



Cellular availability of *all-trans*-retinoic acid in mice: a pilot study

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ABSTRACT

Retinoic acid signaling is essential for mammalian development and health. The genetic manipulations that enable visualization of this signal have revealed its involvement in many organs, including the eyes, colon, and trunk regions of mouse embryos. The *in vivo* cellular availability of the ligand, *all-trans*-retinoic acid (ATRA), is, however, still unclear, even though it, along with receptor expression, constitutes an essential part of signal transduction. In this pilot study, we sought to identify cells with ATRA availability by introducing a fusion protein comprising the mouse retinoic acid receptor alpha ligand-binding domain and the yeast Gal4 DNA-binding domain in mice. By crossing with other reporter mice with Gal4-responsive Cre expression and subsequent tandem DsRed expression to track ATRA availability through adulthood, we labeled the *in vivo* cellular availability of ATRA in two out of 14 mice from three F1-generation lines. As expected, we observed prior ATRA availability in organs associated with this signaling pathway, including the eye and colon. In addition, we observed prior ATRA availability in gastric epithelial cells and hepatocytes, which originates from the intestinal region where ATRA availability has not been determined, suggesting that ATRA's involvement in development or physiology may be greater than previously thought. Unfortunately, prior ATRA availability was limited to a small population of cells in the animals owing to low expression of the fusion receptor, as determined by quantitative reverse-transcription PCR. In summary, this pilot study suggested extensive effects of ATRA, underscoring its potential importance in developmental biology and clinical nutrition. In addition, this study encourages careful transgene design by reporting our experience with a low success rate in transgene expression.

1. Introduction

Retinoic acid signaling (RA signaling) plays multiple roles from the embryonic stage to adulthood in mammals. For example, it is associated with the development of many organs, including the eye [1] and colon [2] [3], and with the physiological maintenance of the eye and gastrointestinal tract, since its deficiency causes eye symptoms and diarrhea [4]. In the colon, RA levels were also associated with the progression of human and mouse colitis and colorectal cancers [4]. The physiologically active ligand molecule (*all-trans*-retinoic acid, ATRA) is derived from retinol (Vitamin A) in two enzymatic steps and is inactivated by CYP enzymes [1]. In the absence of ATRA, retinoic acid receptors (RAR α , RAR β , RAR γ) are bound with multiple co-repressors. ATRA releases

these co-repressors to induce gene expression under the retinoic acid-responsive element (RARE). Notably, retinoic acid receptors have their own DNA-binding domain (DBD) apart from their ligand-binding domain (LBD) [5]. This resembles a yeast transcription factor, Gal4, which has its DBD and activation domain at the N- and C-termini, respectively [6].

RA signaling has been detected using a transgenic mouse in which β -galactosidase is expressed under the control of a RARE (RARE-lacZ mice) [7]. How signaling is regulated *in vivo*, however, remains unclear, as the timing and location of ligand availability have not been documented despite many attempts to estimate them by visualizing ATRA-producing or ATRA-inactivating enzymes [1]. The embryonic lethal phenotypes in knockout mice of ATRA-synthesizing or -catalyzing

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genes also make it difficult to elucidate the regulation of RA signaling from the embryonic stage to adulthood [5]. To directly observe ATRA availability at single-cell resolution, we attempted to generate transgenic mice expressing a fusion protein comprising the RAR α ligand-binding domain (RaraLBD) and the Gal4 DNA-binding domain (Gal4DBD), which has been shown to induce Gal4-dependent gene expression in response to ATRA *in vitro* [8]. We crossed these mice with two transgenic lines that comprise a Gal4-responsive Cre expression element (UAS-Cre mice) [9] and a Cre-dependent tandem DsRed (tDsRed) reporter element (R26GRR mice) [10] (Fig. 1). In this system, prior and ongoing ATRA availabilities are labeled with tDsRed and Cre, respectively. Here, we report tDsRed observations in two of 14 mice across three F1-generation lines. We observed tDsRed expression in both well-documented cells (e.g., retinal cells and cells in the muscle layer of the colon) and obscure cells (e.g., gastric epithelial cells and hepatocytes), suggesting a broader involvement of this signaling in development or physiology than previously considered.

2. Materials and methods

2.1. pcDNA3_Gal4DBD-RaraLBD construction

For Gal4DBD, we used the first 147 amino acids [6] of the *Saccharomyces cerevisiae* S288C strain acquired from the Saccharomyces genome database (<https://www.yeastgenome.org/locus/S000006169/>

sequence). We planned to use (1) CMV immediate early (IE) enhancer and promoter, human β -globin/IgG chimeric intron and Kozak sequence in its N-terminus, and (2) SV40 nuclear localization sequence (NLS), 3 $\times$ FLAG sequence between Gal4DBD and RaraLBD, and (3) bovine growth hormone poly (A) sequence (bGH pA) in its C-terminus (Supplementary Material 1). The fusion sequence of human β -globin/IgG chimeric intron, Gal4DBD, SV40 NLS, 3 $\times$ FLAG, and RaraLBD was supplied in IDT Vector Amp Blunt by Integrated DNA Technologies (Tokyo, Japan). The sequence from the human β -globin/IgG chimeric intron to RaraLBD was excised with EcoR1 and Not1 and then inserted into the pcDNA3 vector containing the CMV IE enhancer and promoter and the bGH pA. Hereafter, we refer to this plasmid as pcDNA3_Gal4DBD-RaraLBD. We confirmed the correct transgene sequence by direct sequencing (Fasmac, Kanagawa, Japan) with a CMV forward primer (5'-CGCAAATGGGCGGTAGGCGTG-3') and an SP6 primer (5'-ATTTAGGTGACACTATAG-3'). The DNA sequence in the WORD file format is available in Supplementary Material 2. An ApE file for the DNA sequence is available upon request to the corresponding author.

