iCre) under regulation of the Endothelin-2 gene (*edn2*) promoter was developed for the conditional deletion of genes in Endothelin-2 lineage cells and for the spatial and temporal localization of Endothelin-2 expression. Endothelin-2 (EDN2, ET-2, previously VIC) is a transcriptionally regulated 21 amino acid peptide implicated in vascular homeostasis, and more recently in female reproduction, gastrointestinal function, immunology, and cancer pathogenesis that acts through membrane receptors and G-protein signaling. A cassette (*edn2*-iCre) was constructed that contained iCre, a polyadenylation sequence, and a neomycin selection marker in front of the endogenous start codon of the *edn2* gene in a mouse genome BAC clone. The cassette was introduced into the C57BL/6 genome by pronuclear injection, and two lines of *edn2*-iCre positive mice were produced. The *edn2*-iCre mice were bred with ROSA26-lacZ and Ai9 reporter mice to visualize areas of functional iCre expression. Strong expression was seen in the periovarian ovary, stomach and small intestine, and colon. Uniquely, we report punctate expression in the corneal epithelium, the liver, the lung, the pituitary, the uterus, and the heart. In the embryo, expression is localized in developing hair follicles and the dermis. Therefore, *edn2*-iCre mice will serve as a novel line for conditional gene deletion in these tissues.

Keywords

Endothelin-2; Cre recombinase; Transgenic; Ovary; BAC Clone

INTRODUCTION

The Endothelin system is made of three 21 amino acid peptides (EDN1, EDN2, EDN3), two receptors (EDNRA and EDNRB), and several activating peptidases including ECE1 and ECE2. The system is highly conserved throughout mammals, and within vertebrates as a whole (Braasch and Scharf, 2014). Though EDN2 (also seen as ET-2, previously vasoactive intestinal contractor VIC) differs from EDN1 by two amino acids and has the same receptor

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affinities, and differs from EDN3 by six amino acids and has the same affinity to EDNRB, there now exists a substantial body of evidence suggesting that EDN2 acts through unique regulation and synthesis from its familial isoforms (Ling *et al.*, 2013). Previously, approaches using Cre/loxP-mediated recombination have demonstrated the importance of EDN1, EDN3, ENDRA, and EDNRB in mice, though with phenotypes unique from newly generated EDN2 deficient mice. It is now apparent that EDN2 has a unique role within the Endothelin system in female reproduction (Ko *et al.*, 2012), and there is also strong evidence that it is involved in gastrointestinal function (Chang *et al.*, 2013b; Takizawa *et al.*, 2005), immune cell function (Grimshaw *et al.*, 2002b), heart failure (Brown *et al.*, 2000), and various cancers (Ling *et al.*, 2013). It has been previously demonstrated that *edn2* is expressed at low levels in the cerebellum, cerebrum, and lung; that it is more highly expressed in the testes and stomach, and it is particularly highly expressed in the intestines, ovary, and uterus (Uchide *et al.*, 2002; Uchide *et al.*, 1999). Embryonic expression begins by embryonic day 15 (e15) and persists until at least e17 (Chang *et al.*, 2013b; Uchide *et al.*, 1999). Global loss of *edn2* is lethal, wherein mice expire around three weeks of age from internal starvation, hypothermia, and emphysema (Chang *et al.*, 2013b) and also exhibit ovulatory defects (Cacioppo *et al.*, 2014), thus demonstrating a critical functional role in the brain, lungs, GI tract, and ovary. Although a floxed *edn2* mouse became recently available to selectively ablate *edn2* (Rattner *et al.*, 2013), no tool has yet been available to remove genes in those cells with *edn2* expression. Thus we aimed to generate a novel mouse model that expresses codon-improved Cre recombinase (iCre) driven by the natural promoter for the *edn2* gene. Two lines of *edn2*-iCre mice were produced and the sites of functional iCre expression and, presumably, localization of endogenous *edn2* expression was determined using two lines of reporter mice: ROSA26-lacZ (Soriano, 1999) and Ai9 (Madisen *et al.*, 2010).

