in vivo *28*: 709-718 (2014)

Mutation of Murine *Sox4* Untranslated Regions Results in Partially Penetrant Perinatal Lethality

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Abstract. *Background: Sox4 is an essential gene, and genetic deletion results in embryonic lethality. In an effort to develop mice with tissue-specific deletion, we bred conditional knockout mice bearing LoxP recombination sites flanking the Sox4 gene, with the LoxP sites located in the Sox4 5'UTR and 3'UTR. Results: The number of mice homozygous for this LoxP-flanked conditional knockout allele was far below the expected number, suggesting embryonic lethality with reduced penetrance. From over 200 animals bred, only 11% were homozygous* $Sox4^{flox/flox}$ *mice, compared to the expected Mendelian ratio of 25% ($p<0.001$). Moreover, there was a significant reduction in the number of female* $Sox4^{flox/flox}$ *mice (26%) relative to male* $Sox4^{flox/flox}$ *mice ($p=0.0371$). Reduced Sox4 expression in homozygous embryos was confirmed by in-situ hybridization and Quantitative real-time polymerase chain reaction (QPCR). Conclusion: LoxP sites in the 5' and 3' UTR of both alleles of Sox4 resulted in reduced, but variable expression of Sox4 message.*

This research was supported by NIH R01CA106826.

Abbreviations: BAC: bacterial artificial chromosome; H.E.: hematoxylin and eosin; ISH: *In Situ* Hybridization; LoxP: locus of X-over P1; MEF: Mouse Embryonic Fibroblast; QPCR: Quantitative Polymerase Chain Reaction; SOX4: Sex Determining Region Y Box 4; UTR: Untranslated Region.

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Key Words: Mouse, *Sox4*, perinatal lethality, transcription, ISH.

The sex-determining region Y-box 4 (*SOX4*) transcription factor is a developmental transcription factor that regulates progenitor development and Wnt signaling (for review see (1)). SOX4 is a 47-kDa protein that contains a highly conserved high-mobility group (HMG) DNA binding domain related to the TCF/LEF family of transcription factors that play important roles in the Wnt pathway. Although the role of SOX4 in the Wnt pathway is still unclear, SOX4 can interact directly with β-catenin and cooperate with β-catenin to activate gene expression (2, 3) and can interact with plakoglobin in a Wnt3a-dependent manner (4).

Embryonic knockout of Sox4 is lethal around E14 due to cardiac developmental defects and these embryos also show impaired lymphocyte development (5). In adult mice, Sox4 is expressed in the gonads, thymus, T- and pro-B-lymphocyte lineages and to a lesser extent in the lungs, lymph nodes and heart (6). Tissue-specific knockout of *Sox4* leads to developmental defects of the pancreas (7), and *Sox4*-heterozygous mice have impaired bone development (8). In contrast, prolonged ectopic expression of *Sox4* inhibits correct neuronal differentiation (9).

Analysis of *SOX4* together with the related SOXC family members *SOX11* and *SOX12* has determined that these factors are essential survival factors for neural and mesenchymal progenitors during organogenesis (10). These studies have suggested that SOX4 may promote survival of progenitor cells by activation of *Tead2*, a mediator of the Hippo pathway. Additional studies have examined the role of

Sox4 in development of hippocampal neurogenesis (11), spinal cord development (12), and the sympathetic nervous system (13). In each case, *Sox4* together with *Sox11* is crucial for organogenesis and proliferation and survival of differentiating cells, with co-deletion of both factors having a more severe phenotype than either one alone. These studies have used one strain of LoxP-flanked homozygous 'flox/flox' *Sox4* mice that shows no developmental defects in the absence of active Cre recombinase (14).

Several years ago, before a conditional *LoxP*-flanked *Sox4* mouse was published in 2007 (14), we generated a different flox/flox mouse strain. Herein we describe this different strain of flox/flox Sox4 mice that contains *LoxP* sites in slightly different regions of the 5' and 3' untranslated regions of the *Sox4* gene than the earlier published strain. These mice exhibit partially penetrant developmental defects even in the absence of *Cre* recombinase, suggesting that the *LoxP* insertion sites themselves within the Sox4 locus affect regulation of *Sox4* gene expression. Interestingly, we observed sex specific differences in both flox/wt and flox/flox mice, with the females being more adversely affected. This was manifested both in mean survival of female flox/flox mice, as well as in generally stunted growth, compared to wildtype of both flox/wt and flox/flox females.