2.2. In vitro experiments

HEK293T cells were cultured using 35 mm glass bottom dishes (Cat #: D11134H, Matsunami Glass, Osaka, Japan) in DMEM (high glucose) (Cat #: 11,960-044, Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (final concentration: 10%v/v) (Cat #: 556-33865,

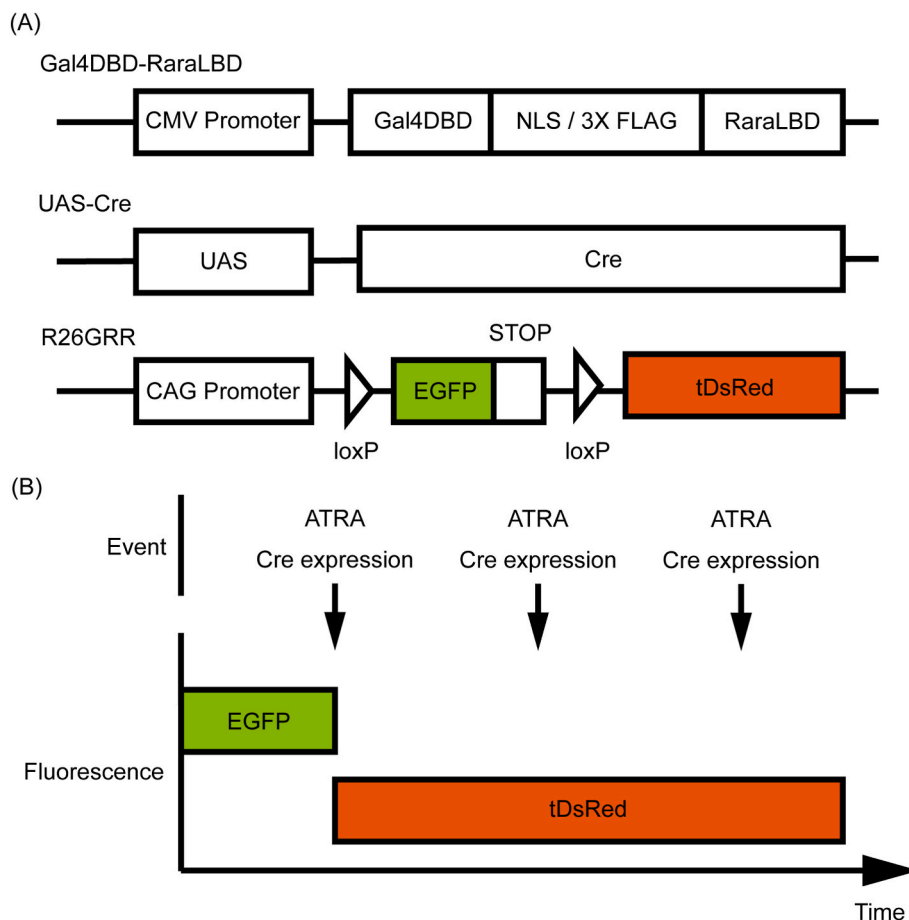


Fig. 1. System for labeling cellular ATRA availability.

(A) The three transgenic mouse lines used in this study. The Gal4DBD-RaraLBD mice were newly generated in this study. Note that we showed only essential gene elements to understand the experimental strategy. In addition, the CMV IE enhancer/promoter is referred to as the “CMV promoter” for brevity. NLS: nuclear localization sequence. (B) Before ATRA binds to Gal4DBD-RaraLBD, EGFP is expressed. The binding of ATRA and Gal4DBD-RaraLBD induces expression of Cre recombinase (ongoing ATRA availability), which converts fluorescence from EGFP to tDsRed (prior ATRA availability). Note that binding of ATRA and Gal4DBD-RaraLBD could occur multiple times.

Fujifilm Wako, Osaka, Japan) and Penicillin-Streptomycin (final concentration: 100 U/mL) (Cat #: 15,140-148, Gibco). We transfected the cells with pcDNA3_Gal4DBD-RaraLBD and UAS-Cre plasmid (Addgene Plasmid #: 193,581) [9] using FuGENE HD Transfection Reagent (Cat #: E2311, Promega, Madison, WI, USA). The next day, we administered either 30 nM *all-trans*-retinoic acid (Cat #: 186-01114, Fujifilm Wako) [11] or vehicle (DMSO) (Cat #: 13,408-64, Nacalai Tesque, Kyoto, Japan) to the medium and cultured for another day. The cells were fixed with 4% Paraformaldehyde Phosphate Buffer Solution (Cat #: 09154-85, Nacalai Tesque), stained for Cre recombinase using rabbit monoclonal *anti*-Cre recombinase antibody (clone: D7L7L, Cat #: 15036 S, RRID: [AB_2798694](#), Cell Signaling Technology, Danvers, MA, USA) (1:500 dilution, 1 h, room temperature) and goat anti-rabbit IgG (H + L) highly cross-adsorbed secondary antibody, Alexa Fluor Plus 555 (Cat #: A32727, RRID: [AB_2633276](#), ThermoFisher Scientific, Waltham, MA, USA) (1:1000 dilution, 1 h, room temperature), counterstained with Hoechst 33,342 (Cat #: 346-07951, Fujifilm Wako) (final concentration: 1 µg/mL, 10 min, room temperature) and photographed using BIOREVO-BZ-X810 (Keyence, Osaka, Japan). We repeated this experiment three times and compared cell numbers in high-power fields using a chi-square test.