RESULTS AND DISCUSSION

Little is known of the regulation of *edn2*, both from the transcriptional and peptide conversion perspectives. Although *edn1* has been shown to be largely transcriptionally regulated through cis-acting promoter elements, and *edn2* was thought to have similar regulation, it has been shown previously to be regulated by transcription factors such as epidermal growth factor, TNF- α , forskolin, HIF1- α , and pituitary gonadotropins within the reproductive system (Ko *et al.*, 2012; Palanisamy *et al.*, 2006). Hence a strategy that encompassed all potentially involved regulatory DNA regions was mandated to ensure correct mimicry of *edn2* expression by Cre recombinase.

To this end, we chose to use a bacterial artificial chromosome (BAC) clone that contained the entire *edn2* gene and neighboring sequences as the vector for iCre insertion (Figure 1). A cassette containing iCre, a polyadenylation sequence, and a neomycin selection marker was inserted in front of the ATG start codon of the *edn2* gene (Figure 1). Following removal of the neomycin selection marker by FLP-mediated recombination, the entire 200Kbp vector was inserted into the C57BL/6 genome by pronuclear injection. Sixty-nine pups were produced, of which 12 contained the *edn2*-iCre vector in their genome (Figure 1). Copy number was not evaluated. Two of these 12 *edn2*-iCre positive mice, *edn2*-iCre#9 and *edn2*-iCre#12, showed no health issues or major fertility defects and were chosen for follow-up

characterization of iCre expression. To validate that iCre expression mimics *edn2* expression in *edn2*-iCre mice, we examined the ovaries of 28 day old mice following gonadotropin stimulation to induce ovulation. Expression of *edn2* specifically occurs between 11 and 12 hours after hCG injection (Ko *et al.*, 2006; Palanisamy *et al.*, 2006), and we expected iCre expression to be limited to this time frame. Ovaries were examined at 0, 6, 12, 16, and 24 hours after hCG injection for iCre RNA in wild type (WT) mice and the two founder lines of transgenic mice. As expected, both founder lines demonstrated iCre expression only 12 hours after hCG injection concurrent with their own and WT *edn2* expression (Figure 2). The gene *Rpl19*, encoding murine ribosomal 60S protein L19, was chosen as an internal control based on previous publications (Al-Bader and Al-Sarraf, 2005; Szabo *et al.*, 2004). From these data, we parsimoniously conclude that iCre expression mimics *edn2* temporally in the ovary, and that in addition to removing floxed genes at the times and locations of *edn2* expression, *edn2*-iCre tissues can also be used to localize *edn2* expression sites.

To typify expression, transgenic mice were crossed with ROSA26-lacZ reporter mice, which express β -galactosidase under a universal promoter with a floxed stop codon (Soriano, 1999). Both founder lines demonstrated similar staining patterns with X-gal. Of these, *edn2*-iCre#9 was further characterized for localization of *edn2* expression. This line was chosen because it provided an average number of healthy pups per litter for C57BL/6 mice, approximately eight, while line *edn2*-iCre#12 averaged only four pups per litter. All results described below, using either reporter mouse, are from this single founder line; other lines were euthanized and frozen sperm was stored for potential restoration of the line if necessary. Of note, positive staining marks cells that are expressing or have expressed *edn2*, or are descended from cells that have expressed *edn2*. No phenotypes are associated with homozygosity for the transgene insertion in these animals.

Pregnant mice were sacrificed and embryos of varying days of development were analyzed by X-gal staining. Positive staining was not present prior to day e12.5 (Figure 3), though placental tissue stained positively past day e8.5 including the umbilicus (data not shown). By day e13.5, faint positive staining was visible in the skin of the embryos; staining was present in a punctate pattern throughout the skin by day e15.5, and was readily apparent in the sinusoid hair follicles of the rostrum (whiskers). This skin staining consistently was present in the outer layer of cells of the developing dermis. Staining was also present on the surface of the digits of fore and hind limbs, the dorsum, and the tail. By day e17.5 near parturition, staining had expanded throughout the skin of the ventrum and was consistently present in the skin surrounding areas of the eye, mouth, and ear openings, and between digits (Figure 3). Staining eventually expands throughout the entirety of the skin by puberty. It is interesting to note early staining presence in those areas that must undergo early keratinization for function in neonates (Findlater *et al.*, 1993), though these areas coincide with those of hair follicle development. This timing of expression is consistent with earlier findings that concluded EDN2 is important for normal embryonic development (Saida *et al.*, 2000; Uchida *et al.*, 2000a).

