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Utility and limits of *Hprt-Cre* technology in generating mutant mouse embryos

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Abstract

Background—*Hprt-Cre* doubles the prevalence of homozygous null embryos per litter *versus* heterozygous breedings without decreasing litter size. Resulting mutant embryos are genotypically and phenotypically equivalent between strategies. We set out to confirm the effectiveness of this approach with other alleles and hypothesized that it would increase efficiency in generating compound mutants.

Materials and methods—Null mutants for *Cyp26b1*, *Pitx2*, and *Shh* were generated with *Hprt-Cre* from conditional alleles as were double and triple allelic combinations of *Fgfr2IIIb*, *Raldh2*, and *Cyp26b1*. Embryos were genotyped and phenotyped by whole mount photography, histology, and immunohistochemistry.

Results—Fifty percent of *Hprt-Cre* litters were homozygous null for *Cyp26b1* (15/29) and *Pitx2* (75/143), with phenotypic and genotypic equivalence to mutants from standard heterozygous breedings. In multi-allele breedings, mutant embryos constituted half of litters without significant embryo loss. In contrast, *Shh* breedings yielded a smaller ratio of embryos carrying two recombined alleles (6 of 16), with a significant litter size reduction because of early embryonic lethality (16 live embryos from 38 deciduae).

Conclusions—*Hprt-Cre* can be used to efficiently generate large numbers of mutant embryos with a number of alleles. Compound mutant generation was equally efficient. However, efficiency is reduced for genes whose protein product potentially interacts with the *Hprt* pathway (e.g., *Shh*).

Keywords

Hprt-Cre; Homozygous null mutants; Mouse; Efficiency; *Cyp26b1*; *Shh*; *Pitx2*; *Raldh2*; *Fgfr2IIIb*; Compound mutants

1. Introduction

Cre recombinase technologies have been a major advance in the study of epithelial–mesenchymal reciprocal signaling during mouse development. They allow for tissue specific mutations, although preserving normal levels of gene expression in adjacent tissue. Another application of *Cre* technologies is in increasing the efficiency and production of homozygous mutant embryos [1,2]. Increased efficiency is accomplished by introducing the

Cre into the female germ-line under the control of the hypoxanthine-guanine phosphoribosyltransferase (*Hprt*, a housekeeping gene) promoter enhancer. The *Cre* is expressed in oogenesis and is present before ovulation and fertilization. Shortly after fertilization, the *Cre* protein will recombine with a conditional allele introduced by the spermatozoa [2]. If the female carries one copy of a recombined allele and the male is homozygous conditional for that same allele, then 1/2 of the progeny will be homozygous mutants. The targeted gene will be recombined from fertilization through all developmental stages.

The *Hprt-Cre* breeding strategy is significantly more efficient than traditional heterozygous breedings. It reduces the number of matings required to generate a desired number of null embryos by doubling the percentage of mutants per litter. To date, this approach has only been fully characterized with one conditional allele, that is, *Fgfr2IIIb* [1]. We were interested in duplicating this methodology with several early embryonic conditional alleles. We also wanted to determine whether this approach could be used to successfully generate compound mutants from combinations of conditional alleles. Finally, we hypothesized that this technology would not work with alleles that produced proteins that may interact with the *Hprt* pathway. To test this, we generated homozygous null mutants for *Pitx2*, *Cyp26b1*, and *Shh*. Compound mutants homozygous for *Fgfr2IIIb* and heterozygous for either *Raldh2* or *Raldh2* and *Cyp26b1* were generated. We observed that homozygous null mutants could be generated using conditional alleles for *Pitx2* and *Cyp26b1* with preservation of the litter size and 50% of the embryos being homozygous nulls. Double and triple compound mutants were also easily generated and constituted 50% or more of the embryos in a litter. However, when applied to a gene that has potential interactions with the *Hprt* signaling during development, fetal mortality was significant and the number of both heterozygous and homozygous mutants decreased dramatically relative to the number of deciduae.