2.3. Animal experiments

We digested pcDNA3_Gal4DBD-RaraLBD with Pvu1 to obtain the linearized transgene. The transgenic mice (Gal4DBD-RaraLBD) were generated in the ICR background, essentially as in a previous report [9]. F0 generation mice were mated from wild-type ICR mice (Charles River Laboratories Japan, Kanagawa, Japan), and F1 generation mice were examined for germline transmission of the transgene by PCR using BIOTAQ DNA polymerase (Cat #: BIO-21040, Meridian Biosciences, Cincinnati, OH, USA) (Forward primer: 5'-AGTTGACTGTATCGC CAAAGA-3', Reverse primer: 5'-CTCCACAGTCTTAATGATGCACT-3', Annealing temperature: 55°, Product length: 217 base pairs). Among the multiple transgenic mouse lines, we chose a single F1-generation line (line 1711) for the initial analysis because it was the first to successfully mate with other reporter mice, and also examined two additional lines (lines 1679 and 1727). Analysis was conducted in mice after the F2 generation. For immunohistochemistry, we prepared formalin-fixed and paraffin-embedded sections from Gal4DBD-RaraLBD; UAS-Cre; R26GRR mice, carried out heat-induced epitope retrieval using DAKO Target Retrieval Solution (Cat #: S1699, Agilent Technologies, Santa Clara, CA, USA) (105°, 5 min), and incubated the samples with a rabbit polyclonal *anti*-DsRed antibody (Cat #: 600-401-379, Lot #: 46,317, RRID: [AB_2209751](#), Rockland Immunochemicals, Philadelphia, PA, USA) (1:500 or 1:1000 dilution, 1.5 h, room temperature), Histofine Simple Stain Mouse MAX-PO (R) (Cat #: 414,341, Nichirei Biosciences, Tokyo, Japan) (room temperature, 30 min), DAB Substrate Kit (Cat #: 425,011, Nichirei Biosciences), and Mayer's Hematoxylin Solution (Cat #: 131-09665, Fujifilm Wako). Images were acquired with BIOREVO-BZ-X810. We adjusted brightness, but not contrast, in Adobe Photoshop.

For the quantitative reverse transcription (RT)-PCR experiment, RNA from the eye and colon of adult Gal4DBD-RaraLBD or wild-type male mice ($n = 3$ for each group, 9-47 weeks old) was purified using Isogen (Cat #: 317-02503, Fujifilm Wako), and cDNA was prepared using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Cat #: FSQ-301, Toyobo, Osaka, Japan). We used Thunderbird Next SYBR qPCR Mix (Cat #: QPX-201, Toyobo) and the Takara Thermal Cycler Dice Real-Time System (Takara Bio, Shiga, Japan) for RT-PCR. We used two primer sets: one for Gal4DBD-RaraLBD (Forward primer: 5'-AGAAAGTTGGAGGCGGATC-3', Reverse primer: 5'-CCTGTGCATCGT-CATCCTTGT-3') and one for the housekeeping gene HPRT (Forward primer: 5'-TTGTGTTGGATATGCCCTTGACTA-3', Reverse primer: 5'-AGGCAGATGGCCACAGGACTA-3') [12].

2.4. Approval of DNA and animal experiments

Animal experiments were conducted in accordance with institutional and national guidelines. Approval was obtained from the Institutional Animal Care and Use Committee and the DNA Experiment Committee of the University of Tsukuba (Approval Numbers for Animal Experiments: 24-411, 25-160, 26-307) (Approval Number for DNA Experiments: 240208).

3. Results

3.1. Transgene functionality

We briefly checked transgene functionality *in vitro* before injection into mouse zygotes. HEK293T cells were transfected with the Gal4DBD-RaraLBD and UAS-Cre plasmids and treated with either 30 nM ATRA or vehicle (DMSO). Although the UAS-Cre plasmid contained a fluorescent protein, mRFP670, its fluorescence was too dim to detect, warranting signal enhancement by immunofluorescence. Cre expression was observed in 175 cells out of 13,113 Hoechst 33,342-positive cells (1.33%) in the ATRA group, while in 79 cells out of 16,600 Hoechst 33,342-positive cells in the DMSO group (0.48%) ($p < 0.001$ by chi-square test), suggesting that the transgene is functional (Representative images are available in [Supplementary Material 3](#)). Although we did not perform a detailed binding-affinity analysis, we confirmed a statistically significant increase in Cre expression at a previously reported physiological ATRA level [11]. As the sensitivity for ATRA detection should depend on several factors, including the expression levels of Gal4DBD-RaraLBD proteins, we considered that final validation should be conducted *in vivo*.