Twenty-eight day old healthy mice were sacrificed and individual organs previously implicated to be involved in EDN2 signaling were stained with X-gal to localize *edn2* expression. In the majority of the organs examined, staining revealed a punctate pattern

(Figure 4). Brains demonstrated intense staining around the olfactory bulb, although individual neurons stained positively throughout the cerebrum and cerebellum (cerebellum histology pictured). Heart staining revealed that the majority of the epicardium expressed *edn2*, though only a few scattered cardiomyocytes and endothelial cells stained positively in a seemingly random arrangement, complimenting previous findings (Kakinuma *et al.*, 1999; Plumptre *et al.*, 1993). A similar stippled pattern was visualized in the lung throughout the pneumocytes of the parenchyma and the respiratory epithelium. In the kidney, staining was present in only some renal corpuscles and tubules, although staining was limited to the cortex and not present in the medulla. However, cells expressing iCre in the kidney may become more prevalent during renal stress or disease (Bot *et al.*, 2012; Miyauchi *et al.*, 2012). Interestingly, the entirety of the epidermis stained positively, as well as the cells surrounding the hair follicles and the inner root sheath of the follicles themselves. Beyond hair follicles (Chang *et al.*, 2013a), this is a novel area of EDN2 expression, and presents a potential unique application for future gene removal. In the gastrointestinal tract, cross-sectional staining was limited to individual villi or to individual tubular glands, and was also present in patches on the exterior surface, which may be a reflection of the enteric nervous system as Endothelins are known to be involved in neural crest cell migration (Kunieda *et al.*, 1996). Ovarian staining was highly prevalent, present throughout the organ stroma, capsule, and small follicles, in addition to large follicles and corpora lutea as previously reported (Ko *et al.*, 2006; Palanisamy *et al.*, 2006). Punctate staining was present in the epithelium of the oviduct, while individual myofibers of uterine myometrium stained to create a stippled appearance.

X-gal staining may occur as a false positive in adult tissues through endogenous beta-galactosidase activity (Burn, 2012; Odgren *et al.*, 2006), and may additionally create 'edge-effect' artifact staining on any tissue. Although the majority of tissues of interest showed little or no negative staining in mice lacking the *edn2*-iCre mutation, false positive staining was present in the gross view of the kidney, GI tract, portions of the uterus, and exterior of the skin (Figure S1) though little staining was present histologically. Additional false positive staining was present in the bone, adrenal, tarsal gland, and liver tissue (not pictured), though decreasing incubation temperature significantly reduced gross background x-gal staining. Similarly, control embryos showed no x-gal staining at any age except for slight edge-effect staining of the skin which does also not appear histologically (Figure S1).

To verify the sites observed as positive for X-gal staining and to identify other novel sites of *edn2* expression, *edn2*-iCre mice were crossed with Ai9 reporter mice that produce red fluorescent protein in cells with functional iCre expression. Tissues from 28 day old males and females were visualized grossly under fluorescent light during dissection, and histological images were compared with H&E staining (Figure 5). Of interest, fluorescence was seen in the anterior and intermediate pituitary, the cornea and photoreceptors of the eye, the detrusor muscle of the bladder, the male coagulation gland (anterior prostate), and the interstitium of the testes. The majority of expression appears in a punctate manner, excluding the epidermis, ovary, testicular interstitium, and coagulation gland. These areas are potential key areas for the utilization of the *edn2*-iCre transgenic mouse outside of localization of *edn2* expression and removal of potential downstream Endothelin target

genes. Eye expression is particularly interesting, given recent work by Rattner et al. showing that EDN2 can override VEGF signaling to inhibit retinal angiogenesis (Rattner *et al.*, 2013). Expression of *edn2* may act in the cornea and retina continually to inhibit angiogenesis, whereas loss of *edn2* could eventually lead to vascularization and visual impairment. A summary of fluorescence observed grossly and histologically is listed in Table 1. No fluorescence was observed in control tissues.

