Endocrine Journal 2016, **63** (10), 897-904

ORIGINAL

Extra-adrenal induction of *Cyp21a1* ameliorates systemic steroid metabolism in a mouse model of congenital adrenal hyperplasia

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Abstract. Congenital adrenal hyperplasia (CAH) due to steroid 21-hydroxylase (21-OH) deficiency (21-OHD) is an autosomal recessive disorder, in which *CYP21A2* mutations or deletions result in underproduction of glucocorticoid and mineralocorticoid, and overproduction of androgens. Patients with CAH are treated with oral steroid supplementation, but optimal control of blood steroid levels remains difficult. Thus, new therapeutic approaches are still needed. Previously, adenovirus-mediated administration of human *CYP21A2* to adrenal glands rescued the phenotype of a mouse model of 21-OHD. In this study, we examined whether transduction of murine *Cyp21a1* in extra-adrenal tissues could rescue steroid metabolism in 21-OHD mice. We transduced primary fibroblasts obtained from 21-OHD mice with a retroviral vector containing *Cyp21a1*. *In vitro* assays demonstrated that *Cyp21a1*-expressing fibroblasts can uptake progesterone from the culture media, convert it to deoxycorticosterone (DOC), and subsequently release DOC back into the media. Autotransplantation of *Cyp21a1*-expressing fibroblasts into the subcutaneous tissues of the back resulted in a significant reduction in the serum progesterone/DOC ratio in four of six 21-OHD mice at 4 weeks after injection. We also directly injected an adeno-associated viral vector containing *Cyp21a1* into the thigh muscles of 21-OHD mice. Serum progesterone/DOC ratios were markedly reduced in all four animals at 4 weeks after injection. These results indicate that extra-adrenal induction of *Cyp21a1* ameliorates steroid metabolism in 21-OHD mice. This study suggests a novel therapeutic strategy for congenital adrenal hyperplasia, which warrants further investigations.

Key words: Gene therapy, Congenital adrenal hyperplasia, 21-hydroxylase deficiency, Retroviral vector, Adeno-associated viral vector

CONGENITAL ADRENAL HYPERPLASIA (CAH) is a relatively common autosomal recessive disorder that occurs in 1/10,000–20,000 births [1]. More than 90% of CAH cases are caused by mutations or deletions in *CYP21A2* encoding steroid 21-hydroxylase (21-OH) [1]. Salient endocrinological findings of patients with 21-OH deficiency (21-OHD) include underproduction of glucocorticoid and mineralocorticoid that leads to salt-wasting, and overproduction of androgens that leads to virilization of genetic females [2]. Although therapeutic regimens of oral steroid supplementation for 21-OHD patients have been well established, optimal control of blood steroid levels remains unattainable in several cases [2]. Thus, new therapeutic approaches are still required for CAH.

Two previous studies have tested the efficacy of

Submitted Mar. 11, 2016; Accepted Jun. 21, 2016 as EJ16-0112
Released online in J-STAGE as advance publication Jul. 14, 2016
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Abbreviations: AAV, adeno-associated virus; CAH, congenital adrenal hyperplasia; CMV, cytomegalovirus; DOC, deoxycorticosterone; DMEM, Dulbecco's modified Eagle's medium; IRES/GFP, internal ribosomal entry site/green fluorescence protein; LTR, long terminal repeat; RT-PCR, reverse transcription polymerase chain reaction; RV, retrovirus; 21-OH, 21-hydroxylase; 21-OHD, 21-OH deficiency.

gene therapy in *H-2*^{aw18} mice, a naturally occurring animal model of 21-OHD. Gotoh *et al*. showed that the mice are rescued by transducing a *Cyp21a1*-containing DNA fragment into embryos [3]. Tajima *et al*. demonstrated that intra-adrenal injection of an adenoviral vector harboring human *CYP21A2* ameliorates biochemical, endocrinological, and histological findings of adult 21-OHD mice [4]. These results suggest that gene therapy might be a novel option for treatment of CAH. However, less invasive gene transfer approaches such as subcutaneous or intramuscular injections of viral vectors, have not been tested in 21-OHD mice. Although *Cyp21a1/a2*-dependent enzymatic conversion of progesterone to deoxycorticosterone (DOC) in wildtype mice occurs almost exclusively in adrenal glands [5], it remains possible that artificially induced expression of *Cyp21a1* in extra-adrenal tissues also mediates systemic steroid metabolism. Since steroids are lipid-soluble molecules that can diffuse through the cell membranes, various tissues are likely to passively uptake blood steroids [6]. Furthermore, cytochrome P450 oxidoreductase, an indispensable co-factor for *CYP21A1/A2*, is ubiquitously expressed [7]. Therefore, we investigated whether the extra-adrenal expression of *Cyp21a1* improves steroid metabolism in 21-OHD mice.

