Application of a Novel Strategy of Engineering Conditional Alleles to a Single Exon Gene, Sox2

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Abstract

**Background:** The Conditional by Inversion (COIN) method for engineering conditional alleles relies on an invertible optimized gene trap-like element, the COIN module, for imparting conditionality. The COIN module contains an optimized 3’ splice site-polyadenylation signal pair, but is inserted antisense to the target gene and therefore does not alter transcription, until it is inverted by Cre recombinase. In order to make COIN applicable to all protein-coding genes, the COIN module has been engineered within an artificial intron, enabling insertion into an exon.

**Methodology/Principal Findings:** Therefore, theoretically, the COIN method should be applicable to single exon genes, and to test this idea we engineered a COIN allele of Sox2. This single exon gene presents additional design challenges, in that its proximal promoter and coding region are entirely contained within a CpG island, and are also spanned by an overlapping transcript, Sox2OT, which contains mmu-miR1897. Here, we show that despite disruption of the CpG island by the COIN module intron, the COIN allele of Sox2 (Sox2COIN) is phenotypically wild type, and also does not interfere with expression of Sox2OT and mir1897. Furthermore, the inverted COIN allele of Sox2, Sox2INV, is functionally null, as homozygotes recapitulate the phenotype of Sox2ßgeo/ßgeo mice, a well-characterized Sox2 null. Lastly, the benefit of the eGFP marker embedded in the COIN allele is demonstrated as it mirrors the expression pattern of Sox2.

**Conclusions/Significance:** Our results demonstrate the applicability of the COIN technology as a method of choice for targeting single exon genes.

Introduction

Intronless (single exon) genes are thought to be evolutionary innovations, whose formation via reverse transcription–mediated mechanisms represents an important route of evolution for tissue-specific functions in animal cells [1,2]. Approximately 12% of the human and 13.4% of the mouse protein-coding genes are intronless [3–6], and include genes that encode for regulatory proteins and components of signal transduction pathways [7], histones [8–11], G Protein-coupled Receptors [3] and transcription factors such as the Sox (SRY-related HMG box) family [12–14].

Sox2 is a well-characterized and important example of a single exon gene. Sox2 pairs with tissue-specific partners [15] to impart and maintain pluripotency [16] and multipotency [14,17] during development and homeostasis [18]. Sox2 null embryos fail to form the epiblast and die at E5.5 [19]. However, even reduction in Sox2 levels to 25–30% relative to the wild type leads to pathological phenotypes in mice. These include neurodegeneration in the cortical region and hippocampus [20], hypoplasia of optic nerves and chiasmata and variable microphthalmia [21], failure of nasal placode induction [22], failure of taste buds to mature [23], malformation of the epithelium lining the conducting airways in the lung [24], enlargement of the lateral ventricles at E14.5 [25], and immature differentiation of cochlea hair follicles [26].

From a gene structure standpoint, Sox2 presents a complex locus rich in genetic elements, including an overlapping transcript [27], a putative microRNA [28], and a CpG island [29–31] [32]. The combination of a well-conserved compact locus with overlapping transcripts and regulatory elements [33–39], together with the apparent need to maintain proper levels of Sox2 for organogenesis and homeostasis, underscore the difficulties associated with designing conditional alleles for Sox2. We hypothesized that a recently developed method for generating conditional alleles – Conditional by Inversion (COIN) – might present a better choice over simple floxing of
Sox2, and generated the corresponding conditional-null allele, Sox2\textsuperscript{COIN}. We show that this method is successful in that the Sox2\textsuperscript{COIN} allele starts as wild type, and it is converted into a null by the action of Cre, at which point, the expression of Sox2 is replaced by that of a marker, eGFP. This work indicates that the COIN method can be applied to single exon genes and provide a new design modality that can be adopted for other genes like Sox2.