METHODS

Ethics Statement

This study was carried out in tight accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocol was approved by the University of Illinois Animal Care and Use Committee (Protocol: 11184), and all efforts were made to minimize animal suffering. Animal models generated in this study will be made readily available to the research community.

Construction of the iCre-polyA-FRT-neo-FRT cassette

E. coli SW105, pL253, pL451, and pICGN21 were obtained from Frederick National Laboratory for Cancer Research (Frederick, MD <http://ncifrederick.cancer.gov/>). An iCre-polyA-FRT-neo-FRT cassette was constructed by cloning the following DNA fragments between HindIII restriction site and AatII restriction site of pGEM7Zf(+) (Promega, Madison, WI): HindIII-EcoRI fragment (1100 bp) containing the coding region of the codon-improved Cre recombinase (iCre) from pBluescript KS(+)-iCre, EcoRI-KpnI fragment (283 bp) containing SV40 late polyadenylation signal sequence from pCS2+MT, KpnI-SfiI fragment (1500 bp) containing FRT-neo-FRT from pICGN21 and SfiI/AatII adaptor sequence (5'-CGGCCCTGATCAGTGCTAGCGACGT-3' annealed with 5'-CGCTAGCACTGATCAGGGCCGCCT-3').

Insertion of iCre-polyA-FRT-neo-FRT cassette into the *edn2* BAC clone

The iCre-pA-FRT-neo-FRT cassette was inserted into exon 1 of the *edn2* gene of the BAC clone (ID RP23-98J9) that was purchased from Invitrogen (Carlsbad, CA). Recombineering was performed according to protocols provided by Frederick National Laboratory for Cancer Research (Jahrling *et al.*, 2014; Parkitna *et al.*, 2009; Thomason *et al.*, 2014). Briefly, the iCre-polyA-FRT-neo-FRT cassette was inserted in front of the ATG initiation codon in the BAC plasmid (Gebhard *et al.*, 2007). A homology arm (upstream from ATG) was amplified using primer pairs (5'-ACGCGTTCCTGAAGGTGTTGCAGAGAA-3', 5'-AAGCTTAGCAGCAGCGGCAGAGTG-3') and cloned between the MluI site and the HindIII site upstream of iCre-polyA-FRT-neo-FRT cassette. Another homology arm downstream from ATG was amplified using primer pairs (5'-GGCCGAGGCGGCCATGGTCTCCGCCTGGTGT-3', 5'-GACGTCCCTTGGTGTTCAGGAACCAC-3') and cloned the between SfiI site and the AatII site after the cassette. The cassette with the two homologies was cut with MluI and AatII and gel purified. Approximately 15ng of the gel-purified cassette was electroporated into red-induced *E. coli* SW105 carrying the BAC clone. The recombinants were selected on LB agar plates supplemented with kanamycin (25 µg/ml). The selected bacterial cells were

then treated with arabinose (0.1%) for one hour to delete FRT-neo-FRT from the BAC. The recombinant BAC plasmid purification, plasmid integrity test, and pronuclear microinjection were performed at the Transgenic Animal Model Core of University of Michigan. Fertilized eggs from C57BL/6 mice were microinjected with the BAC plasmid and implanted into pseudopregnant foster mothers (C57BL/6). Genomic DNA was extracted from the tails of 69 offspring from the pseudopregnant mothers. The presence of iCre was determined by PCR using the following primer pairs: Cre-F (5'-TCTGATGAAGTCAGGAAGAACC-3') and Cre-R (5'-GAGATGTCCTTCACTCTGAATC-3') (Bridges *et al.*, 2008).