3.2. ATRA availability *in vivo*

To maximize the chance of labeling ATRA availability, we permanently labeled prior ATRA availability using Cre recombinase (Fig. 1). We examined tDsRed expression in adult mice, focusing on the eye and colon, where ATRA is suggested to act during embryogenesis and adulthood. We observed tDsRed expression in some retinal (Fig. 2A and A', 2B, and 2B') and colonic (Fig. 2C and C', 2D, and 2D') cells in two mice (Animal IDs: 2092 and 2111) from the same F1 founder (line 1711). In the colon, we observed tDsRed expression in the muscle layer cells as well as in epithelial cells, whereas previous studies have suggested ATRA action in neurons [2] and immune cells [3]. Given RA is a diffusible molecule, our results were consistent with previous studies, suggesting that tDsRed signal reflects prior ATRA availability, albeit with low efficacy. Unfortunately, we did not observe Cre recombinase expression (ongoing ATRA availability) in these samples. This incompleteness is due to low expression of the Gal4DBD-RaraLBD protein, as Gal4DBD-RaraLBD mRNA was not detected by RT-PCR ([Supplementary Material 4](#)).

RA signaling acts as a caudalizing factor along the anteroposterior axis during gut development. In the chick embryo, the thyroid develops in the absence of, or at very low levels of, RA, although RA is required for midgut (the future stomach, duodenum, liver, and pancreatic region) development [13]. In contrast to the well-characterized action in chick, RA signaling in mammalian midgut development was unclear. One study showed that RA signaling is present in the liver bud on embryonic day 9.5 in RARE-lacZ mice and is required for hepatic induction from the midgut endoderm [14]. Another study suggested that liver mesothelial cells produce ATRA during development [15]. As RA action throughout the midgut in mammals remains unclear despite extensive examination of the liver, we performed immunohistochemistry for tDsRed in the liver and found tDsRed expression in hepatocytes even distant from the mesothelium (Fig. 3), supporting RA in the liver bud, but not excluding its presence in the liver mesothelium. Current knowledge of ATRA in the stomach is more elusive, as RA production in gastric epithelial cells was

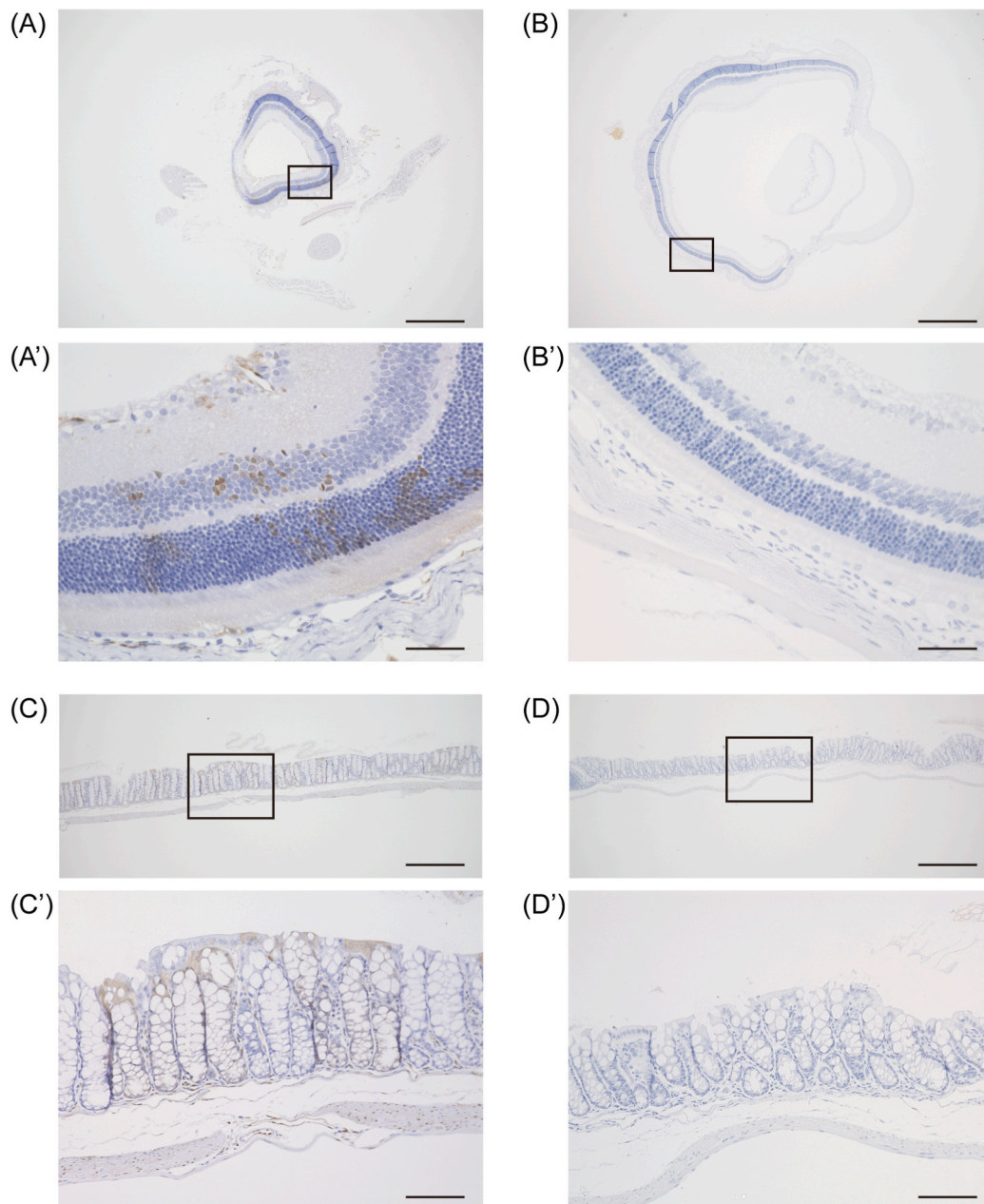


Fig. 2. Prior ATRA availability (tDsRed) in the eye and colon.

