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Esrrb-Cre Excises loxP-Flanked Alleles in Early Four-Cell Embryos

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Abstract

Among transgenic mice with ubiquitous Cre recombinase activity, all strains to date excise *loxP*-flanked (floxed) alleles, either at or before the zygote stage or at nondescript stages of development. This manuscript describes a new mouse strain, in which Cre recombinase, integrated into the *Esrrb* locus, efficiently excises floxed alleles in pre-implantation embryos at the onset of the four-cell stage. By enabling inactivation of genes only after the embryo has undergone two cleavages, this strain should facilitate *in vivo* studies of genes with essential gametic or zygotic functions. In addition, this study describes a new, highly pluripotent hybrid C57BL/6J × 129S1/SvImJ mouse embryonic stem cell line, HYB12, in which this knock-in and additional targeted alleles have been generated.

Keywords

Cre recombinase; *Esrrb*; knock-in; four-cell embryos; hybrid ES cells

INTRODUCTION

Since its inception nearly two decades ago, Cre-*lox* technology has facilitated investigation of mammalian gene function in numerous adult and embryonic cell types (Wang, 2009). Several Cre transgenes are known to excise *loxP*-flanked (floxed) genes throughout the entire organism, some by acting in the germ-line of either parent or shortly following fertilization and some through ubiquitous deletion at nondescript stages of development (Notable examples: Schwenk et al., 1995; Lakso et al., 1996; O'Gorman et al., 1997; Lewandoski et al., 1997a; Lewandoski et al., 1997b; Hayashi et al., 2002; Liu et al., 2011). A few recent developments may enable ubiquitous allele excision in a temporally-controlled manner, using either tamoxifen-inducible Cre or *ex vivo* manipulation (e.g., Tabansky et al.,

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2013; Ryder et al., 2014). However, these methods are either insufficiently temporally penetrant (tamoxifen) or technically cumbersome (*in vitro* manipulation). To date, no strain that enables temporal distinction of ubiquitous gene function between the zygote and blastocyst stages as is, without further experimental intervention, has been reported. This type of capability may help to streamline inactivation of genes whose essential functions in the gamete or zygote prohibit study of their ubiquitous or specialized functions in the multicellular embryo. Here, we describe a new Cre strain that leaves floxed alleles intact through the first two egg cleavages, excising them efficiently in all blastomeres of the nascent four-cell embryo.

The work leading to the establishment of this mouse was originally designed in pursuit of a robust, specific pan-placental Cre strain, which is still at large. An ongoing limitation to this type of effort has been the elusiveness of information on genes that are expressed in all trophoblasts or their progenitors but not in any cells of the embryo proper or their precursors. We honed in on *Esrrb* (*ERRβ*; *Nr3b2*) – an orphan nuclear receptor whose ablation results in mid-gestation lethality due to severe placental defects (Luo et al., 1997). During early mouse embryonic development, *Esrrb* is expressed in a restricted subset of extraembryonic cells and, later, in the chorionic layer of the nascent placenta, followed by a precipitous decline by E8.5, foreshadowing definitive placentation (Luo et al., 1997). Consistent with its expression in the chorion, which harbors presumptive trophoblast progenitor cells that populate the placenta, *Esrrb* is expressed abundantly in undifferentiated trophoblast stem cell cultures and is suppressed within one day of their differentiation (Tanaka et al., 1998). Additional studies found that *Esrrb* is also expressed and carries out discrete biological functions in several nonessential adult tissues that would not interfere with the interpretation of trophoblast-specific functions, including germ cells, inner ear, endometrium, and rod photoreceptors (Mitsunaga et al., 2004; Chen and Nathans, 2007; Bombail et al., 2008; Onishi et al., 2010). Of some concern was a body of reports that identified *Esrrb* in embryonic stem cells (ESC) and implicated it as a crucial determinant in their self-renewal (Festuccia et al., 2012; Martello et al., 2012, and references therein). However, considering that *Esrrb* is entirely dispensable outside the placenta for embryonic survival (Luo et al., 1997), we gambled on its behavior in ESC culture not carrying over to the whole organism and on its expression in epiblast progenitors being meager enough to avoid significant Cre activity in these cells. While this expectation was overoptimistic in hindsight, the resulting mouse turned out useful in an unintended fashion.