Results

Generation of the Sox2\textsuperscript{COIN} Allele

Sox2 (NM_001084) is a single exon gene encoding a 319 amino acid protein. The Sox2 locus contains several features that render it complex from the standpoint of engineering modified alleles (Fig. 1A). To begin with, Sox2’s proximal promoter and coding region comprise a CpG island [15]. Furthermore, the Sox2 exon is contained with the intron of a long non-coding RNA (ncRNA), termed Sox2 overlapping transcript (Sox2ot) or “non-protein coding RNA 43”, which also contains mmu-miR1897 (miR1897) [40]. Sox2ot is transcribed from the same strand as Sox2 but its molecular and biological functions remains elusive. Sox2ot transcript is expressed in mouse embryonic stem cells and in other tissues, including the nervous system, where Sox2 is also highly expressed [41], while an isoform of Sox2ot, Sox2otb, located around 500 base pairs upstream of Sox2, was detected exclusively in adult mouse brain [27]. Because of this complexity, Sox2 is a challenging locus to apply conditional mutagenesis, and therefore presents a stringent test for new methods of allele design, such as COIN.

The COIN method relies on an optimized gene trap-like element, referred to as the COIN module [42]. The COIN module is comprised of a 3' splice region-reporter cDNA-polyadenylation region optimized to function as an efficient transcriptional block, and it is flanked by Sox71 and Lox66 sites are in a mirror image configuration to enable Cre-mediated inversion [43]. In order to generate conditional null alleles, the COIN module is placed in a position antisense to the target gene, either within a native intron, or an exon. The latter is made possible by embedding the COIN module within an artificial intron – the COIN module intron – and using that intron to split the target exon into two operational halves [42].

To generate Sox2\textsuperscript{COIN}, the COIN module intron was inserted directly into the single exon of Sox2 (Figure 1B), after the 30\textsuperscript{th} nucleotide of Sox2’s open reading frame, splitting the single Sox2 exon into two exons. The COIN module lies internally within the antisense strand of Sox2, stealth to transcription. Upon Cre-mediated inversion into the sense orientation, the Sox2\textsuperscript{COIN} allele is converted into a null allele, Sox2\textsuperscript{INV}. This is accomplished by the COIN module abrogating transcription of full length Sox2, effectively replacing it with expression of the COIN module’s eGFP reporter. The expression of eGFP in place of Sox2 is controlled by Sox2’s promoter and regulatory elements, and enables visual identification of the inversion event at the tissue and cellular level. The functionality of the allele was assessed \textit{in vivo} in a series of experiments that assessed whether Sox2\textsuperscript{COIN} is a truly wild type allele, and whether Sox2\textsuperscript{COIN/INV} recapitulate the null phenotype, while providing a useful marker that faithfully reproduces the expression profile of Sox2.

Sox2\textsuperscript{COIN} is Wild Type in Homozygosis

Offspring of Sox2\textsuperscript{COIN/+} intercrosses were born in Mendelian ratios and no lethality was observed in embryos, newborn pups and adults (Table 1). Homozygote mice fed normally, showed no abnormal behavior and they had normal weight in adulthood (data not shown). Macroscopic analysis of E14.5 Sox2\textsuperscript{+/-}, Sox2\textsuperscript{INV/INV}, and Sox2\textsuperscript{COIN/COIN} mice showed that the COIN module does not affect normal embryonic mouse development (Figure 2C). These phenotypic observations are further corroborated by the result that Sox2\textsuperscript{COIN/geo+/+} E6.5 embryos were morphologically indistinguishable from Sox2\textsuperscript{+/-} or Sox2\textsuperscript{geo/+} embryos derived from a Sox2\textsuperscript{COIN/COIN} cross (Figure 3B, F), where Sox2\textsuperscript{geo/+} is a null allele of Sox2 [19] (see below). Furthermore, examination of Sox2 miRNA (Figure 2F) and Sox2 protein (Figure 2H) expression levels show no apparent difference between the three genotypic classes, Sox2\textsuperscript{+/-}, Sox2\textsuperscript{INV/INV}, and Sox2\textsuperscript{COIN/COIN}, demonstrating that the COIN module has no effect on the expression of Sox2. Thus, by all of these criteria – heritability, phenotype, expression of mRNA and protein – Sox2\textsuperscript{COIN} behaves as a wild type allele.