Materials and Methods

Animals

This study was approved by the Animal Care and Use Committee at the National Research Institute for Child Health and Development (Project Number: 2009-009). All mice were handled according to institutional guidelines.

Fertilized eggs of *H-2*^{aw18} in C57BL/10SnSlc (*H-2*^{b}) [8], a naturally occurring mouse model of 21-OHD, was kindly provided by Dr. T. Shiroishi. The eggs were transferred to a pseudo pregnant mother. Mice were genotyped by Southern blotting using a *Cyp21a1* cDNA probe labeled with digoxigenin (Roche diagnostics, Basel, Swiss) (Supplemental Fig. 1A). Homozygous mutant mice were obtained by mating heterozygous pairs. Heterozygous pregnant mothers received daily injections of 5 μg dexamethasone from late pregnancy to the day of delivery, to prevent deaths of newborn pups. Homozygous newborn mice received daily injections of 5 μg corticosterone (Sigma-Aldrich, St Louis, MO) and 0.025 μg fludrocortisone during the first 3 weeks after birth [9]. Afterwards, the mice were maintained under standard conditions with regular rodent diet and water *ad libitum*. Progesterone and DOC in serum samples were measured by liquid chromatography tandem mass spectrometry (ASKA Pharmaceutical Medical Corporation, Kawasaki, Japan) [10].

Cyp21a1 induction by an ex vivo protocol using an retroviral (RV) vector

Murine *Cyp21a1* cDNA was PCR-amplified and cloned into the pCR-BluntII-TOPO vector (Open Biosystems, Lafayette, CO). The cDNA fragment was inserted into *Xho*I sites of pGCDNsap-IRES/GFP with the LTR derived from a murine stem cell virus [11] (Fig. 1A). The vector was co-transfected with a vesicular stomatitis virus -G protein expression plasmid into 293gpg cells. The FuGENE HD transfection reagent (Promega, Madison, WI) was used tor transfection. The cells were cultured for 24 hours in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, tetracycline, puromycin and G418 (Sigma-Aldrich). Then, culture medium was replaced by tetracycline-free medium. After 72-hour incubation, supernatant of the culture medium containing viral particles was collected, filtered with 0.45 μm filters, and centrifuged. Viral pellets were re-suspended with STEMPRO (Thermo Fisher Scientific, Yokohama, Japan).

Primary tail fibroblasts were obtained from the homozygous mice and cultured for 30 to 90 days in 6-well plates with DMEM containing 10% fetal calf serum and antibiotics. For transduction, fibroblasts were collected and resuspended in 10 μL STEMPRO solution containing the RV vector and protamine sulfate (final concentration, 10 μg/mL; Sigma-Aldrich). Then, the cells were centrifuged and reseeded in 6-well plates. Seventy-two hours after infection, fluorescence signals were detected by with a fluorescence microscope (Olympus, Tokyo, Japan), and the expression levels of *Cyp21a1* was assessed by real-time RT-PCR. In addition, steroidogenic capacities of the fibroblasts were assessed by measuring DOC levels in the culture media. In this experiment, 2 μM progesterone (Sigma-Aldrich) was added to the media 24 hours prior to sampling.

Transduced fibroblasts were autotransplanted into the homozygous mice (n = 7, 6 for RV vector containing *Cyp21a1* and 1 for RV vector without *Cyp21a1* as