RT-PCR

Of those offspring that had correct construct insertions, founder lines #9 and #12 were used for further evaluation for ovarian *edn2* expression. It is known *edn2* is induced during ovulation; thus female 28-day old pre-pubertal *edn2*-iCre mice of these lines and control WT mice were injected in the intra-peritoneal cavity with 5 IU of pregnant mare serum gonadotropin (PMSG) at 25 days old to induce follicle development, and then 48 h later with 5 IU of human chorionic gonadotropin (hCG) to induce ovulation. Mice ovulate 12–14 hrs after hCG injection, and ovaries were collected at 0, 6, 12, 16, and 24 hrs after hCG injection (n=5). Animals were euthanized by CO₂ asphyxiation and cervical luxation, ovaries were removed, and homogenized in Trizol solution (Life Technologies, Grand Island, NY) for RNA extraction. RNA was purified with an RNeasy kit (Qiagen, Germantown, MD), and cDNA was generated using a reverse transcription superscript VILO synthesis kit (Invitrogen). For RT-PCR, *iCre* was amplified using the above primer set; *edn2* was amplified with (5'-CTCCTGGCTTGACAAGGAATG-3') and (5'-GCTGTCTGTCCCGCAGTGTT-3'), and *L19* was amplified as a control housekeeping gene with (5'-CCTGAAGGTCAAAGGGAATGTG-3') and (5'-GTCTGCCTTCAGCTTGTGGAT-3'). PCR products were visualized on an agarose gel; band intensity was analyzed using ImageJ software (NIH, Bethesda, MD) for relative intensity.

Visualization of Transgene Expression

Animals were bred with B6;129S4-*Gt(Makinoda et al.)26Sor^{tm1Sor}/J* (ROSA26-lacZ) reporter mice to visualize *edn2*-expressing tissues by X-gal staining (Soriano, 1999). Mice were euthanized by CO₂ asphyxiation; perfused intracardially with cold PBS solution and then a 4% paraformaldehyde (PFA) solution to assist in fixation. Tissues were then collected and washed 2 times with cold PBS; large and lipid-dense tissues (brain, liver, lungs, heart, stomach, kidney, tubular organs) were cut into sections with a scalpel to allow better fixative penetration. Tissues were placed in 4% PFA on ice for 1 hour. They were then washed twice with PBS and placed into individual vials with X-gal stain solution (Millipore, Billerica, MA), diluted 1:40 according to the manufacturer's instructions. Tissues were incubated in the dark in at 4°C on a shaker for 24–48 hours, washed 3 times with PBS, incubated in the dark at room temperature for 1 hour with X-gal holding solution, and then fixed overnight in 4% PFA at 4°C. Embryos were similarly fixed and stained. Gross images of tissues were taken of tissues following this final fixation. Tissues were then dehydrated, embedded in paraffin blocks, and sectioned by microtome at 5µm thickness. Background staining was performed with nuclear fast red (Fisher Scientific, Pittsburgh, PA) and images were taken

with an Olympus BX51 microscope. The *edn2-iCre* animals were also bred with B6;129S6-*Gt(Makinoda et al.)26Sor^{tm9(CAG-tdTomato)Hze/J}* (Ai9) reporter mice (Madisen *et al.*, 2010). Gross images were taken immediately after euthanasia under a Zeiss SV11 fluorescent microscope. Tissues were fixed overnight, embedded, and serially sectioned at 5µm; one section was used for fluorescence visualization and an adjacent slide was stained with hematoxylin and eosin (Thermo Scientific, Kalamazoo, MI).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Transgenic vector construct and screening of *edn2*-iCre transgenic mouse

(a) Schematic diagram of a part of chromosome 4 and the *edn2*-iCre BAC transgenic vector. *Edn2* and its neighboring genes are shown. Exons are shown in blue boxes. Positions of PCR primers for screening transgenic mice are indicated by horizontal black arrows. The cassette containing the coding region of iCre, SV40 late polyadenylation signal, and frt-neo-frt cassette was inserted in front of ATG of *edn2* by homologous recombination. The frt-neo-frt cassette was then deleted by Flp recombinase. (b) Screening of transgenic mice carrying the transgene *iCre* (a representative gel image). The transgene was inserted into the mouse genome by pronuclear injection to C57B/6 blastocysts. Lanes 1–8: founder mouse template genomic DNA (gDNA) from one litter; Lane 9: iCre sequence positive control; Lane 10: WT gDNA negative control; Lane 11: No template DNA negative control; Lane 12: 100bp ladder (Invitrogen).