RESULTS AND DISCUSSION

Having routinely used the 129S4/SvJae-derived ESC line J1 for gene targeting (e.g., Barak et al., 1999; Kim et al., 2007), this report marks the establishment and first use of HYB12, a new WT C57BL/6J (B6) × 129S1/SvImJ (129Sv) hybrid ESC line. Several basic rationales guided this strategic retooling: (a) Hybrid B6129Sv ESC are significantly superior to either 129Sv or B6 inbred ESC in terms of both overall pluripotency and their ability to sustain it throughout prolonged growth in culture (Eggan et al., 2001; Schwenk et al., 2003). (b) Most state-of-the-art mouse genetics studies are conducted on a B6 background after a multi-generation introgression (Seong et al., 2004; Bouabe and Okkenhaug, 2013); when loci targeted in inbred 129Sv ESC are backcrossed onto B6, 129Sv sequences surrounding the

targeted locus are perpetually retained. A hybrid configuration, which carries one whole copy of the desired B6 genome, allows the targeting construct to integrate into the B6 chromosome and circumvent lingering genetic contamination, as well as providing a one-generation head start in the introgression process. (c) At the start of this study, the superior annotation of B6 genomic sequences in public databases and of B6-derived mouse genomic bacterial artificial chromosome (BAC) libraries archived at the Children's Hospital of Oakland Research Institute (CHORI) repository favored design and construction of targeting vectors based on B6 templates. Considering sequence variations between mouse strains, which might impinge on homologous recombination (HR) efficiency (Deng and Capecchi, 1992; te Riele et al., 1992), a recipient ESC carrying the B6 genome is a more prudent choice for HR of such vectors. (d) Hybrid ESC lines generated previously (You et al., 1998; Rideout et al., 2000) were only available at a disconcertingly late passage, and the specimens that we were able to obtain presented with mycoplasma and/or a faulty karyotype.

With these considerations in mind, and to facilitate this and additional gene targeting efforts, we derived permanent ESC lines from B6129Sv hybrid blastocysts. Several isolates gave rise to permanent clones, designated HYB10, -11, and -12, which exhibited typical ESC-like morphology (Fig. 1a,b). Of these, HYB10 and HYB12 were male (Fig. 1c) with a predominantly euploid karyotype (Fig. 1d,e); HYB11, a female hybrid clone, has subsequently differentiated and was not pursued further. Most pertinently, when microinjected into B6 blastocysts, both HYB10 and HYB12 yielded abundant chimeric males with near-complete agouti coat color (Fig. 1f and data not shown), which transmitted this pigmentation to approximately 50% of all progeny (Fig. 1g), indicating a near-100% ESC-derived germ-line. To date, five different targeting vectors, including the one described here, were successfully introduced into mice via the HYB12 cell line, all yielding high-percentage chimeras with a cumulative germ-line transmission rate of ~100% (115/227 Agouti-colored F1 progeny, and independently, 116/227 heterozygous progeny).

Because the *Esrrb* promoter has not been characterized, we opted to design the *Esrrb*-driven Cre *via* knock-in to ensure preservation of the entire regulatory information necessary and sufficient for faithful Cre expression. In addition, because *ESRRB* is a crucial regulator of placental development (Luo et al., 1997), we sought to mitigate concerns about potential contributions of *Esrrb* haploinsufficiency to any observed phenotype by expressing Cre with minimal disruption of the integrity or expression of the host gene. Guided by these considerations, an internal ribosome entry sequence (*IRES*)-driven Cre was inserted at the start of the long 3' non-coding sequence (3'-NCS) of *Esrrb*. This design, shown in Fig. 2a, preserved the entire *Esrrb* coding sequence alongside Cre, while at worst, compromising putative attributes requiring an intact 3'-NCS. A different design, in which Cre was integrated in lieu of the first coding exon of *Esrrb*, was also generated and targeted successfully into mouse ESC (Fig. S1), but has not been pursued at this point.

The targeting vector was constructed by means of recombineering (see Methods), and HYB12 cells were transfected with the linearized plasmid. HR was observed in 40 out of 96 ESC clones, of which 12 were expanded and reconfirmed for accurate targeting using probes outside both the 5' and 3' genomic arms of the construct (Fig. 2b,c shows four such clones).