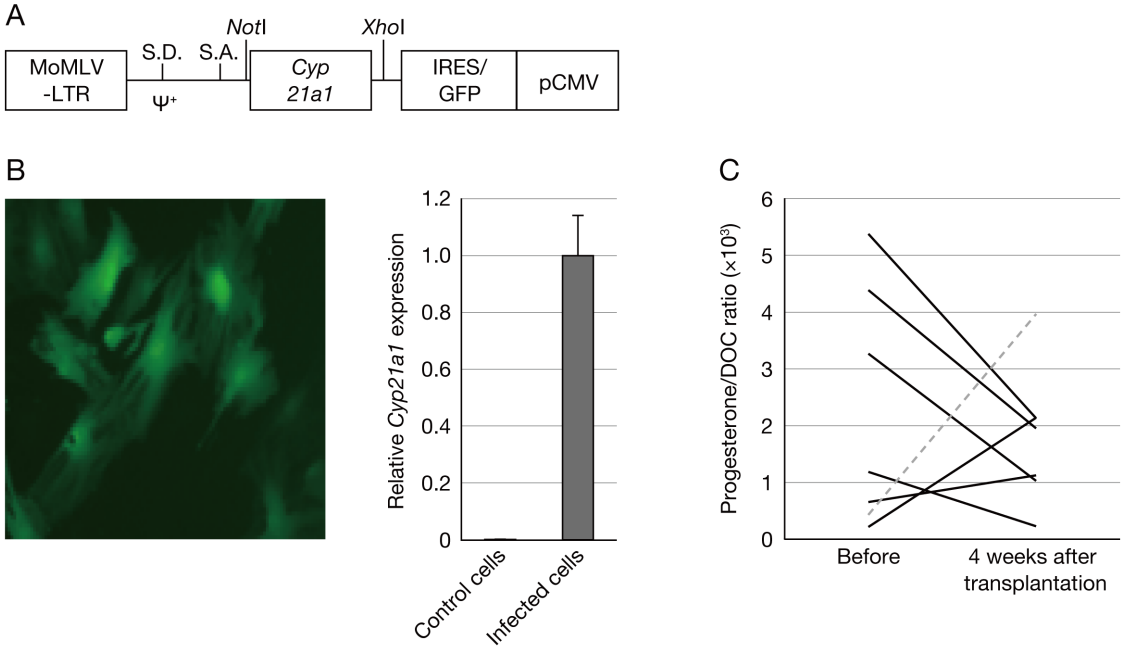


Fig. 1 *Cyp21a1* induction using a retroviral (RV) vector

A, Schematic representation of the RV vector. The vector contains Moloney murine leukemia virus long-terminal repeats (MoMLV-LTR), and extended packaging signal (Ψ^+), splice donor (S.D.), splice acceptor (S.A.), cytomegalovirus promoter (pCMV), mouse 21-hydroxylase cDNA (*Cyp21a1*), internal ribosomal entry site/green fluorescence protein cDNA (IRES/GFP).

B, Fluorescence images of the infected primary fibroblasts obtained from 21-hydroxylase deficiency mice (left panel). Signals of RV were detected in the cytoplasm. *Cyp21a1* mRNA levels in the fibroblasts (right panel). Target mRNA expression relative to that of *Gapdh* is shown. The results are expressed as the mean ± SEM. The average of mRNA levels in infected cells was defined as 1.0.

C, The serum progesterone/deoxycorticosterone (DOC) ratio in mice autotransplanted with *Cyp21a1*-expressing fibroblasts. Solid lines indicate results of *Cyp21a1*-expressing fibroblasts and broken gray line shows that of fibroblast infected with RV vector without *Cyp21a1* cDNA as a control.

a control, aged 7 weeks to 12 months). In each animal, 5×10^5 fibroblasts were injected into the subcutaneous tissues of the back. Serum levels of progesterone and DOC were measured before and 4 weeks after transplantation. Mice were sacrificed 6 weeks after transplantation, and subcutaneous tissues at the injection sites were examined histologically.

Cyp21a1 induction by an in vivo protocol using an adeno-associated virus (AAV) vector

A serotype-2 AAV vector containing *Cyp21a1* cDNA and a cytomegalovirus promoter was constructed and that containing *GFP* cDNA as a control by Applied Viromics (Fremont, CA) (Fig. 2A). Methods for the vector construction were described previously [12]. The transduction activity of the AAV vector was validated by *ex vivo* experiments. Primary tail fibroblasts of the homozygous mice were infected with 10^9 copies of the AAV vector by centrifugal enhancement with protamine sulfate (final concentration, 10 μg/mL; Sigma-Aldrich). The expression levels of *Cyp21a1* were assessed by real-time RT-PCR 72 hours after infection.

We injected the AAV vector to the homozygote mice aged between 3 and 10 months ($n = 4$ for *Cyp21a1*-containing vector and $n = 2$ for control GFP vector). A 100 μL PBS with 0.01% Pluronic F-68 (Sigma-Aldrich) containing 1.0×10^{11} vector particles was injected into the bilateral thigh muscles. Serum progesterone and DOC levels were measured before and 4 weeks after injection. Tissues of liver, heart, injected muscles from a mouse 4 weeks after AAV vector injection and adrenal glands of wild type mouse as a control were collected for RT-PCR. In addition, one AAV-injected mouse was subjected to monthly blood sampling during 7 months after injection.