Figure 2. Temporal mRNA expression of *Edn2* and *iCre* in the *edn2-iCre* mouse ovary
 Twenty-five day old *edn2-iCre9* and *Edn2iCre12* mice were injected with PMSG/hCG for superovulation induction. Ovaries were collected at the indicated hours (h) after hCG injection, and mRNA expression levels were measured by semi-quantitative RT-PCR. Shown is a representative image of n=5 mice of each genotype. Note the temporal expression pattern of *Edn2* and *iCre* mRNA. *L19* was used as internal control. Bar graph indicates quantification at 12 hours after hCG administration and was normalized to *L19*. No significant differences were present between groups for *edn2* expression.

Figure 3. X-Gal characterization of *edn2-iCre* embryos

(A) X-Gal staining detects iCre expression and successful recombination in whole mount embryos of *edn2-iCre* mice crossed with ROSA26-lacZ reporter mice. Faint positive staining is consistently observed as early as e12.5 in the skin of the ventrum. It becomes noticeable throughout much of skin in a fragmented pattern by day e15.5. (B) Higher magnification of dissecting scope images of *edn2-iCre* fetuses at approximately embryonic day e15.5 after X-Gal staining. Images show a) rostrum, b) dorsal skin and tail, c) whole-mount histological staining 1.25 $\times$, and d) magnified histological staining of skin of embryo

40×. Staining of sinus follicles of rostrum is visible by day e15.5, while wide-spread staining of ventrum begins one to two days later. By day 17.5, staining is also visible at opening of mouth, eyes, and ears, and between digits. Closed arrowheads: sinus hair follicles; open arrows: staining in developing dermis/epidermis.

Figure 4. Localization of functional iCre expression in adult *edn2*-iCre mouse organs

X-Gal staining detects iCre expression and successful recombination in whole organs of *edn2*-iCre mice crossed with ROSA26-lacZ reporter mice that have previously been shown to produce EDN2 peptide. Gross images are shown at left and histology images with nuclear fast red background staining are at right. Kidney tissue was bisected, ovary was punctured, and heart and colon were flushed prior to staining. The oviduct is visible in the bottom portion of the gross ovarian image. Symbol labeling is as follows: Brain: open arrowhead: olfactory bulb; open arrow: molecular layer; closed arrow: granule cell layer; solid arrowhead: purkinje cell layer. Heart: open arrowhead: epicardium; closed arrowhead: cardiomyocyte. Lung: solid arrow: pneumocytes of alveoli; open arrow: respiratory epithelium of bronchiole. Kidney: open chevron: cortex; closed chevron: medulla; closed arrowhead: renal corpuscle; open arrowhead: tubules of cortex. Skin: solid arrow: epidermis; open arrow: bulge stem cells; closed chevron: dermis. Colon: open arrowhead: muscularis interna; closed arrowhead: goblet cells of longitudinal mucosal fold. Ovary: closed arrowhead: oviduct; open arrowhead: oocyte of secondary follicle; open arrow: secondary follicle granulosa cells; closed arrow: theca cell layer and stromal cells. Uterus: solid arrowhead: endometrium; open arrowhead: uterine glands; open arrow: smooth muscle cells of myometrium.