2. Materials and methods

2.1. Animals

Institutional Animal Care and Use Committee (IACUC) approval for these studies was obtained from the University of Wisconsin School of Medicine and Public Health (P.F.N. protocol #M02258). All animals were maintained in a clean facility with *ad libitum* access to fresh food and water, and kept on a 12 h alternating light–dark cycle. *Hprt^{Cre/Cre}* females were bred to *flox/flox* males for each gene of interest (*Pitx2*, *Cyp26b1*, or *Shh*) [3–5]. Male pups were sacrificed and female pups were genotyped by toe clipping before postnatal (P) day 10 for *Hprt-Cre*, *wild-type*, *null*, and *floxed* alleles for each gene. The genotype of all female pups was *rec/+* for the gene of interest and *Hprt^{Cre/+}*. *Rec/rec* embryos for each gene of interest were generated by breeding mature *rec/+*; *Hprt^{Cre/+}* females to *flox/flox* males for each gene of interest. Genotyping of embryos for each allele was performed as described previously [3–5]. Ratios of *rec/rec* and *rec/+* embryos were determined. For double and triple compound mutants *Fgfr2IIIb^{flox/flox}*; *Raldh2^{flox/flox}* or *Fgfr2IIIb^{flox/flox}*; *Raldh2^{flox/flox}*; *Cyp26b1^{flox/flox}* males were bred to *Fgfr2IIIb^{rec/+}*; *Hprt^{Cre/+}* females to generate *Fgfr2IIIb^{rec/rec}*; *Raldh2^{rec/+}* or *Fgfr2IIIb^{rec/rec}*; *Raldh2^{rec/+}*; *Cyp26b1^{rec/+}* embryos. All embryos were genotyped by polymerase chain reaction [6–9].

2.2. Morphologic studies

Embryos were harvested at E12.5, E13.5, E16.5, E17.5, or E18.5. Whole mount photographs were taken of embryos under a stereoscopic dissecting microscope. For assessment of cleft palate, the mandible was removed after fixation and the palate was photographed.

2.3. Histology

Harvested embryos were dissected free of the yolk sac and fixed overnight at 4° C in 4% paraformaldehyde. They were then dehydrated through a series of escalating methanol and PBST washes, isopropyl alcohol, and xylene. The embryos were washed three times in paraffin at 60° C in a vacuum oven and then embedded head up in paraffin. Sections of 7- μ m thickness were taken. They were stained with standard hematoxylin and eosin and photographed under a light microscope (Olympus BX41 DP25) running CellSens Standard (OlympusAmerica, version 1.7.1, Center Valley, PA).

2.4. Immunohistochemistry

Heterozygous and homozygous embryos were harvested, fixed, and embedded in paraffin as described previously. Transverse sections of 7- μ m thickness were generated and processed for detection of PITX2 protein. Immunohistochemistry procedure is published elsewhere [10]. Briefly, we used a 10 mmol/L citric acid epitope recovery with primary antibody Anti-PITX2 (Abcam, Cambridge, MA [ab111266]) at 1:100 in RGBT (blocking buffer and secondary antibody Goat anti-rabbit Alexa Fluor 594 (Jackson ImmunoResearch, West Grove, PA [111-585-144]) at 1:250 in RGBT (normal goat sera, 1% bovine serum albumin fraction 5) blocking buffer. Slides were counter stained with Vectashield Hard Set containing DAPI (4',6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA [H-1500]), cover slipped, and allowed to set overnight at 4° C. Processed sections were then imaged using a fluorescent microscope (Olympus BX53 outfitted with a Olympus DP80 camera, x-Cite series 120Q light source [Lumen Dynamics, Mississauga, Ontario, Canada]) and CellSens Dimension software (OlympusAmerica, version 1.8.1) for DAPI to visualize DNA and Alexa594 to visualize PITX2 protein (at 10 $\times$ magnification).

2.5. Statistical analysis

Statistical analysis was performed with *P* values calculated by Fisher Exact test for percentage homozygous embryos and for embryonic lethality for each allele.

3. Results

It has been previously reported that homozygous null embryos can be generated with greater efficiency than the standard heterozygous breedings using *Hprt-Cre* in combination with a conditional allele [1]. This was fully characterized for only one gene, that is, *Fgfr2IIIb*. To determine if this could be done with other genes, we obtained conditional alleles for *Pitx2* and *Cyp26b1* and used the *Hprt-Cre* breeding strategy. We were able to consistently generate 50% null embryos without an appreciable reduction in the litter size (Table 1).