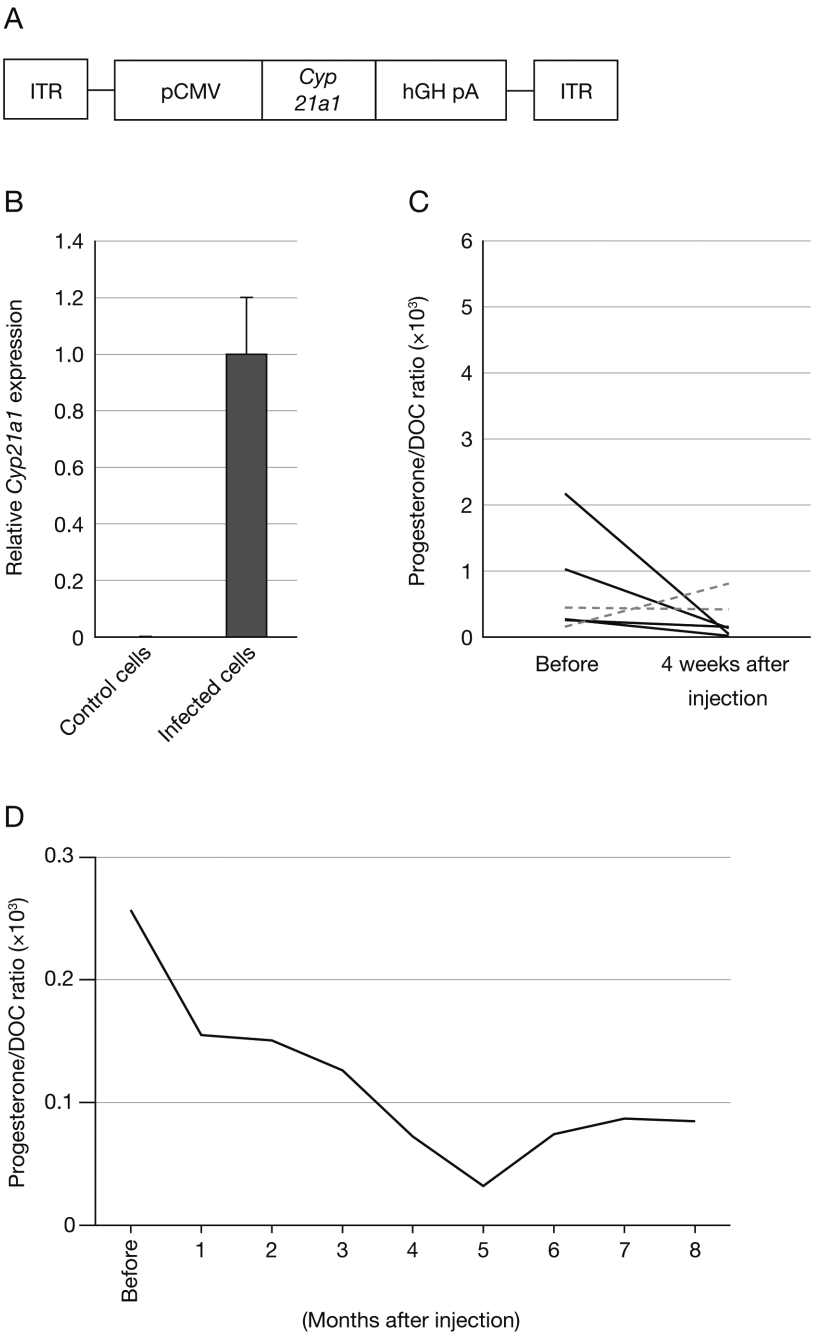


Fig. 2 *Cyp21a1* induction using an adeno-associated virus (AAV) vector
A, Schematic representation of the AAV vector. The vector utilized a cytomegalovirus promoter (pCMV), human growth hormone polyA signal (hGH pA), mouse 21-hydroxylase cDNA (*Cyp21a1*) and AAV inverted terminal repeats (ITR).
B, *Cyp21a1* mRNA expression in primary fibroblasts. The results are expressed as the mean ± SEM. The average of mRNA levels in infected cells was defined as 1.0.
C, The serum progesterone/deoxycorticosterone (DOC) ratio in mice infected with the AAV vector. Solid lines indicate results of AAV vector containing *Cyp21a1* cDNA and broken gray lines show those of AAV vector containing GFP cDNA as a control.
D, Time course of changes in the serum progesterone/DOC ratio of a mouse after AAV vector injection.