The Expression of Sox2ot and miR1897 are Unaffected in Sox2\textsuperscript{COIN/COIN} Mice

To assess whether the COIN module affects Sox2ot RNA expression, we isolated RNA from E14.5 mouse embryos from different intercrosses and quantified Sox2ot RNA levels by Taqman Real-Time PCR analysis (Figure 2G). No significant difference was detected in the expression of Sox2ot in Sox2\textsuperscript{+/-}, Sox2\textsuperscript{INV/INV}, and Sox2\textsuperscript{COIN/COIN}, demonstrating that the COIN module has no effect on the expression of Sox2ot, at least prior to inversion. Identical observations where made for miR1897, which is embedded in Sox2ot (Figure 2H).

Sox2\textsuperscript{COIN} is Efficiently Inverted by Cre to Generate Sox2\textsuperscript{INV}

To assess whether we could trigger COIN inversion upon Cre expression, Sox2\textsuperscript{COIN/+} adult mice were intercrossed with Sox2\textsuperscript{+/-} (Sox2:CRE) transgenic mice to generate Sox2\textsuperscript{INV/+} embryos and adult mice (Figure 2D, E). In contrast to the partial infertility phenotype that has been observed with Sox2\textsuperscript{INV/+} mice [19], Sox2\textsuperscript{INV/+} adult mice exhibited no obvious phenotypes and transmitted the inverted allele in Mendelian ratios (Table 2), irrespective of whether the Sox2\textsuperscript{INV} allele is transmitted via the male or female germline (data not shown). More importantly, E14.5 Sox2\textsuperscript{INV/+} embryos (Figure 2E) displayed vivid eGFP expression in the cerebral cortex, retina, olfactory bulb, hair follicles, olfactory epithelium and spinal cord (Figure 2E), mirroring what has been observed with X-gal stained E14.5 Sox2\textsuperscript{geo} embryos [44]. In addition, the presence of eGFP protein can be detected by Western blotting in protein extracts derived from Sox2\textsuperscript{INV} embryos, and appears to be accompanied by a reduction in the levels of Sox2 protein, similar to what has been observed in the Sox2\textsuperscript{geo} embryos (Figure 2F).

Sox2\textsuperscript{INV} is a Null Allele of Sox2

Mice carrying a loss of function mutation in the Sox2 locus have been generated by the insertion of a β-gal cassette into the Sox2 locus (Sox2\textsuperscript{geogeo} [19], and Sox2\textsuperscript{geogeo} [44]). Upon homozygosis, both alleles yield Sox2-null embryos that fail to form the epiblast and die around implantation. To test whether Sox2\textsuperscript{INV/INV} phenocopy Sox2\textsuperscript{geogeo}, we performed Sox2\textsuperscript{INV/INV} intercrosses (Table 3). No Sox2\textsuperscript{INV/INV} offspring were born. More specifically, Sox2\textsuperscript{INV/INV} mutants failed to survive shortly after implantation (Figure 3I), phenocopying Sox2\textsuperscript{geogeo} and Sox2\textsuperscript{geogeo} embryos. Only Sox2\textsuperscript{INV/INV} and Sox2\textsuperscript{INV+/-} embryos reach the embryonic stage of E6.5 (Figure 3A, G). Histological examination of whole decidual swellings harvested at 6.5 dpc revealed that 25% of decidua carried abnormal implants, which had no egg cylinder structure and lacked the epithelial cells typical of eclipatation (Figure 3I).
A. Sox2 genomic locus

![Schematic representation of the mouse Sox2 locus indicating the relative location of the exon on chromosome 3, as well as that of mir1897, the non-coding RNA Sox2ot, and CpG islands in the genomic region. The degree of conservation of the locus sequence between mammalian species (ECRs) is indicated. Adapted from http://genome.ucsc.edu.](image)