Among these, two clones were injected into B6 blastocysts, both yielding high-grade chimeras that transmitted the allele through the germ-line. Live progeny of one clone were introgressed onto B6 and analyzed. In addition, mice homozygous for the allele were born at expected Mendelian frequencies, confirming that the targeting strategy had no major impact on the essential placental functions of *Esrrb*; however, attempts to breed males and females homozygous for the allele were unsuccessful, leaving their fertility status in question. This potential infertility could reflect interference of the *Esrrb-IRES-Cre* knock-in with the known functions of *Esrrb* in male and female germ cell proliferation, manifested in reduced sperm counts in *Esrrb*-null males and infertility of *Esrrb*-null females (Mitsunaga et al., 2004). For example, the interruption of the 3'-NCS by *IRES-Cre* or the ectopic presence of the *PGK-neo* expression cassette within the locus might have disrupted aspects of *Esrrb* expression regulation that are either germ cell-specific or ubiquitous, but with functional consequences in germ cells and not trophoblasts.

To evaluate activity patterns of *Esrrb-IRES-Cre*, heterozygous males were bred with females homozygous for *mT/mG* – a reporter driven by a CMV β -actin enhancer-promoter integrated into the ubiquitously expressed ROSA26 locus, in which a floxed membrane-tethered *TdTomato* (mT) gives way to membranous GFP (mG) upon excision by Cre (Muzumdar et al., 2007). At E9.5 and beyond, both fetal and placental tissues of Cre-positive embryos were uniformly GFP-positive (Fig. 3a-d). Moreover, blastocysts from similar crosses partitioned between entirely GFP-positive and TdTomato-positive (Fig. 3e), consistent with Cre-mediated excision in the entire embryo prior to blastocyst formation.

To determine the onset of *Esrrb-IRES-Cre* action, *in vitro* cultures of pre-implantation embryos were imaged by time-lapse video confocal microscopy. Clear GFP fluorescence could be observed in several 8- to 16-cell embryos retrieved from homozygous *mT/mG* females mated with *Esrrb-IRES-Cre* males, indicating that Cre acts prior to that juncture; the signal was uniform across all cells of these embryos and intensified as they developed further (Fig. 3f). Next, imaging was performed, starting at the two-cell stage. Combined red and green fluorescence at 45-min intervals (Fig. 3g), reinforced by careful inspection of mG (Fig. 3h), enabled determination of the earliest emergence of GFP and concomitant cessation of TdTomato accumulation. The earliest definitive mG signal was detected in one embryo approximately 1.5h after it cleaved into four cells (Fig. 3h, 08:15, green arrowhead; contrast with a Cre-negative embryo, red arrowhead). In a second embryo, cleaved later, mG was first observed approximately 0.75h post-cleavage (Fig. 3h, 12:45, green arrowhead). These observations suggested that *Esrrb-Cre* excises floxed alleles very close to the onset of the four-cell stage.

Considering the time required for synthesis and detectability of mG after Cre-mediated *mT/mG* excision, one may conceivably argue that Cre action may have taken place prior to the second cleavage, at the end of the two-cell stage. However, the former of the two embryos shown, which provides a rare example of incompletely penetrant *Esrrb-IRES-Cre* action, counters this concern. This embryo harbors three mG⁺ blastomeres (Fig. 3g, 18:45, open green arrows) and one mG⁻/mT⁺ blastomere (open red arrow), a partition ratio highly consistent with excision after cleavage into four cells; earlier failure of Cre would have resulted, if only temporarily, in two mG⁺ plus two mG⁻ blastomeres. In the only other

example of incompletely penetrant *Esrrb-IRES-Cre* action, an eight-cell embryo was retrieved as a mosaic of two mG^+ and six mG^-/mT^+ cells that developed into a mosaic blastocyst (data not shown). This 3:1 Cre failure ratio further reinforces the notion of excision after the onset of the four-cell stage.

Nevertheless, mosaic excision of *mT/mG* was relatively infrequent, having been observed in only two of 28 GFP-positive pre-implantation embryos analyzed, including the one in Fig. 3g, and in none of the 14 Cre-positive post-implantation embryos and placentas imaged (Table 1). These data suggest that *Esrrb-IRES-Cre* excises floxed alleles in the entire four-cell embryo over 90% of the time.