Pitx2^{rec/rec} and *Cyp26b1^{rec/rec}* mutants appeared externally identical to the homozygous nulls described in the literature (Figs. 1 and 2) [4,11]. Additionally, genotyping of the embryos demonstrated the absence of the floxed allele for either gene in both heterozygous and homozygous null embryos (Figs. 1 and 2). Histologic analysis of phenotype for *Cyp26b1^{rec/rec}* embryos demonstrated abnormal limbs, epidermis, and whole mount photographs demonstrated a cleft of the secondary palate (Fig. 3) as have been described previously for these mutants [7,12,13]. Hematoxylin and eosin staining at E13.5 of *Pitx2^{rec/rec}* mutants showed incomplete closure of the abdominal wall with ectopic viscera including the lungs, heart, and intestine as has been reported previously for these mutants [14]. Furthermore, immunostaining demonstrated PITX2 around the neural tube of heterozygous *Pitx2* embryos, but a lack of the protein around the neural tube of *Pitx2* homozygous embryos (Fig. 4).

The *Hprt-Cre* breeding strategy was less successful in generating both homozygous and heterozygous mutants for *Shh* (Table 2). Homozygous mutants were observed at all stages between E12.5 and E18.5, but comprised a smaller percentage of the embryos compared with *Cyp26b1* or *Pitx2*. This, however, did not reach statistical significance ($P = 0.5$). Pups displayed abnormalities in head, limbs, and notochord development as has been reported for these mutants. More notably, there was a significant increase in embryo loss compared with *Cyp26b1* and *Pitx2* with viable embryos present in only 42% of deciduae indicating significant in utero lethality after placentation ($P = 5.312 \times 10^{-9}$).

Finally, we wanted to determine whether the *Hprt-Cre* breeding strategy could be successfully used to generate either double or triple compound mutants as originally postulated [1]. Double and triple homozygous-heterozygous compound mutants *Fgfr2IIIb^{rec/rec}; Raldh2^{rec/+}* and *Fgfr2IIIb^{rec/rec}; Raldh2^{rec/+}; Cyp26b1^{rec/+}* were generated and constituted 50% of the embryos per litter (Table 3). This was achieved without a significant decrease in the litter size.

4. Discussion

With intensifying federal oversight and reductions in federal funding, the cost of vertebrate, and in particular mammalian, research continues to increase. Investigators are being pushed to reduce costs, minimize animal usage, and maximize efficiency. We have previously reported that the efficiency of generating homozygous null mutants can be significantly increased by using the *Hprt-Cre* breeding strategy (Fig. 5) [1]. This approach can generate homozygous null embryos effectively identical to those generated using a standard heterozygous breeding strategy. The drawback of the original study was that it was conducted using a conditional allele for only one gene, that is, *Fgfr2IIIb*. A true demonstration of the efficacy of this approach depends on reproducing these results with other conditional alleles. We also wanted to determine (1) if this approach would work with alleles for genes that could potentially interact with the *Hprt* pathway during development and (2) whether compound alleles could be generated with greater efficiency than through the standard heterozygous breeding strategy.

We observed with conditional alleles for *Pitx2* and *Cyp26b1* that homozygous nulls were easily generated and constituted 50% of the litters without a significant reduction in the litter size. In contrast, using the *Hprt-Cre* breeding strategy with a condition allele for *Shh* resulted in a significant reduction in the litter size, in comparison with the other alleles. There was a smaller number of homozygous mutants at all points examined compared with the other genes; however, this did not reach statistical significance. These data suggest that the loss of *Shh* signaling possibly in combination with *Hprt* heterozygosity results in significant *in utero* lethality. The extent of this interaction and how *Hprt-Cre* contributes is not entirely clear. However, haplo-insufficiency of *Shh* results in impaired maternal cholesterol synthesis, which is critical in *Shh* pathway signaling. Furthermore, decreased maternal levels of cholesterol increase the rate of preterm delivery [15]. As *Hprt* is a housekeeping gene in the placenta [16], the large number of deciduae without viable embryos suggests that the increase in *in utero* mortality is the murine equivalent of human preterm delivery. Whether direct interaction between the products of these two genes is responsible for the high rate of embryos loss is speculative. In any event, when using the *Hprt-Cre* breeding strategy one should keep in mind the possibility that any interactions between the gene of interest and *Hprt* may significantly alter the efficiency and success of this approach in generating null embryos.