Gal4DBD-RaraLBD; UAS-Cre; R26GRR (A, A', C, and C') and UAS-Cre; R26GRR (negative control) samples were stained for tDsRed. (A) A low-magnification image of a Gal4DBD-RaraLBD; UAS-Cre; R26GRR retina. (A') A high-magnification image of the boxed area in (A). (B) A low-magnification image of a UAS-Cre; R26GRR retina. (B') A high-magnification image of the boxed area in (B). (C) A low-magnification image of a Gal4DBD-RaraLBD; UAS-Cre; R26GRR colon. (C') A high-magnification image of the boxed area in (C). (D) A low-magnification image of UAS-Cre; R26GRR colon. (D') A high-magnification image of the boxed area in (D). Scale bars: 500 μ m (A, B, C, and D), 50 μ m (A' and B'), and 100 μ m (C' and D').

expected given the presence of retinol (an ATRA precursor) and RA biosynthesis genes in those cells [16], although direct evidence of ATRA or RA signaling remains lacking. Our immunohistochemistry for tDsRed in the gastric cardia showed tDsRed expression in gastric epithelial cells (Fig. 4), providing the first *in vivo* observation.

Finally, to provide systemic screening results of prior ATRA availability, we performed immunohistochemistry for tDsRed in the heart (Supplementary Material 5), lung (Supplementary Material 6), pancreas (Supplementary Material 7), small intestine (Supplementary Material 8), kidney (Supplementary Material 9), and testis (Supplementary Material 10; $n=1$, as Animal ID 2092 was female). For details, see each figure legend.

4. Discussion

Signal transduction begins with ligand binding to its receptor, so understanding its regulation requires elucidating three components (ligand, receptor, and signal). In RA signaling, the RARE-lacZ mice helped elucidate signal transduction [7], and many attempts have been made to elucidate the expression of enzymes involved in metabolism or catabolism [1]. Elucidation of the ligand (ATRA) distribution is, however, challenging as it is an unstable small molecule. Our approach is unique in that we focus on the cellular availability of ATRA, as we consider it a biologically meaningful amount *in vivo*. To this aim, we attempted to generate transgenic mice that convert ATRA availability to labelable gene expression (Cre expression).

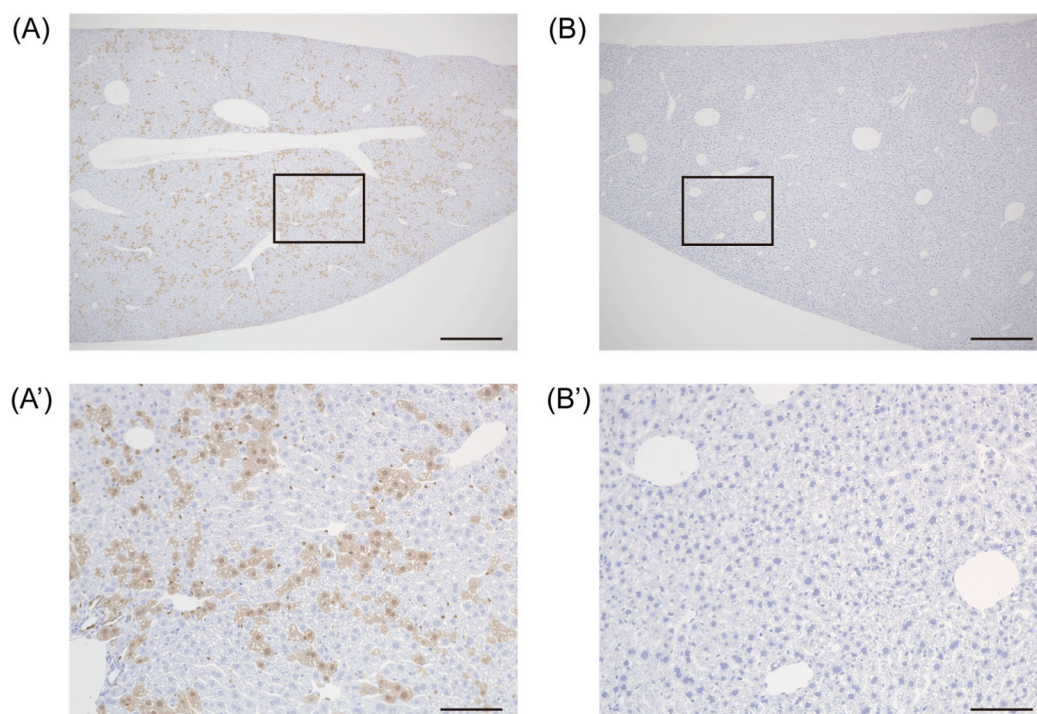


Fig. 3. Prior ATRA availability (tDsRed) in the liver.

Gal4DBD-RaraLBD; UAS-Cre; R26GRR and UAS-Cre; R26GRR (negative control) samples were stained for tDsRed. (A) A low-magnification image of a Gal4DBD-RaraLBD; UAS-Cre; R26GRR liver. (A') A high-magnification image of the boxed area in (A). (B) A low-magnification image of a UAS-Cre; R26GRR liver. (B') A high-magnification image of the boxed area in (B). Scale bars: 500 μ m (A and B) and 100 μ m (A' and B').