The developmental expression and functions of *Esrrb* have not been studied nearly as extensively as those of the canonical trophoblast master regulator *Cdx2*. It is well established that while *Cdx2* is restricted to the extraembryonic lineage from the blastocyst stage onward, its expression is first detected, at least semi-ubiquitously, in 8-cell embryos and only later segregates strictly to the trophectoderm (Ralston and Rossant, 2008; Skamagki et al., 2013). This expression pattern makes *Cdx2* inadequate for driving trophoblast-restricted Cre. In contrast, the whereabouts of *Esrrb* during this critical period have not been reported. Insofar as the temporal Cre activity pattern seen here faithfully reflects endogenous *Esrrb* expression dynamics and is not an artifact of its 3'-NCS destruction or an effect of the *PGK-neo* insert, it invokes an early *Cdx2*-like induction pattern during pre-implantation development. This raises the possibility that the two could be conjointly regulated, epistatic and/or interdependent during embryonic cleavage and compaction. Irrespective of the underlying mechanism, this expression pattern undermined the original goal of harnessing *Esrrb* to drive a pan-placental Cre recombinase transgene.

Nevertheless, *Esrrb-IRES-Cre* mice are uniquely useful. As detailed earlier, to the best of our knowledge no Cre strain to date offers unambiguous temporal distinction of gene function between the zygote and blastocyst stage. Such a distinction is especially crucial in the case of genes that are essential for gametic or early zygotic or postzygotic processes, but are either nonessential or possess distinct functions at the four-cell stage or later. *Esrrb-IRES-Cre* mice provide a highly efficient deleter strain for distinguishing and functionally analyzing such genes. Notably, in the case of genes that play important roles at the immediate aftermath of *Esrrb-IRES-Cre* action in the pre-implantation embryo, allele excision is mitigated by the stability of extant mRNA and protein products and might therefore fail to exert an immediate effect.

METHODS

Establishment and validation of hybrid ESC lines

ESC lines were derived from B6 females mated with 129Sv males without hormonal stimulation, essentially as described (Chawla et al., 2001; Abbondanzo et al., 1993). Briefly, blastocysts were flushed in M2 medium (Sigma-Aldrich) from both uterine horns at E3.5 (0.5 designating the noon of copulation plug detection). Individual blastocysts were cultured in separate chambers of gelatin-coated four-well dishes layered with γ -irradiated mouse embryonic fibroblasts in standard ESC medium containing 15% ESC-grade Fetal Bovine

Serum (Invitrogen) and leukemia inhibitory factor (LIF, Chemicon). By the fourth day of culture, most blastocysts had yielded small cellular outgrowths, which were disaggregated and re-cultured. Within the next 4-7 days some of the wells contained ES-like colonies, which were expanded for two more passages and then cryopreserved.

Clones with persistent ES-like morphology were karyotyped as described (Navara et al., 2007). Sex of each clone was determined by PCR for the autosomal gene *LH β* (control; Chr. 7) and the Y chromosome-resident gene *SRY*, using the following oligonucleotides: *mLH β* forward – TGGCCGCAGAGAATGAGTTC; *mLH β* reverse – TCACAGGCCATTGGTTGAGTC-3'; *mSRY* forward – ATTTATGGTGTGGTCCCGTG; *mSRY* reverse – AAGCTTTGCTGGTTTTTGA. To confirm germ-line competence, single-cell suspensions of clones HYB10 and HYB12 were microinjected into B6 blastocysts and transferred to the uteri of pseudopregnant CD-1 females. Chimerism rate was determined from the ratio of agouti and black coating of the resulting pups. Several high-grade chimeras from each clone were mated with B6 females, and the ratio of agouti-colored to black progeny determined (whereby 100% transmission of the hybrid ESC-derived germ-line equals 50% agouti pups). Both ESC lines are available to the research community upon request.