Finally, we wanted to determine if this approach could be used to successfully mutate multiple genes simultaneously. We were able to generate double and triple compound

mutants that were homozygous null for *Fgfr2IIb* and heterozygous for *Raldh2* alone or a compound mutation of *Raldh2* and *Cyp26b1*. We observed a minimal reduction in the litter size with 50% of the litters being homozygous for *Fgfr2IIb* and all embryos being heterozygous for the additional genes of interest. Thus, one of every two embryos in the litter was a compound mutant. This indicated that compound mutants can be easily generated with this approach. The efficiency of this approach increases logarithmically (Fig. 6) as alleles are added. For example, when using the heterozygous breeding strategy, only one in eight embryos would be a double compound homozygous-heterozygous mutant. For three alleles only, one in 16 embryos would be a triple compound homozygous/heterozygous-heterozygous mutant. In contrast, with the *Hprt-Cre* breeding strategy homozygous/heterozygous double and triple compound mutants constituted 50% of all litters.

One caveat to the *Hprt-Cre* approach is that it cannot be used with other *Cres* as the gene(s) of interest are mutated at conception. Thus, mutation of the target gene will occur at the single-cell zygote stage ensuring that all cells in the embryo carry the mutation and rendering subsequent Cre activity obsolete. Another caveat is that there is no clear way to use this technology in a tissue-specific manner. Addition of a tissue-specific enhancer in the *Hprt* locus upstream of the Cre would potentially inhibit Cre expression at conception without conveying any tissue-specific advantage. If there was tissue-specific expression then the Cre, under optimal circumstances, would have equivalent function to a knock-in tissue-specific Cre such [17] or a tissue-specific trans-Cre [18].

We strongly encourage the use of this breeding approach, although keeping in mind that there will be an occasional conditional allele for which it will not be effective. Nevertheless, this approach significantly reduces overall animal colony size, whereas improving the efficiency in generating null mutants.

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Fig. 1. *Cyp26b1*^{rec/rec}; *Hprt*^{Cre/+} fetuses appear identical to *Cyp26b1*^{-/-} fetuses. (A) *Cyp26b1*^{rec/+} control and (B) *Cyp26b1*^{rec/rec}; *Hprt*^{Cre/+} fetuses at E18.5. Close up photographs of the (C) eyes, upper (D) and lower (E) limbs of the *Cyp26b1*^{rec/rec}; *Hprt*^{Cre/+} fetuses. (F) Polymerase chain reaction demonstrating the absence of the floxed and wild-type allele in the *Cyp26b1*^{rec/rec}; *Hprt*^{Cre/+} mutant (*rec/rec*).

Fig. 2. *Pitx2*^{rec/rec}; *Hprt*^{Cre/+} fetuses appear identical to *Pitx2*^{-/-} fetuses. (A) *Pitx2*^{rec/+}; *Hprt*^{Cre/+} control and (B) *Pitx2*^{rec/rec}; *Hprt*^{Cre/+} at E12.5. (C) Polymerase chain reaction demonstrating the absence of the floxed and wild-type allele in the *Pitx2*^{rec/rec}; *Hprt*^{Cre/+} mutant (*rec/rec*). White arrow indicates ectopic viscera herniating through defect in the body wall.

Fig. 3. *Cyp26b1* Heterozygous and homozygous embryo histologic analysis. (A) *Cyp26b1* heterozygous embryos demonstrate normal limb development, (B) whereas *Cyp26b1* homozygous mutants have malformed, small limbs (limbs indicated by orange arrowhead). (C) The epidermis of the *Cyp26b1* heterozygous embryos exhibit normal developing hair follicles (green arrowhead), (D) whereas the *Cyp26b1* homozygous mutants have a lower instance, or lack of, hair follicles when viewing similar-sized regions of epidermis. (E) Whole mount, gray-scaled, coronal images of the *Cyp26b1* heterozygous mice show phenotypically normal closure of the palate. (F) Whole mount, gray-scaled, coronal images of *Cyp26b1* homozygous embryos indicate a cleft of the secondary palate. The palatal shelves of the homozygous embryos have not elevated or closed, unlike the normally developing palates of the heterozygous embryos. PP = primary palate, PS = palatal shelves. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Fig. 4. *Pitx2* Heterozygous and homozygous mutant histologic analysis. (A) Hematoxylin and eosin staining of heterozygous *Pitx2* embryos show a phenotypically normal thoracic region, (B) whereas homozygous *Pitx2* mutants exhibit incomplete closure of the thoracoabdominal wall (green arrowhead). (C) The heart of heterozygous *Pitx2* embryos appears normal, (D) whereas the heart of homozygous *Pitx2* mutants is asymmetrical. (E) Immunostaining for PITX2 protein indicated the presence around the neural tube of heterozygous *Pitx2* embryos (yellow arrowheads), (F) but a distinct lack of PITX2 around the neural tube of the homozygous *Pitx2* mutants. The scale bar for the immunostaining images is 200 μ m. For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.