Methods

In situ hybridization (ISH). A fragment of DNA was amplified from mouse genomic DNA using polymerase chain reaction (PCR) and cloned into a pGEMT plasmid (Promega, Madison, WI, USA). This fragment of DNA corresponds to nucleotide position 664-2047 of mouse *Sox4* gene (GenBank accession number NM_009238.2). *In vitro* transcription for generating DIG-labeled antisense and sense RNA probes was performed using DIG Northern starter kit (Roche Diagnostics, Basel, Switzerland). Antisense probe was used to detect *Sox4* mRNA. Sense probe was used as a control.

Mouse embryos at E12.5 were cryosectioned at 8 and 10 μm in the midsagittal plane. The 8 μm sections were used for hematoxylin and eosin (H.E.) staining. The 10-μm sections were used for *in situ* hybridization (ISH). The ISH method was modified from published protocols (15). The sections were fixed in 4% paraformaldehyde for 10 min. The sections were washed twice for 15 min in phosphate-buffered saline (PBS) containing 0.1% active Diethylpyrocarbonate (DEPC) and equilibrated in 5X saline-sodium citrate (SSC) for 15 min. The sections were prehybridized at 70˚C for 1 h in hybridization solution containing 5X SSC, 50% formamide and 40 μg/ml salmon sperm DNA. Denatured RNA probes were added to hybridization solution at 500 ng/ml and the hybridization reaction was carried out at 70˚C overnight. The sections were washed 2-times in 50% formamide, 5X SSC, 1% SDS 30 min each at 65˚C; 3 times in 50% formamide, 2X SSC 30 minutes each at 65˚C; 3 times in washing buffer containing 100 mM Tris, 150 mM NaCl, pH 7.5, 5 minutes each at room temperature. The sections were blocked in 1X blocking solution (DIG Northern starter kit, Roche) for 30 min at room temperature. The sections were incubated with anti-DIG antibody at 1/5000 dilution in 1X blocking solution for 2 h at room temperature. After incubation, the sections were washed for 3 times in washing buffer, 10 min each; and the sections were equilibrated for 5 min in detection buffer containing 100 mM Tris, 100 mM NaCl and 50 mM $MgCl_2$, pH 9.5. Detection reaction was performed by incubating section in nitro-blue tetrazolium chloride and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt (NBT/BCIP) solution (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) for 20 min at room temperature. The reaction was stopped by a 10 min wash in Tris/EDTA (10/1 mM, pH 8.0). Sections were mounted in mounting medium (VectaMount, Vector Laboratories, Burlingame, CA, USA).

For control staining of *Msx1* expression, 10-micron sections were fixed in 4% paraformaldehyde for 10 min and stained as described (16). The sections were washed 3 times for 3 min in PBS-DEPC, acetylated 10 min in acetylation buffer (triethanolamine, hydrochloric acid, acetic anhydride in H_2O-DEPC), and rinsed 3 times for 5 min in PBS-DEPC. The sections were prehybridized at room temperature for 2 h in hybridization solution. Denatured DIG-labeled RNA probes were added to hybridization solution and the hybridization reaction was carried out at 70˚C overnight. The sections were rinsed in 70˚C 5X SSC, and then incubated in 0.2X SSC for 1 h at 70˚C. The sections were washed in 0.2X SSC at room temperature for 5 min, followed by a 5 min wash with buffer B1 (0.1M TrisCl pH 7.5, 0.15M NaCl). The sections were blocked 1 h at room temperature in buffer B1 with 10% heat-inactivated serum. Sections were incubated with anti-DIG antibody at 1/1000 dilution in buffer B1 with 1% heat-inactivated serum overnight at 4˚C. After incubation, sections were washed 3 times for five minutes in buffer B1 and then equilibrated in buffer B3 (0.1M TrisCl pH 9.5, 0.1M NaCl, 50 mM $MgCl_2$). Detection was performed by incubating the sections in NBT/BCIP solution for 3 h at room temperature. The staining reaction was stopped by a rinse in Tris/EDTA and sections were mounted in 80% glycerol.