As suggested in prior literature [8], the Gal4DBD-RaraLBD fusion protein could induce downstream gene expression at a physiological level (30 nM) of ATRA [11] *in vitro* (Supplementary Material 3). Indeed, we observed tDsRed expression that suggests prior ATRA availability in the RA signal-requiring organs, including the eye and colon, in two mice. Unfortunately, we were no longer able to reproduce the result, despite increasing the number of mice examined to 14 from three F1-generation lines. This does not seem to be due to the toxic action of Gal4DBD-RaraLBD because transgene transmission rates were close to the expected value from Mendel's principles of inheritance (50%): 93/183 (50.8%) for line 1711, 28/53 (52.8%) for line 1679, and 21/46 (45.7%) for line 1727. In addition, one limitation in this study is that we did not establish functional Gal4DBD-RaraLBD mouse lines. RT-PCR results suggested that this is partly owing to failure of constitutive expression of the Gal4DBD-RaraLBD fusion protein (Supplementary Material 4). In retrospect, we speculate that this failure could be caused by unfavorable selection of the promoter and enhancer. One study suggested that GC-content significantly influences gene expression levels [17]. Specifically, this study showed that the addition of CpG-island fragments from the HNRPA2B1/CBX3 genomic locus enhanced transgene expression from the CMV promoter/enhancer, which is susceptible to silencing, in mammalian cells. In addition, expression from the CMV promoter/enhancer could be highly dependent on the cell types, compared with EF1 α or CAGG promoters [18]. Therefore, refining the promoter would improve Gal4DBD-RaraLBD expression and effectively validate this study's results. This report encourages future studies to design transgenes carefully. In addition, although we sacrificed the F1 generation without examining Gal4DBD-RaraLBD expression because we did not expect low transgene expression, this would have shown whether that risk was possible.

Although this study has such limitations, we report the first attempt to observe ATRA availability. Because our strategy is constitutive expression of an "ATRA-catching" exogenous receptor (Gal4DBD-

RaraLBD), the interpretation of the results should differ from that of endogenous gene promoter-driven strategies. Indeed, tDsRed-expressing cells (e.g., retinal neurons, hepatocytes, and gastric epithelial cells) were in line with the literature, suggesting the soundness and potential of our strategy. Overcoming the low expression levels of Gal4DBD-RaraLBD would validate this study's results and further clarify the overlooked availability of ATRA from the embryonic stage to adulthood, which might be associated with human development and health.

5. Conclusion

We reported the first observation of ATRA availability at single-cell resolution in mice, although further investigation is required to validate the result.

CRediT authorship contribution statement

Masaharu Yoshihara: Writing – original draft, Visualization, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Yoshitoku Watabe:** Writing – original draft, Investigation. **Baoshuo Ning:** Investigation. **Hayate Suzuki:** Resources. **Seiya Mizuno:** Resources. **Satoru Takahashi:** Supervision.

Ethics declaration

This study was conducted in accordance with the following guidelines for animal welfare and/or reporting: Animal experiments were conducted in accordance with the institutional or national guidelines of the University of Tsukuba and Japan. This study was approved by the University of Tsukuba. (Approval No. 24-411, 25-160, 26-307 (animal experiments), and 240208 (DNA experiments))