Recombineering and Gene Targeting

Esrrb-IRES-Cre and *Esrrb-Cre* targeting vectors were constructed *via* recombineering methodology (Warming et al., 2005), with a few modifications. In short, 150 to 500bp-long homology boxes (HBs), corresponding to the extremities of the genomic arms of each design, were cloned in place of the floxed neo cassette of the vector PGKneolox2DTA (Soriano, 1997). The resulting plasmids were linearized between the two HBs, electroporated into heat-induced SW106 *E. coli* bacteria containing a BAC spanning the B6 *Esrrb* locus, and selected on LB-ampicillin plates for retrieval of the intervening genomic fragment by recombineering gap repair. Next, *IRES-Cre-Neo* or *Cre-Neo* cassettes flanked with PCR-amplified HBs corresponding to each side of the chosen insertion site were cloned into shuttle plasmids. Single contiguous restriction fragments encompassing the HBs and Neo cassettes were electroporated into heat-induced SW106 bacteria containing the respective *Esrrb* genomic fragments, selected with ampicillin and Kanamycin for cassette recombination, and verified by restriction digestion.

Targeting vectors were purified by CsCl banding followed by extraction with phenol/chloroform. Prior observations in our laboratory revealed that this preparation method yields 10-25 $\times$ higher HR efficiency in ESC compared to column-based purification and 4-7 $\times$ higher efficiency compared to CsCl banding without subsequent organic extraction; we have no compelling technical explanation for this empirical enrichment effect. Vectors were linearized, electroporated into HYB12 ESC, and selected as described (Kim et al., 2007), except that negative selection was based on diphtheria toxin A (DTA) exclusion rather than thymidine kinase activity. HR was determined by Southern blotting with probes residing outside one of the homology arms of either vector, and positive clones were expanded and revalidated with probes external to both arms. Several confirmed clones of *Esrrb-IRES-Cre*

were microinjected into B6 blastocysts to establish gene-targeted founders. *Esrrb-IRES-Cre* mice are now available through The Jackson Laboratory (Jax Stock Number: 28575).

Animals

B6 and 129Sv mice used here were from standing colonies propagated off stocks purchased from The Jackson Laboratory. *mT/mG* mice were purchased from The Jackson Laboratory (Strain #007676) and maintained as a homozygous stock. Experiments described here were performed with heterozygous *Esrrb-IRES-Cre* sires bred with homozygous *mT/mG* dams. Carriers of the targeted allele were introgressed onto a B6 background for several generations. Initial genotyping of the targeted allele was by general PCR for Cre DNA (He et al., 2003). A locus-specific PCR protocol that enables distinction from other Cre strains, as well as determination of homozygosity for the allele, uses the following primers: Common – CCAAAGCCGTGCAACACTTCTACAGT; WT – CTGTCCCAGGAGTCTGCACATG; insert-specific – GGCGGAATTCATATGCGTGTCCGTC – yielding products of 243bp (WT) and 135bp (targeted allele). All mouse studies were approved by the Animal Care and Use Committees of the University of Pittsburgh and Magee-Womens Research Institute.

Imaging

Mid-gestation embryos were fixed for 24h in fresh, neutralized 4% paraformaldehyde, equilibrated in 30% sucrose/PBS and cryoembedded in OCT. Direct emission of GFP and TdTomato was imaged using a Nikon Eclipse E400 upright microscope equipped with the corresponding fluorescence filters. Whole-mount pre-implantation embryos were imaged live using a Zeiss Axiovert 40 CFL epifluorescent inverted microscope equipped with the requisite filters. Time-lapse confocal video microscopy was performed using a fluorescent inverted Nikon Eclipse T_i microscope equipped with Perfect Focus™ to prevent Z-axis drift for the duration of live imaging. Pre-implantation embryos were incubated in sterile oil-covered KSOM medium in 35-mm glass-bottom microwell (14mm) petri dishes (MatTeK) and kept at 37°C and 5% CO₂ for the duration of imaging using a Tokai HIT stage incubator system. Images were captured with a Plan Apo ×20 differential interference contrast (DIC) objective (NA=0.75), a Lumencor Spectra X Fluorescence Light Source (25% power level), and an Andor Zyla sCMOS monochrome camera and archived using Nikon Elements software. Image z-stacks (9 loops at 15-μm steps) were collected sequentially for GFP (exposure time, 90 ms), TdTomato (50 ms), and DIC (6 ms) every 45 min for 22-44h.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Establishment of B6129 hybrid ESC clones HYB10 and HYB12