Gene targeting and generation of mutant mice. A 129 bacterial artificial chromosome (BAC) library (CT7, Invitrogen) was screened with a *Sox4* genomic probe to identify a BAC clone containing the *Sox4* genomic locus (clone no. 220M4). A smaller 12.4-kb fragment containing *Sox4* was subsequently retrieved from clone 220M4 in EL350 cells through recombineering into an ES cell targeting vector PL253 as previously described (17). A single *loxP* and a *loxP* plus *Frt*-flanked neo cassette were targeted to the resulting construct in two steps in EL350 cells through recombineering. First, to insert the single 5' *loxP* site, a targeting cassette containing *Pgk-em7*-neo flanked by homology arms to regions 5'UTR of *Sox4* was constructed in PL400. The homology arms are PCR amplified using the following primers: 5'-arm sense, 5'-CGC GGA TCC CCG CCT CCC GCC GCC AAC CT-3'; 5'-arm antisense, 5'-CGG CGG CCG CCT AGA GAC GAT GTC GCT TTC-3'; 3'-arm sense, 5'-CGC GAA GCT TGT CCG AGC TCT CCC CGG CCA-3'; 3'-arm antisense, 5'-CGC GGA ATT CCG CCC CTG GCC CGC GCG C-3'. The homology arms were sequence verified, restriction digested, and cloned into PL400 *via* four-way ligation. The targeting cassette was released by *Not*I/*Hind*III double digest and targeted through coelectroporation into heat shock-induced EL350 cells. The *Pgk-em7-neo* sequence was then removed by electroporation into arabinose-induced Cre-expressing EL350 cells, leaving behind a single *loxP* site. To insert the second *loxP* site in 3'UTR of *Sox4*, a targeting cassette containing *frt-Pgk-Em7-neo-frt-loxP* flanked by homology arms to targeting site was constructed in PL451.

Homology arms were amplified using the following primers: 5'-arm sense, 5'-CGC GGA TCC AGA TCT CAG CCC TGA GAG TGG GGG AT-3'; 5'-arm antisense, 5'-CGG CGG CCG CCA AGA GGC AGG AGA GGA GAG-3'; 3'-arm sense, 5'-AGG GAA AGA TTC CTT CGG TC-3'; 3'-arm antisense, 5'-CGC GGA ATT CGG GAC TCG AAG GAG GCG GA-3'. The targeting cassette was released by *Not*I/*Hind*III double digest and targeted similarly as described above. The conditional targeting vector was then linearized by *Cla*I digestion and electroporated into 129-derived CJ7 embryonic stem (ES) cells, using standard procedures. G418 (180 μg/ml) and Ganciclovir (2 μM) double-resistant clones were analyzed by Southern blotting hybridization, using both 5' and 3' external probes. External probes were PCR amplified using the following primers: 5' probe, sense, 5'-TTG GAG TCC CAG GAG GCA AG-3'; 5' probe, antisense, 5'-GTA GGG CAC TCA CTA CGT TG-3'; 3'-probe, sense, 5'-GAA ACC CTG CCT CGG AAA AC-3'; 3'-probe, antisense, 5'-ACT TAG CTA GCG TGC TAG AG-3'. Correctly targeted clones were then injected into C57BL/6 blastocysts using standard procedures, and the resulting chimeras were mated with C57BL/6 females to obtain germline transmission of the targeted allele. The Neo cassette was removed by crossing to the Flp recombinase strain and deletion from the 3'UTR was verified by PCR and DNA sequencing. All mice were maintained on a mixed 129/Sv/C57BL/6J genetic background. This strain will be available to the research community upon request.

Genotyping of Sox4^{Flox} mice. Sox4^{flox} mice and embryos were genotyped by standard protocol from purified tissues (tail, toe, ear clip, or liver from embryos). PCR primers were as follows:
SoxKO reverse: 5'-GCA TCT CTA ACC TGG TCT TCA CCT-3'
Sox KO forward 5' GCA TCT CTA ACC TGG TCT TCA CCT- 3'
Mouse XY forward: 5' CTG AAG CTT TTG GCT TTG AG 3'
Mouse XY reverse: 5' CCA CTG CCA AAT TCT TTG G 3'
Sox4 PCR reactions were performed using a 63˚C extension step for 30 seconds with 35 cycles. XY reactions were performed at 60˚C for 45 seconds with 35 cycles. The Sox4 PCR yields a wildtype band of 550 bp, and a mutant band at 650 bp. The mouse XY reaction gives a 340 bp band for the X chromosome, and a 320 bp band for the Y chromosome.