Figure 5. Localization of iCre expression by red fluorescence in adult *edn2-iCre* mouse organs
Edn2-iCre mice were crossed with *Ai9* reporter mice for rapid visualization. Fluorescence intensity at low magnification (row 2) correlates with the number of *edn2-iCre*-expressing cells. Red fluorescence indicates areas of cell lineage expression of iCre (row 4). Top rows: gross images and fluorescence comparison. Bottom rows: histological images and fluorescence comparison. A–D: pituitary (female, PND28); E–F: Eye; G–H: Cornea of eye; I–L: Bladder (detrusor muscle); M–N: Gastrointestinal tract; O–P: Jejunum; Q–R: Male reproductive organs; S–T: Seminiferous tubules; U–V: Female reproductive organs; W–X: Ovary. Symbols are used to mark specific organs or cell types. Image A, B, C, D: solid arrow: anterior pituitary (adenohypophysis); open arrow: posterior pituitary (neurohypophysis). Image G, H: Solid arrowhead: corneal epithelium (anterior epithelium, outside of eye). Image K, L: solid arrow: smooth muscle cells of detrusor muscle (3 layers); open arrow: transitional epithelium of bladder facing lumen. Image M, N: Solid arrow: small intestines; open arrow: liver; solid arrowhead: large intestines. Image O, P: Solid arrow head: luminal epithelium of jejunum. Image Q, R: solid arrow: seminal vesicle; open arrow: ductus (vas) deferens; solid arrowhead: coagulation gland (anterior prostate); open arrowhead: testis; solid chevron: epididymis; open chevron: bladder. Image S, T: solid arrow: interstitial cells of testes. Image U, V: Solid arrowhead: ovary; open arrowhead: oviduct; open chevron: uterus. Image W, X: Solid arrowhead: secondary follicle; solid arrow: antral follicle.

Table 1

Summary of Cre expression in multiple adult organs as determined by localization of red fluorescence after crossing with an Ai9 reporter mouse line.

Tissue	Relative Fluorescence	Notes	Relevant Previous Literature
Adrenal	+++	Punctate fluorescence throughout adrenal cortex; little to none in medulla	(Davenport <i>et al.</i> , 1996)
Aorta	+	Very limited punctate fluorescence in tunica media in smooth muscle cells	(Bacon and Davenport, 1996; Howard <i>et al.</i> , 1992; O'Reilly <i>et al.</i> , 1993a; Yanagisawa <i>et al.</i> , 1988)
Bladder	+	Punctate fluorescence in smooth muscle cells of detrusor muscle, not in epithelium	–
Blood (circulating)	–	No fluorescence seen (in all RBC and WBCs observed)	(Grimshaw <i>et al.</i> , 2002a; Grimshaw <i>et al.</i> , 2002b)
Bone (femur)	–	No fluorescence seen in osteocytes or bone marrow	(Briggs <i>et al.</i> , 1998)
Brain	+	Mild fluorescence throughout cortex and cerebellum, extremely limited, 1–2/100 neurons. More intense in hypothalamus and optic chiasm. Greatest fluorescence in olfactory bulb.	(Masuo <i>et al.</i> , 2003; Saida <i>et al.</i> , 2002)
Coagulation gland (anterior prostate)	+++	Fluorescence seen in all secretory cells	–
Colon	+++	Punctate fluorescence throughout mucosa; cells of mucosa will either all fluoresce or not within one tubular gland (all or none expression by gland); not present in submucosa or muscularis; punctate pattern seen in mesothelial cells of serosa.	(Chang <i>et al.</i> , 2013b; McCartney <i>et al.</i> , 2002; Wang <i>et al.</i> , 2013)
Duodenum	+++	Fluorescence in groups of epithelial cells of individual villi with punctate expression of some submucosal glands; punctate serosal fluorescence present.	(Bianchi <i>et al.</i> , 2012; Chang <i>et al.</i> , 2013b; Takizawa <i>et al.</i> , 2005)
Epididymis	±	0–1 cells/100 of epithelial cells in lumen of epididymis	–
Esophagus	–	No fluorescence visualized	–
Eye	+	Punctate/striped fluorescence in most outer cellular layer of corneal epithelium; fluorescence in photoreceptor layer with fluorescence extending through all retinal layers in striped pattern in limited areas of retina approx. 0–1 per section, fluorescence in tarsal gland, no fluorescence in lens or iris	(Bramall <i>et al.</i> , 2013; Braunger <i>et al.</i> , 2013; Rattner <i>et al.</i> , 2013)
Heart	+	Punctate fluorescence in cardiomyocytes (2–5/100) and the majority of epicardial cells	(Kakinuma <i>et al.</i> , 1999; O'Reilly <i>et al.</i> , 1993b; Plumpton <i>et al.</i> , 1996; Plumpton <i>et al.</i> , 1993; Uchida <i>et al.</i> , 2000a)
Ileum	+++	Fluorescence seen in villi similar to duodenum – punctate in groups of cells and often near crypts, fluorescence in individual cells within Peyer's patches (1–2/100), fluorescence in serosa cell layer in patches	(Bloch <i>et al.</i> , 1991; Chang <i>et al.</i> , 2013b; Fu <i>et al.</i> , 1989)
Jejunum	+++	Punctate fluorescence seen in villi and serosa similar to duodenum, ileum; may not extend entire villi if fluorescence seen in crypt	(Chang <i>et al.</i> , 2013b)
Kidney	+	Limited punctate fluorescence in a few individual cells of corpuscle or proximal tubules, entirely limited to cortex	(Hoche <i>et al.</i> , 1996; Karet and Davenport, 1996; Ohkubo <i>et al.</i> , 1990)