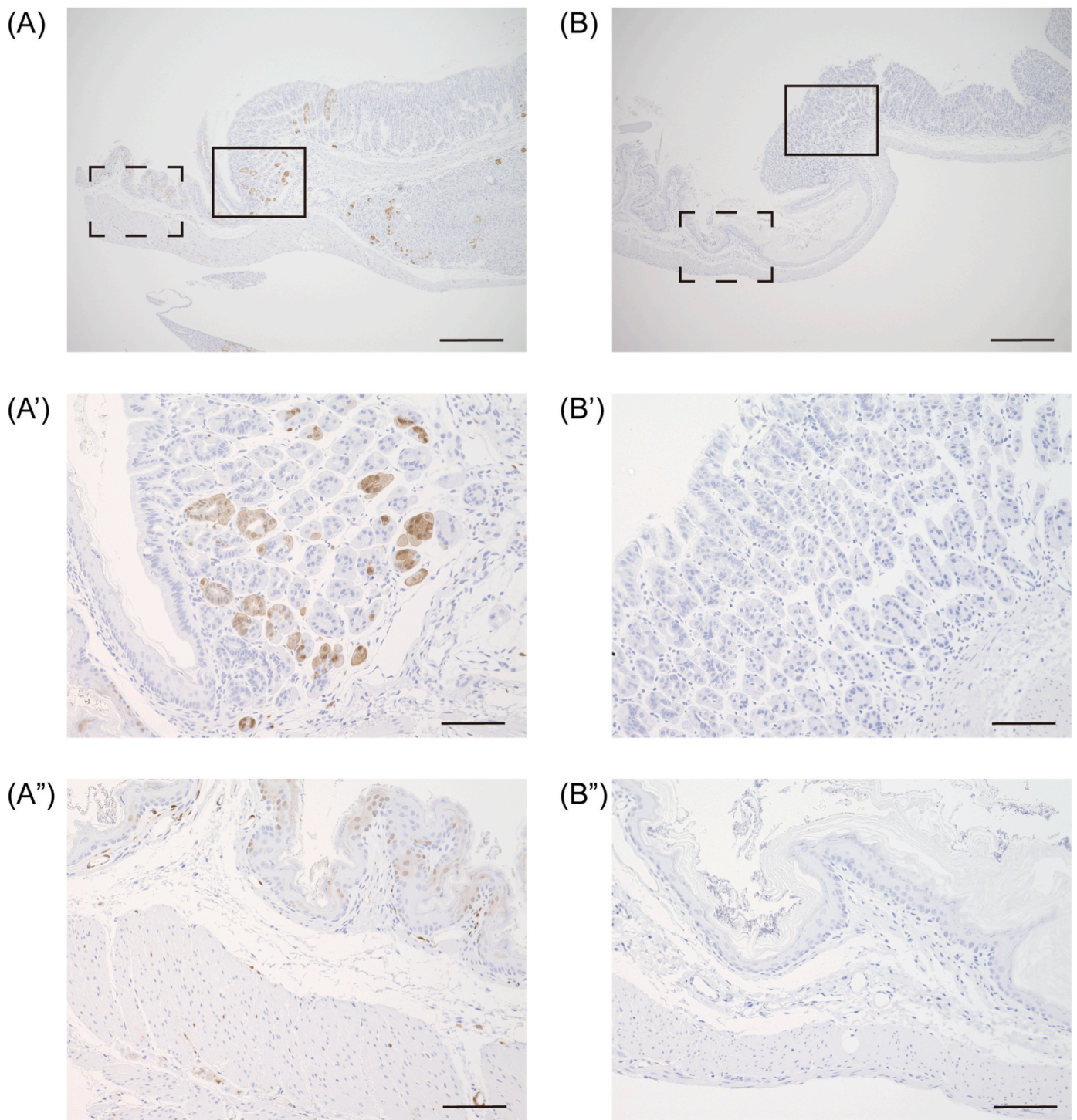


Fig. 4. Prior ATRA availability (tDsRed) in the cardia region of the stomach. Gal4DBD-RaraLBD; UAS-Cre; R26GRR and UAS-Cre; R26GRR (negative control) samples were stained for tDsRed. (A) A low-magnification image of a Gal4DBD-RaraLBD; UAS-Cre; R26GRR sample. (A') A high-magnification image of the boxed area (cardia) in (A). (A'') A high-magnification image of the area surrounded by a dashed line (esophagus) in (A). Note that multilayered squamous epithelial cells indicate the esophagus. (B) A low-magnification image of a UAS-Cre; R26GRR sample. (B') A high-magnification image of the boxed area (cardia) in (B). (B'') A high-magnification image of the area surrounded by a dashed line (esophagus) in (B). Note that multilayered squamous epithelial cells indicate the esophagus. Scale bars: 500 μm (A and B) and 100 μm (A', A'', B', and B'').

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Masaharu Yoshihara reports financial support was provided by Japan

Society for the Promotion of Science. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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List of abbreviations

All-trans-retinoic acid (ATRA), bovine growth hormone poly A sequence (bGH pA), cytomegalovirus (CMV), DNA-binding domain (DBD), Gal4 DNA-binding domain (Gal4DBD), ligand-binding domain (LBD), retinoic acid receptor alpha ligand-binding domain (RarαLBD), retinoic acid (RA), reverse transcription polymerase chain reaction (RT-PCR), SV40 nuclear localization sequence (NLS), tandem DsRed (tDsRed), upstream activating sequence (UAS).

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2026.102839>.

Data availability

Data will be made available on request.