Fig. 5.

Heterozygous breeding strategy *versus* *Hprt-Cre* strategy. (A) In standard heterozygous breeding a male mouse heterozygous for *Gene A* is mated to a wild-type female. This results in 50% heterozygous progeny and 50% wild-type progeny. Following genotyping, the wild-type pups are sacrificed and the mature male and female heterozygous animals are bred to each other. From this mating 25% of pups will be homozygous nulls for *Gene A*. (B) Using the *Hprt-Cre* strategy, a female mouse homozygous for *Hprt-Cre* is mated to a male mouse homozygous for a conditional allele *Gene A*. Fifty percent of progeny will be heterozygous for *Hprt-Cre* and *Gene A*. The male progeny are sacrificed, whereas the female progeny are mated back to the original male that is homozygous for the conditional allele *Gene A*. Fifty percent of the resulting progeny from this second mating are homozygous recombined (null), for *Gene A*.

Fig. 6.

Hprt-Cre breedings also increase in efficiency with compound mutations. (A) In a heterozygous breeding strategy, a heterozygous female mouse for *Gene A* is bred to a heterozygous male for *Gene B*. This yields progeny where 25% are heterozygous for both genes. Resulting double heterozygous males are bred to females heterozygous for *Gene A*, whereas the other animals are sacrificed. Of the resulting progeny, 12.5% will be homozygous for *Gene A* and heterozygous for *Gene B*. (B) In contrast, generating homozygous null mutants through the *Hprt-Cre* breeding strategy is more efficient. A female mouse homozygous for *Hprt-Cre* is mated to a male homozygous for a conditional allele *Gene A*. This results in 50% of progeny as females heterozygous for *Hprt-Cre* and the recombined allele for *Gene A*. These female mice are bred to a male that is double homozygous for conditional alleles A and B. Within resulting progeny, 50% are homozygous for *Gene A* and heterozygous for *Gene B*.

Table 1

Comparison of *Pitx2* and *Cyp26b1* embryos from the *Hprt-Cre* breeding strategy.

For the *Pitx2* *Hprt-Cre* matings, there were 16 litters with an average litter size of 8.94. Within that group, 52.45% (75/143) of viable embryos were null mutants for *Pitx2*. Only 13.86% (23/166) of the deciduae lacked a viable pregnancy. For the *Cyp26b1* matings, there were three litters with an average litter size of 9.67. Within that group, 51.72% (15/30) of viable embryos were null mutants for *Cyp26b1*. Only 3.3% (1/30) of the deciduae lacked a viable pregnancy.

Table 2

Shh embryos from the *Hprt-Cre* breeding strategy.

For the *Shh Hprt^{Cre}* matings, there were five litters with an average litter size of 5.33. The percentage of mutants from viable embryos from all matings was 37.5% (6/16). However, the number of deciduae lacking a viable pregnancy across all matings ranged from 37.5% (3/8) to 85.71% (6/7).

Table 3

Compound mutants generated using the *Hprt-Cre* breeding strategy.

For the *Fgfr2IIIb; Raldh2 Hprt^{Cre}* matings, there were five litters with an average litter size of 8.20. Within that group, 48.78% (20/41) of viable embryos were null mutants for *Fgfr2IIIb^{rec/rec}; Raldh2^{rec/+}*. Only 8.89% (4/45) of the deciduae lacked a viable pregnancy. For the *Fgfr2IIIb; Raldh2; Cyp26b1; Hprt^{Cre}* matings, there were four litters with an average litter size of 8.50. Within that group, 61.76% (21/34) of viable embryos were null mutants for the *Fgfr2IIIb^{rec/rec}; Raldh2^{rec/+}; Cyp26b1^{rec/+}*. Only 8.1% (3/37) of the deciduae lacked a viable pregnancy.