B. Targeted allele

![Schematic representation of the Sox2COIN allele. The COIN module intron is inserted after the 30th nucleotide of Sox2's coding region (i.e. coordinate 34549367 on Chromosome 3) splitting the single exon of Sox2 into two exons and also dividing the CpG island. The COIN module is comprised of an optimized gene trap-like element composed of the 3' splice region of the rabbit beta globin gene (HBB_RABIT), followed by eGFP (lacking an initiating ATG) and the polyadenylation region from HBB_RABIT, all placed in the antisense strand. The COIN module has been flanked with Lox71 and Lox66 sites in a](image)

Figure 1. Targeting strategy generating a COIN allele of Sox2. (A) Schematic representation of the mouse Sox2 locus indicating the relative location of the exon on chromosome 3, as well as that of mir1897, the non-coding RNA Sox2ot, and CpG islands in the genomic region. The degree of conservation of the locus sequence between mammalian species (ECRs) is indicated. Adapted from http://genome.ucsc.edu. (B) Schematic representation of the Sox2COIN allele. The COIN module intron is inserted after the 30th nucleotide of Sox2's coding region (i.e. coordinate 34549367 on Chromosome 3) splitting the single exon of Sox2 into two exons and also dividing the CpG island. The COIN module is comprised of an optimized gene trap-like element composed of the 3' splice region of the rabbit beta globin gene (HBB_RABIT), followed by eGFP (lacking an initiating ATG) and the polyadenylation region from HBB_RABIT, all placed in the antisense strand. The COIN module has been flanked with Lox71 and Lox66 sites in a
Instead, many trophoblast giant cells could be identified (Figure 3C, H, I). The same phenotype is observed in Sox2INV/INV embryos (Figure 3H). These results demonstrate the failure of Sox2INV/INV embryos to develop an epiblast similarly to the Sox2INV/neo and Sox2INV/neo2 mutants. Thus, the inverted COIN cassette generates a true Sox2 null phenotype.

Discussion

We show here the application of COIN technology to generate a conditional-null allele for a single exon gene, Sox2. The COIN method was invented at least in part to overcome the challenges and limitations of traditional site-specific recombinase-based strategies such as Cre/Lox for designing conditional alleles [45]. These include the placement of Lox sites as well as the distance between them [46], defining critical exons (i.e. the exons of the gene that need to be deleted by Cre in order to bring about the desired allelic state) [47,48], and the lack of unified strategy for including a reporter that can mark those cells that harbor the post-recombinase allele. To date, COIN has been successfully applied in generating conditional alleles for more than twenty-five protein-coding genes [42], but its applicability to single exon genes has not been tested.

Single exon genes present a design challenge for engineering conditional alleles by traditional, e.g. simple floxing, methods. First, the Lox sites should be placed in a position that does not affect the expression of the target locus, a design decision that can be complicated by the lack of specific knowledge of the exact position of promoters and regulatory elements. An additional design challenge is presented if a reporter that marks the conversion from ‘wild-type’ to null is desired. The COIN method addresses both of these challenges irrespective of gene structure by avoiding the placement of Lox and FRT sites, reporters, and other functional elements within regions upstream of the target gene’s coding sequence [42]. Instead, COIN employs an ‘exon-splitting’ artificial intron to place an optimized reporter that is activated after Cre acts on the allele. There is the single cell level; at the population level and at steady state, the level of Sox2 mRNA as expressed from the Sox2COIN locus does not appear different to that of wild type. Lastly, because in COINs the Lox and FRT sites are placed within the artificial intron, they do not disrupt of promoters or regulatory sequences, and are also not incorporated into mRNA.