References

- [1] T.J. Cunningham, G. Duyster, Mechanisms of retinoic acid signalling and its roles in organ and limb development, *Nat. Rev. Mol. Cell Biol.* 16 (2) (2015) 110–123, <https://doi.org/10.1038/nrm3932>.
- [2] E.C. Wright-Jin, J.R. Grider, G. Duyster, et al., Retinaldehyde dehydrogenase enzymes regulate colon enteric nervous system structure and function, *Dev. Biol.* 381 (1) (2013) 28–37, <https://doi.org/10.1016/j.ydbio.2013.06.021>.
- [3] N. Bhattacharya, R. Yuan, T.R. Prestwood, et al., Normalizing microbiota-induced retinoic acid deficiency stimulates protective CD8(+) T cell-mediated immunity in colorectal cancer, *Immunity* 45 (3) (2016) 641–655, <https://doi.org/10.1016/j.immuni.2016.08.008>.
- [4] G.A. Stevens, J.E. Bennett, Q. Hennocq, et al., Trends and mortality effects of vitamin A deficiency in children in 138 low-income and middle-income countries between 1991 and 2013: a pooled analysis of population-based surveys, *Lancet Global Health* 3 (9) (2015) e528–e536, [https://doi.org/10.1016/S2214-109X\(15\)00039-X](https://doi.org/10.1016/S2214-109X(15)00039-X).
- [5] K. Niederreither, P. Dollé, Retinoic acid in development: towards an integrated view, *Nat. Rev. Genet.* 9 (7) (2008) 541–553, <https://doi.org/10.1038/nrg2340>.
- [6] J. Ma, M. Ptashne, Deletion analysis of GAL4 defines two transcriptional activating segments, *Cell* 48 (5) (1987) 847–853, [https://doi.org/10.1016/0092-8674\(87\)90081-x](https://doi.org/10.1016/0092-8674(87)90081-x).
- [7] J. Rossant, R. Zirmgibl, D. Cado, et al., Expression of a retinoic acid response element-hsplaZ transgene defines specific domains of transcriptional activity during mouse embryogenesis, *Genes Dev.* 5 (8) (1991) 1333–1344, <https://doi.org/10.1101/gad.5.8.1333>.
- [8] M.D. Huq, N.P. Tsai, S.A. Khan, et al., Lysine trimethylation of retinoic acid receptor-α: a novel means to regulate receptor function, *Mol. Cell. Proteomics* 6 (4) (2007) 677–688, <https://doi.org/10.1074/mcp.M600223-MCP200>.
- [9] M. Yoshihara, T. Nishino, N. Sambe, et al., Generation of a Gal4-dependent gene recombination and illuminating mouse, *Exp. Anim.* 71 (3) (2022) 385–390, <https://doi.org/10.1538/expanim.21-0202>.
- [10] Y. Hasegawa, Y. Daitoku, K. Sekiguchi, et al., Novel ROSA26 cre-reporter knock-in C57BL/6N mice exhibiting green emission before and red emission after Cre-mediated recombination, *Exp. Anim.* 62 (4) (2013) 295–304, <https://doi.org/10.1538/expanim.62.295>.
- [11] S.B. Kurlandsky, J.H. Xiao, E.A. Duell, et al., Biological activity of all-trans retinoic acid requires metabolic conversion to all-trans retinoic acid and is mediated through activation of nuclear retinoid receptors in human keratinocytes, *J. Biol. Chem.* 269 (52) (1994) 32821–32827.
- [12] S. Mitsutake, K. Zama, H. Yokota, et al., Dynamic modification of sphingomyelin in lipid microdomains controls development of obesity, fatty liver, and type 2 diabetes, *J. Biol. Chem.* 286 (32) (2011) 28544–28555, <https://doi.org/10.1074/jbc.M111.255646>.
- [13] E. Bayha, M.C. Jørgensen, P. Serup, et al., Retinoic acid signaling organizes endodermal organ specification along the entire antero-posterior axis, *PLoS One* 4 (6) (2009) e5845, <https://doi.org/10.1371/journal.pone.0005845>. Published 2009 Jun 10.
- [14] T.M. Guertin, A. Palaria, J. Mager, et al., Deciphering the role of retinoic acid in hepatic patterning and induction in the mouse, *Dev. Biol.* 491 (2022) 31–42, <https://doi.org/10.1016/j.ydbio.2022.08.003>.
- [15] T. Brade, S. Kumar, T.J. Cunningham, et al., Retinoic acid stimulates myocardial expansion by induction of hepatic erythropoietin which activates epicardial Igf2, *Development (Camb.)* 138 (1) (2011) 139–148, <https://doi.org/10.1242/dev.054239>.
- [16] D. Bimczok, J.Y. Kao, M. Zhang, et al., Human gastric epithelial cells contribute to gastric immune regulation by providing retinoic acid to dendritic cells, *Mucosal Immunol.* 8 (3) (2015) 533–544, <https://doi.org/10.1038/mi.2014.86>.
- [17] S. Williams, T. Mustoe, T. Mulcahy, et al., CpG-island fragments from the HNRPA2B1/CBX3 genomic locus reduce silencing and enhance transgene expression from the hCMV promoter/enhancer in mammalian cells, *BMC Biotechnol.* 5 (2005) 17, <https://doi.org/10.1186/1472-6750-5-17>. Published 2005 Jun 3.
- [18] J.Y. Qin, L. Zhang, K.L. Clift, et al., Systematic comparison of constitutive promoters and the doxycycline-inducible promoter, *PLoS One* 5 (5) (2010) e10611, <https://doi.org/10.1371/journal.pone.0010611>. Published 2010 May 12.
- [19] K. Niederreither, J. Vermot, N. Messaddeq, et al., Embryonic retinoic acid synthesis is essential for heart morphogenesis in the mouse, *Development (Camb.)* 128 (7) (2001) 1019–1031, <https://doi.org/10.1242/dev.128.7.1019>.
- [20] S. Malpel, C. Mendelsohn, W.V. Cardoso, Regulation of retinoic acid signaling during lung morphogenesis, *Development (Camb.)* 127 (14) (2000) 3057–3067, <https://doi.org/10.1242/dev.127.14.3057>.
- [21] M. Martín, J. Gallego-Llamas, V. Ribes, et al., Dorsal pancreas agenesis in retinoic acid-deficient Raldh2 mutant mice, *Dev. Biol.* 284 (2) (2005) 399–411, <https://doi.org/10.1016/j.ydbio.2005.05.035>.
- [22] H.B. Jijon, L. Suarez-Lopez, O.E. Diaz, et al., Intestinal epithelial cell-specific RARα depletion results in aberrant epithelial cell homeostasis and underdeveloped immune system, *Mucosal Immunol.* 11 (3) (2018) 703–715, <https://doi.org/10.1038/s41385-017-0091-1>.
- [23] C. Rosselot, L. Spraggon, I. Chia, et al., Non-cell-autonomous retinoid signaling is crucial for renal development, *Development (Camb.)* 137 (2) (2010) 283–292, <https://doi.org/10.1242/dev.040287>.
- [24] N. Vernet, C. Dennefeld, F. Guillou, et al., Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells, *EMBO J.* 25 (24) (2006) 5816–5825, <https://doi.org/10.1038/sj.emboj.7601447>.
- [25] E.J. Jauregui, D. Mitchell, T. Topping, et al., Retinoic acid receptor signaling is necessary in steroidogenic cells for normal spermatogenesis and epididymal function, *Development (Camb.)* 145 (13) (2018) dev160465, <https://doi.org/10.1242/dev.160465>. Published 2018 Jul 9.