Real-time RT-PCR

Total RNA samples were extracted using an RNeasy Mini Kit (QIAGEN, Venlo, The Nederland) and reverse transcribed using a High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Carlsbad, CA). Levels of *Cyp21a1* mRNA were measured by real-time RT-PCR using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) and a TaqMan Assay Kit (#4331182, Mm00487230_g1). The housekeeping gene *Gapdh* (#4308313) was used as an internal control.

Statistical analysis

Statistical analysis were performed by Wilcoxon signed-rank test with GrapPad Prism 5 (GraphPad Software, La Jolla, CA).

Results

Animals

Homozygous mice were smaller than wildtype littermates (Supplemental Fig. 1B and C). At 6 weeks of age, the serum progesterone/DOC ratio was higher in homozygous mice than in wildtype animals (Supplemental Fig. 1D).

Cyp21a1 induction by an ex vivo protocol using an RV vector

Expressions of *Cyp21a1* in RV-infected primary fibroblasts were confirmed by fluorescence imaging (Fig. 1B) and real-time RT-PCR (Fig. 1B). DOC concentrations in the culture media of the infected and uninfected (control) cells were 192.095 ng/mL and 0.018 ng/mL, respectively. This indicated that in RV-infected cells, 27.5% of the progesterone added to the culture media was converted to DOC within 24 hours, while this conversion in the uninfected fibroblasts was negligible.

Serum levels of progesterone and DOC were measured in mice before and 4 weeks after autotransplantation (Fig. 1C). Progesterone/DOC ratios were reduced in four of six mice at 4 weeks after autotransplantation. In the remaining two mice, the progesterone/DOC ratio either remained unchanged or slightly increased. These changes were statistically insignificant. Histological findings of the tissues at the injection sites were unremarkable at 6 weeks after autotransplantation (data not shown). Macrophage infiltration indicative of local inflammation was absent.

Cyp21a1 induction by an in vivo protocol using an AAV vector

Primary fibroblasts infected by the *Cyp21a1*-containing AAV vector expressed *Cyp21a1*, indicating high activity of the vector (Fig. 2B). Direct injection of the AAV vector into the thigh muscles resulted in a decrease in the serum progesterone/DOC ratio in all four mice at 4 weeks after infection (Fig. 2C). The hormonal changes were statistically insignificant because of the small number of samples. In these mice, *Cyp21a1* was weakly expressed in the thigh muscle (0.03% of wildtype adrenal), and at lower levels in the heart and liver (Supplemental Fig. 1E). The serum progesterone/DOC ratio of an AAV-injected mouse remained relatively low during 7 months after injection (Fig. 2D). Serious adverse events, either local or systemic, were not observed in mice after injections.

Discussion

We report here that extra-adrenal induction of *Cyp21a1* can ameliorate systemic steroid metabolism in mice with 21-OHD. Two protocols for gene induction were tested in this study. First, we transduced *Cyp21a1* into primary fibroblasts of the 21-OHD mice using an RV vector. *In vitro* assays demonstrated that the *Cyp21a1*-expressing fibroblasts uptake progesterone from the culture media, metabolize it into DOC, and then release DOC back into the media. Transduced fibroblasts were autotransplanted into the subcutaneous tissues of 21-OHD mice. Four weeks after autotransplantation, the serum progesterone/DOC ratio was decreased in four of six animals. These results indicate that autotransplantation of *ex vivo* transduced fibroblasts represents a potentially effective but limited treatment protocol. The number of infected fibroblasts or the titer of the viral vector may be insufficient to exert significant effects of systemic steroid metabolism in all mice. In addition, this protocol required several weeks to obtain sufficient numbers of the primary fibroblasts from mouse tail samples. Thus, we tested another protocol consisting of direct injection of the *Cyp21a1*-containing AAV vector into the thigh muscles. Four weeks after injection, the serum progesterone/DOC ratio was significantly reduced in all four mice. No adverse events were observed in the mice. Although our sample size was small, these findings indicate for the first time that extra-adrenal induction of *Cyp21a1* improves

systemic steroid metabolism in CAH animals.