The particular choice of Sox2 to test the COIN method's applicability to single exon genes presented additional challenges in that the majority of Sox2’s single exon is contained within a CpG island, and there is also an overlapping non-coding transcript Sox2ot. Due to design constraints – specifically the need to place the COIN module as near the initiating ATG as possible – the COIN module intron was inserted into Sox2’s exon in manner that disrupts the CpG island. However, this had no apparent effect on Sox2 expression and had not apparent phenotypic consequences in the mouse. Sox2ot levels also remained unaltered, indicating that at least in the antisense position the presence of the COIN module has no effect on the expression of Sox2ot. This was evident by the normal phenotype of Sox2COIN/neo2 embryos, in which only the COIN allele can generate wild type mRNA. This genotype should sensitize the embryo to any reduction in Sox2 levels, and thus provides a stringent comparison between the wild type allele and the COIN allele prior to inversion.

Equally important is the fact that post-inversion of the COIN module, the resulting allele, Sox2COIN phenocopies the previously generated null alleles upon homozygosis. In addition, the reporter embedded in the COIN module, is expressed in a manner representative of Sox2 expression, thereby generating a tool to visualize Sox2 expression and to follow the conversion of the COIN allele into a null by Cre.

In addition to the conditional-null allele presented here, four other conditional-null alleles of Sox2 have been published [21,52–54]. All four rely on floxing of the single exon of Sox2, though the placement of the LoxP sites and selection cassettes (and their retention) varies among alleles. One of the main differences between these alleles and Sox2COIN, is that they do not incorporate a reporter that is activated after Cre acts on the allele. There is however a paucity of published data such as expression analysis of Sox2, Sox2ot, and miR1897 to allow further comparisons between Sox2COIN and the previously described conditional-null alleles. Given the increasing evidence for roles that Sox2 plays in a wide range of pathological and patho-physiological conditions, assays for the normal regulation of Sox2 expression need to be conducted in a variety of cell types. Overall, our results highlight the importance of the Conditional by Inversion technology as a method of choice in targeting intronless (single exon) genes, especially when complexity of the locus and desire for inclusion of a reporter are taken into consideration.

Methods

Gene Targeting

Targeted Sox2flox/neo+/+ ES cells were generated using VelociGene™ methodology, essentially as described [53]. Briefly, the BAC-based targeting vector was assembled on bacterial artificial chromosome (BAC) RP23_406a6 that encompasses the single protein-coding exon of Sox2 flanked by approximately 95 and 71 kb upstream and downstream respectively. The COIN module

Table 1. Analysis of progeny from Sox2COIN/– intercrosses*.

| Genotypic distribution in live progeny* | Live | Dead | Sox2INV/– | Sox2COIN/– | Sox2COIN/COIN |
|--------------------------------------|------|------|-----------|------------|--------------|
| Age; No | No | No | % | No | % | No |
| P21 95 | 0 | 27 (25%) | 40 (50%) | 28 (25%) |

Genotyping of Sox2COIN/– heterozygous intercross progeny.

Genotypes were assessed by PCR of genomic tail DNA.

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intron was introduced by bacterial homologous recombination after the 30th nucleotide of Sox2’s coding region (i.e. coordinate 34549367 on Chromosome 3, as annotated on Ensembl release 67), splitting the single coding exon of Sox2 into two exons of 441 bp and 2016 bp respectively (Figure 1B).

Experimental Animals

Sox2COINneo/+ mice were bred with Tg(ACTB:FLPe) mice (Flp-deleter mice) to excise the Neo cassette and generate Sox2COIN/+ Tg(ACTB:FLPe) mice. These were bred with C57BL6 mice to bring the Sox2COIN/+ allele into the germline. Sox2COIN/+ mice were in turn bred with Tg( Sox2:CRE) mice to generate Sox2COIN-INV/+ mice. All animals were handled in strict accordance with good animal practice as defined by the Animals Act 60/03.05.1991 applicable in Greece, revised according to the 86/609/EEC/24.11.1986 EU directive regarding the proper care and use of laboratory animals and in accordance to the Hellenic License for Animal Experimentation at the BSRC “Alexander Fleming” (Prot. No. 767/28.02.07) issued after protocol approval by the Animal Research Committee of the BSRC “Alexander Fleming” (Prot. No. 2762/03.08.05).