a,b. Morphologies of HYB10 and HYB12 colonies. c. PCR-based determination of the sex of HYB10-12. C – control DNA from an adult male mouse; M – DNA size marker. d,e. Chromosome number distributions of 20 and 19 karyotype spreads from HYB10 and HYB12 cells, respectively. f. Representative chimeric pups derived from microinjection of HYB10 cells into C57BL/6J blastocysts. g. Coat color counts of F1 progeny of HYB10 and HYB12 chimeric founders and B6 dams. The totals represent 8 litters of 3 different HYB10 chimeras and 6 litters of 2 distinct HYB12 chimeras.

Fig. 2. *Esrrb*-IRES-Cre design and targeting

a. Targeting scheme, indicating to-scale vector structure, including location of relevant exons, knock-in cassette, external probes, restriction sites and predicted sizes of differential restriction fragments expected of homologous recombinant clones. b,c. Southern Blot analysis of targeted clones using external 3' and 5' probes (Top – Clone identifiers).

Fig. 3. *Esrrb-IRES-Cre* excises *loxP*-flanked alleles in the early four-cell-stage embryo

a,b. Direct fluorescence of a section through an E9.5 *mT/mG*; *Esrrb-IRES-Cre* conceptus (a), showing uniform GFP fluorescence throughout the entire embryo, placenta, and extraembryonic membranes, as opposed to a control, *mT/mG*; *Cre*⁰ sibling conceptus (b); TdTomato fluorescence is present but very dim in both deciduas and in the control, *Cre*⁰ embryo. c,d. Direct GFP fluorescence of lung sections from E12.5 *mT/mG*; *Esrrb-IRES-Cre* embryo (c) and an *mT/mG*; *Cre*⁰ littermate (d). e. Brightfield, red and green fluorescent imaging of blastocysts from an *mT/mG* homozygous dam mated with an *Esrrb-IRES-Cre* sire. f-h. Time-lapse video microscopy of pre-implantation embryos retrieved from homozygous *mT/mG* dams mated with *Esrrb-IRES-Cre* sires. Confocal fluorescent images were acquired at 45-min intervals. f. Representative TdTomato/GFP co-fluorescent z-stacked frames of embryos cultured from the 8- or 16-cell to the blastocyst stage. Upon retrieval (00:00), six out of thirteen embryos were already GFP-positive throughout, with the signal intensifying over the culture period. g. Uninterrupted serial images of embryos from the late 2-cell stage (6:00h after retrieval) to the early 8-cell stage (21:00h) showing, at 45-min intervals, the kinetics of Cre-mediated mT inactivation and concomitant mG induction. The embryo on the lower-right corner exhibits a rare case of incomplete Cre-mediated conversion of mT to mG, with three of four blastomeres expressing mG (green arrows at 18:45) and one having retained TdTomato (red arrow); this incomplete penetrance, which

was observed in only 2 of 42 (<5%) GFP-positive embryos, strongly suggests that *Esrrb*-Cre excises floxed alleles in individual 4-cell stage blastomeres. h. Fine timing of Cre-mediated excision. GFP fluorescence of the center slice from the relevant subset of frames from (g) enables determination of the earliest point at which mG is first visualized in each embryo (green arrowheads). The red arrowhead identifies a control, Cre-negative embryonic littermate for clearer contrast of specific vs. background green fluorescence. Scale bars: a,b – 500µm; c-d – 100µm.

Table 1

Embryo imaging summary.

Age	Cre ⁺	Cre ⁻
E15.5	2	2
E12.5	6	6
E9.5	6	6
E3.5 (blastocyst)	6	2
Embryo culture	mG ⁺ (inc)	mG ⁻
E2.5-E4.5	1(1)	3
E2.5-E3.5	6(0)	7
E1.5-E4.5	10(0)	14
E1.5-E3.5	5(1)	5

The number of embryos imaged at each developmental stage (top) or during continuous *in vitro* cultures (bottom). Values in parentheses indicate the number of mG⁺ embryos with incomplete (inc) allele excision resulting in one or more mT⁺ cells; none were observed among embryos harvested between E3.5 and E15.5.