Previously, Tajima *et al.* reported that intra-adrenal administration of a human *CYP21A2*-containing adenoviral vector could compensate enzymatic defects in 21-OHD mice [4]. In their study, the authors described the technical difficulty of intra-adrenal injections. Our data indicate that less invasive methods for gene induction can also ameliorate biological features of 21-OHD mice. It appears that only faint expression of *Cyp21a1* in peripheral tissues is sufficient to reduce the serum progesterone/DOC ratio (Supplemental Fig. 1E). Since the clinical severity of CAH patients reflects the degree of residual activity of the mutant *CYP21A2* [1], a mild increase in enzymatic activity in extra-adrenal tissues may benefit patients with severe enzymatic defects. Indeed maintenance of minimal cortisol production may help to prevent life-threatening adrenal crisis in these patients [13].

This study has some limitations. First, the 21-OHD mice do not produce adrenal androgen, and therefore, they are not strictly comparable to human patients with 21-OHD [8]. Second, the present study focused only on the short term effects of *Cyp21a1* induction. While our preliminary data obtained from one mouse suggest that the effect of *Cyp21a1* induction using AAV vectors may be maintained for more than 7 months, these results need to be validated in further studies. Third, our subject groups consisted of a limited number of animals, whose age and basal serum progesterone/DOC ratio were highly variable. In future studies, mice should be divided into subgroups according to their age and basal hormone values.

To date, a number of viral vectors have been employed for gene therapy [14]. Of these, AAV vectors have yielded particularly promising results in multiple phase I-III clinical trials [15]. For example, in clinical trials for familial lipoprotein lipase deficiency, AAV vector intravenous injection resulted in stable gene expression and protein activity and was approved as a therapeutic option in the European Union [16]. Notably, AAV vectors have only weak immunogenicity and are capable of delivering genes to various tissues to maintain stable expression [15]. In addition, AAV vectors are not integrated into the host genome and have not been associated with vector-induced malignancy [17]. Administration of AAV vectors to skeletal muscle *via* intramuscular injection has several advantages: (i) the procedure is technically uncomplicated; (ii) the risk of vector dissemination outside of the target tissue is relatively low; and (iii) pre-existing anti-AAV humoral immunity does not block transduction [15]. Thus, AAV vectors are good candidates as tools for *Cyp21a1/CYP21A2* induction in extra-adrenal tissues. Moreover, AAV vectors may suffice to deliver *Cyp21a1/CYP21A2* to adrenal glands, because Gong *et al*. successfully transferred *ABCD1* gene into adrenal glands of X-linked adrenoleukodystrophy model mice by intravenous injection of an AAV9 vector [18]. Thus, intravenous administration of AAV9 vectors containing *Cyp21a1/CYP21A2* may be a promising therapeutic option for 21-OHD.

Collectively, our results indicate that AAV-mediated *Cyp21a1* transduction into extra-adrenal tissues can ameliorate systemic steroid metabolism in 21-OHD mice. This suggests a novel therapeutic strategy for CAH, which warrants further investigations.

Acknowledgments

The authors are grateful to Dr. Toshihiko Shiroishi (Research Organization of Information and Systems, National Institute of Genetics) for providing us the *H-2*^{aw18} in C57BL/10SnSlc (*H-2*^{b}) mice. The authors thank Dr. Julian Tang (Department of Education for Clinical Research, National Center for Child Health and Development) for editorial assistance. The authors also thank Ms. Shigeko Honjyo for her secretarial assistance, Ms. Atsuko Nagashima-Miyokawa for her technical assistance, and Dr. Hideki Tsumura for his support on animal handling.