Tissue	Relative Fluorescence	Notes	Relevant Previous Literature
Liver	+	Punctate fluorescence seen in parenchyma, limited to cells appearing stellate	(Battistini <i>et al.</i> , 1994)
Lung	+	Moderate punctate fluorescence in respiratory epithelium; limited in alveoli/pneumocytes (0–1/100)	(Chang <i>et al.</i> , 2013b; Marciniak <i>et al.</i> , 1992; Uchide <i>et al.</i> , 2000a)
Mouth/oral cavity	+	No fluorescence in teeth, present on entirety of gingival surface	–
Nerve (thoracic vagus)	–	No fluorescence visualized	(Yuen <i>et al.</i> , 2013)
Ovary	+++	Fluorescence seen encompassing all of ovarian capsule, majority of cells of stroma, corpora lutea, and granulosa cells; not seen in oocytes	(Cacioppo <i>et al.</i> , 2014; Klipper <i>et al.</i> , 2010; Ko <i>et al.</i> , 2006; Palanisamy <i>et al.</i> , 2006; Uchide <i>et al.</i> , 2000a)
Oviduct	+++	Punctate fluorescence pattern in epithelium; more prevalent fluorescence in epithelium of isthmus	(Al-Alem <i>et al.</i> , 2007; Jankovic <i>et al.</i> , 2010)
Pancreas	+	Seemingly random punctate fluorescence throughout parenchyma, 2–5 cells/100	–
Penis	+	Seen in all cells of transitional epithelium of urethra and in the skin surrounding the prepuce	–
Pituitary	+	Punctate pattern throughout anterior and intermediate pituitary; not present in posterior pituitary	(Masuo <i>et al.</i> , 2003)
Preputial gland	+++	Fluorescence seen in the majority of acini in both basal and secretory cells	–
Rostrum	+	Visible in epidermis, in inner layer of dermal root sheath of large sinusoid hair follicles	–
Salivary gland	+	Punctate pattern present through some cells of acini; more prevalent in mucous than serous glands	(Lam <i>et al.</i> , 2004; Lam <i>et al.</i> , 1991)
Seminal vesicle	–	No fluorescence visualized	–
Skeletal muscle	–	No fluorescence visualized	–
Skin	+++	Visible throughout epidermis, extending into hair follicles	(Chang <i>et al.</i> , 2013a; Tanese <i>et al.</i> , 2010)
Testes	+	Interstitial cells, potentially including Leydig cells; not seen within seminiferous tubules	(Ergun <i>et al.</i> , 1999)
Uterus	+	Seen in some smooth muscle cells of the myometrium throughout the uterus in a banded pattern (Figure 4)	(Cameron <i>et al.</i> , 1993; Uchide <i>et al.</i> , 2000b)