Embryo Processing, Tissue Preparation and Histological Analysis

For staging of the embryos, midday of the vaginal plug was considered as embryonic day 0.5 (E0.5). E6.5 decidua and E14.5 embryos were collected and dissected in cold PBS. Tissues were fixed with 10% formalin for 24 hours at room temperature and then washed several times with 1% PBS, then placed in embedding cassettes. Paraffin sections (10 μm) were stained with Hematoxylin and Eosin (H&E) using standard procedures and mounted with xylene based mounting medium. E14.5 Sox2geo2/+ LacZ staining was performed following standard protocol [19].

Figure 2. Sox2COIN is a functional conditional allele. (A) Efficient removal of the neo cassette by Flpe recombinase to generate a Sox2COIN/+ allele. Sox22+/-(lane 1), Sox22COIN+/-(lane 2), Sox22CON/CON (lane 3), Sox22INV/-/-(lane 4), Sox22geo2+/-(lane 5) E14.5 mice. PCR genotyping of (1) Sox22+/+, (2) Sox22CON/+, Tg(ACTB:FLPe), (3) Sox22COINneo/+, (4) Sox22CON+/+, (5) Sox22INV/-. (B) PCR genotyping of Sox22+/+ (lane 1), Sox22CON/+ (lane 2), Sox22CON/CON (lane 3) (genomic DNA from tail biopsies). (C) E14.5 Sox2COIN/CON embryos are morphologically indistinguishable from Sox22+/+ and Sox22CON/+. (D) Efficient inversion of the COIN module to generate Sox22INV/ mice. PCR-based genotyping of (1) Sox22+/+, and (2) Sox22INV/ (genomic DNA from tail biopsies). (F, G) Sox2 and Sox2tet qPCR analysis in Sox22+/+, Sox22COIN/CON, Sox22COIN/CON, Sox22INV/+, and Sox22INV/ E14.5 embryos. Cyclophilin was used as a control label. Data are presented as the mean±SEM (n = 3-6) for each genotype. The COIN module does not affect expression of Sox2 prior to inversion. Additionally, the COIN module does not appear to affect the expression level of Sox2tet either in Sox2COIN/CON or in Sox2INV/-/embryos. (H) MiR1897 qPCR analysis in Sox22+/+, Sox22COIN/+, Sox22COIN/CON, Sox22INV/+, and Sox22INV/-/E14.5 embryos. (I) Sox2 protein analysis. Western blot showing Sox2 and eGFP protein detected with specific antibodies in whole protein extracts from Sox22+/+, Sox22COIN/CON, Sox22COIN/CON, Sox22geo22+, and Sox22INV/ E14.5 embryos. The COIN module does not affect Sox2 protein levels prior to inversion.

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Genotyping

Tail, yolk sack or embryonic tissues were isolated and processed according to previously described methodology. Deletion of the Em7p-neo-polyA FRT-flanked (FRT6) in the germline dual-purpose antibiotic/drug selection cassette by FLP recombinase was documented by genotyping PCR from adult mice tail. Neo gene was amplified with Neo Frw (5'-CTGAATTCAAGATGAGGAGGACTTATCATCTTCA-3') and Neo Rev (5'-GACTCTGTTGACGGAGGAC-3') (172 bp); FLPc with FLP Frw (5'-GAGGAAAGCGCGCACATAGTGCG-3') and FLP Rev (5'-GACAAGCGGGTATGAGGAGGAC-3') (600 bp). Genomic DNA from E14.5 embryos was isolated from yolk sack. In E6.5 embryos genomic DNA was isolated by scraping carefully under the stereoscope off glass slides after staining with H&E and mounting. Detachment of cover slides was done by embedding mounted slides back in xylene. Detection after staining with H&E and mounting was performed using 0.2 U/L Taq Polymerase, standard PCR conditions, and 1 M betaine.