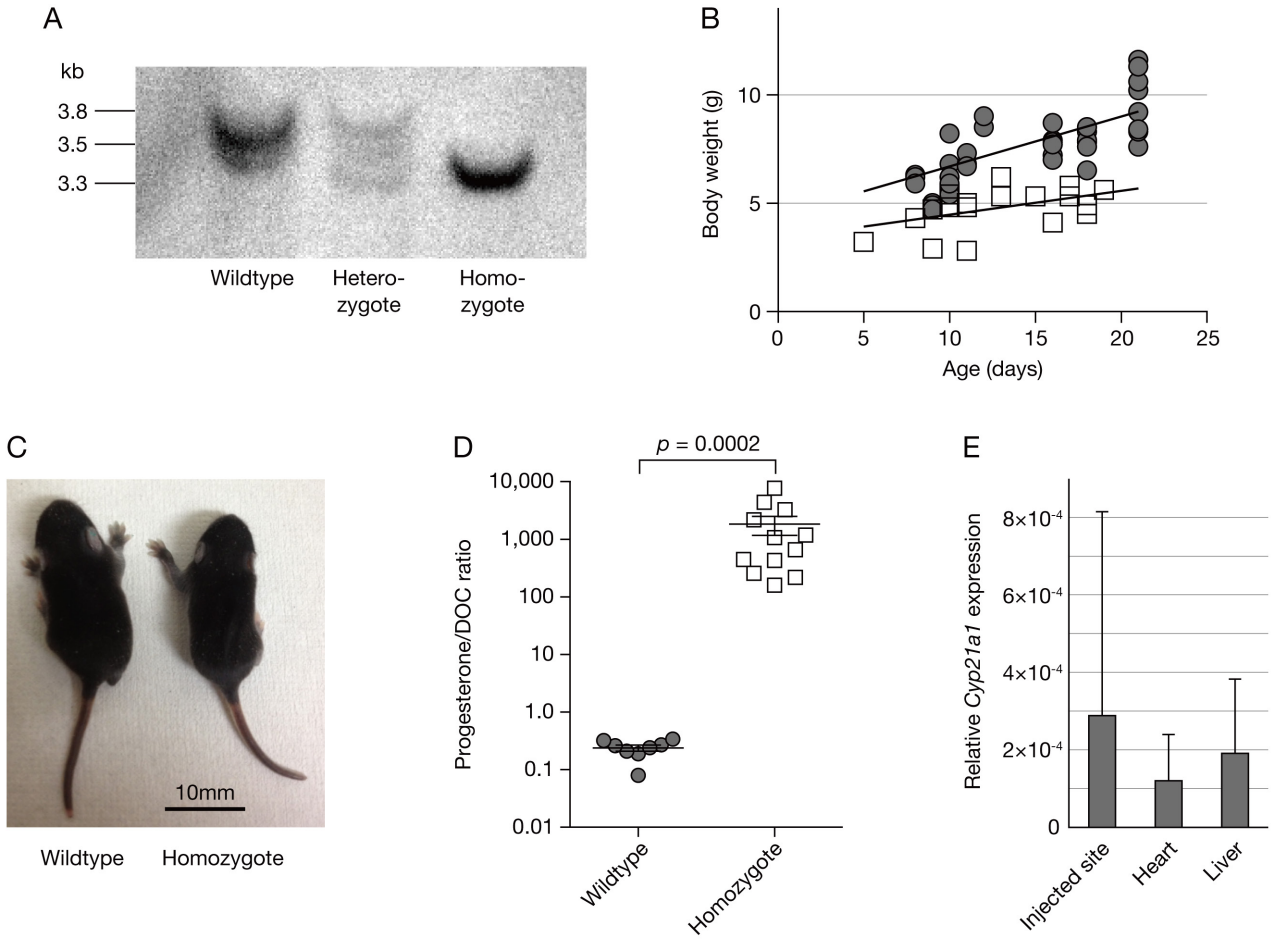
This work is supported by a Grant for Research on Grant-in-Aid for Scientific Research (C) (22591148, 25461575) from Japan Society for the Promotion of Science.

Disclosure

The authors have nothing to disclosure.

Author Contributions

Y.N. and S.P. conceived and designed the study, Y.N., M.M. and N.K. performed experiments and analyzed and interpreted the data, R.H., M.O. and T.O. contributed reagents/materials/analysis tools, Y.N. and M.F. wrote the paper.



Supplemental Fig. 1 Characterization of 21-hydroxylase deficiency mice used in this study

A, Southern blot hybridization of genomic DNA. Genomic DNA samples were digested with *Taq*I and probed with a mouse *Cyp21a1* cDNA.

B, Body weight of mice (solid circles, wildtype mice; open squares, homozygous mutant mice).

C, Appearance of mice at 10 days of age.

D, The Serum progesterone/deoxycorticosterone (DOC) ratio (solid circles, wildtype mice; open squares, homozygous mutant mice).

E, *Cyp21a1* mRNA expression in tissues of mice at 4 weeks after AAV vector injection. Target mRNA expression relative to that of *Gapdh* is shown. Relative mRNA levels were compared to those in the adrenal glands of wildtype mice.

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