Conventional bright field and fluorescence microscopy was performed under a Leica MZ16FA stereoscope, while the dissection of the embryos took place either in 1X cold PBS or in DMEM medium supplemented with 2 mM glutamine and 0.5 mM penicillin and streptomycin.

Western Blotting

E14.5 embryos were lysed by sonication and the resulting pellets were washed with PBS and dissolved in cold buffer A (20 mM Tris-HCl, 420 Mm NaCl, 0.2 Mm EDTA, 0.5 Mm DTT, 25% glycerol, 0.5 Mm PMSE, 1.5 Mm MgCl2, 0.5% NP40). Incubation at 4°C for 15 min and centrifugation for 15 min at 10,000 xg followed. The supernatant was recovered and the protein concentration was determined by BCA Protein Assay Reagent (bicinchoninic acid) according to the instructions of the manufacturer (Thermo Scientific Pierce BCA Protein Assay Kit). Proteins (50 µg per lane) were separated on 10% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and membrane was blocked in western blot blocking buffer (5% milk, 0.1% Tween-20) for 2 h at RT, incubated o/n with the primary antibody at 4°C. Sox2 rabbit polyclonal IgG, goat anti-GFP polyclonal antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and goat β-actin polyclonal antibody (Cell Signaling Technology, Inc, Danvers, MA, USA) were used (1:1000). After extensive washing in TBST (10 mM Tris-HCl, 0.15 Mm NaCl, 0.1% Tween-20), goat anti-rabbit HRP conjugated secondary antibody was applied (1:10,000) for 2 h at RT. Proteins were visualized by chemiluminescence detection using ECL (Cell Signaling Technology, Inc., Danvers, MA, USA).

RNA Analysis

RNA was extracted from E14.5 mouse embryos and subjected to Taqman. Real-time PCR analysis typically, Gapdh was used as a control house-keeping gene, although analysis was also performed using Cyclophilin and β-actin with similar results. For miR1897 analysis, mir16 and Sno135 were used as internal controls. All probes are hydrolysis probes with 5' Fam Fluorophore and 3' quencher (BHQ) (Biosearch Technologies). Probes codes and sequences for each gene are: for mSox2, Applied Biosystems, Inc, MA, USA).

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Table 2. Analysis of progeny from Sox2INV/INV × Sox2INV/+ intercrosses.

| Age | Sox2INV/INV | Sox2INV/+ | Sox2INV/+ |
|-----|-------------|-----------|-----------|
| Live | Dead |
| E6.5 | 17 | 0 | 9 (53%) | 8 (47%) |
| P21 | 73 | 0 | 40 (55%) | 33 (45%) |

Genotyping of Sox2INV/+ heterozygous intercross progeny.

Table 3. Analysis of progeny from Sox2INV/INV × Sox2INV/INV intercrosses.

Genotypic distribution in live progeny.

| Age | Sox2INV/INV | Sox2INV/+ | Sox2INV/+ |
|-----|-------------|-----------|-----------|
| Live | Dead |
| E6.5 | 18 | 6 (33%) | 5 (28%) | 0 | 7 (29%) |
| P21 | 43 | 0 (0%) | 21 (49%) | 22 (51%) |

Genotyping of Sox2INV/INV heterozygous intercross progeny.

Genotypes were assessed by PCR either from tail biopsies or whole embryos. doi:10.1371/journal.pone.0045768.g003
All animals were handled in strict accordance with good animal practice as defined by the Animals Act 160/03.05.1991 applicable in Greece, revised according to the 06/699/EEC/24.11.1996 EU directive regarding the proper care and use of laboratory animals and in accordance to the Hellenic License for Animal Experimentation at the BSRC “Alexander Fleming” (Prot. No. 767/ 28.02.07) issued after protocol approval by the Animal Research Committee of the BSRC “Alexander Fleming” (Prot. No. 2762/ 03.08.05).

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Conceived and designed the experiments: AE. ER. Performed the experiments: NM. JS. LH. AK. PY. Analyzed the data: NM. JS. LH. AK. PY. AE. ER. Wrote the paper: AE. ER.

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