Generation of Murine Embryonic Fibroblast (MEF) cell lines and collection of tissues for pathological analysis. Sox4 wt/flox mice were intercrossed and embryos collected at 13.5 days post-coitum (pc). Individual animals were either processed for histology, or were utilized to generate MEF. All animals were genotyped for Sox4 status and sex as described above. Embryos used to generate MEFs were collected rinsed and then processed by standard techniques. Briefly, the embryos were placed in individual 60-cm plates after removal of the head and internal organs. The remaining tissue was finely chopped with a razorblade and then incubated with 10 ml Trypsin for 10-15 min at 37˚C. The tissue was then mixed with standard DMEM medium, spun down and cells plated. After 2-3 days of expansion the cells were collected, counted and then either frozen down, or utilized in growth assays.

Cell culture. MEF cell lines (passage 1) were plated at 1.5×10^5 cells in individual wells of a 12-well plate. At 24, 48 and 72 h post-seeding the cells were harvested from individual wells and counted. All experimental time points were performed in duplicate, with triplicate counts for each sample.

Transcription factor binding site analysis of Sox4 5'UTR. Genomic DNA was prepared from wild-type C57/Bl6 mice and each of the homozygous *Sox4$^{flox/flox}$* strains. Genomic DNA was PCR amplified and analyzed by Sanger sequencing. PCR primers used to amplify 5' and 3' UTR sequences were: 5' UTR forward (5'-CCAGCAGA GCCTCAGTGTTC-3'); 5' UTR reverse (5'-TTATTGCATCGGG TTCCAAG-3'); 3' UTR forward-1 (5'-TCCCACAGTCTCCT GTCCTC-3'); 3' UTR reverse-1 (5'-TGATGTTGGTGGTGG CTAAA-3'); 3' UTR forward-2 (5'-AGCAAAATTGGGGAGG AAAC-3') and 3' UTR reverse-2 (5'-GGCAGTTTCAGCTCC TCATC-3'). Mouse genomic DNA sequence flanking the LoxP insertion site was submitted to the Uniprobe database of TFBS recognition sequences (18-20)

Institutional board approval. All studies using live vertebrate animals were performed under protocols approved by the Emory University Institutional Animal Care and Use Committee (PHS Assurance: A3180-01). Every effort was made to minimize discomfort, distress, and pain, in experimental animals. Emory is accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC). Euthanasia was performed according to recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

Results

Generation of Sox4^{Flox} mice. Transgenic mice were generated using a murine Sox4 targeting vector containing LoxP recombination sequences in the 5' and 3' untranslated regions (UTR) of the *Sox4* gene (Figure 1A). The targeted insertion sequence in the 5' UTR was 98 bp in length located 117 bp upstream of the start ATG codon and included a LoxP site for Cre recombinase mediated deletion. In the 3' UTR, the insertion sequence contained LoxP site and a neomycin cassette including Flp recombinase recognition sequences for subsequent removal of the neomycin gene. Following germline transmission and crossing to the *Flp* recombinase mouse, the remaining targeted insertion sequence in the 3' UTR was 111 bp in length and located 290 bp downstream of the stop TGA codon. Proper integration of the *Sox4*-targeting construct was verified by Southern blot (Figure 1B). Removal of the neo cassette was verified by DNA Sanger sequencing of Sox4 3'UTR PCR product. All *Sox4^{flox}* mice were maintained on a mixed *129/Sv/C57BL/6J* genetic background.

Breeding of homozygous Sox4$^{flox/flox}$ mice. To attempt tissue-specific deletion of *Sox4*, we bred *Sox4$^{flox/wt}$* mice to a Probasin-Cre (PbCre) transgenic mouse line (21). However, we were unable to efficiently produce *Sox4$^{flox/flox}$* offspring from *Sox4$^{flox/wt}$ PbCre^{+}* x *Sox4$^{flox/flox}$ Pbcre^{wt}* matings regardless of the presence or absence of the *PbCre* transgene in the resultant offspring. This result indicates that the presence of the *PbCre* transgene was not responsible for the loss of flox/flox animals. However, in order to rule-out any possible effect of the *PbCre* transgene, we backcrossed the

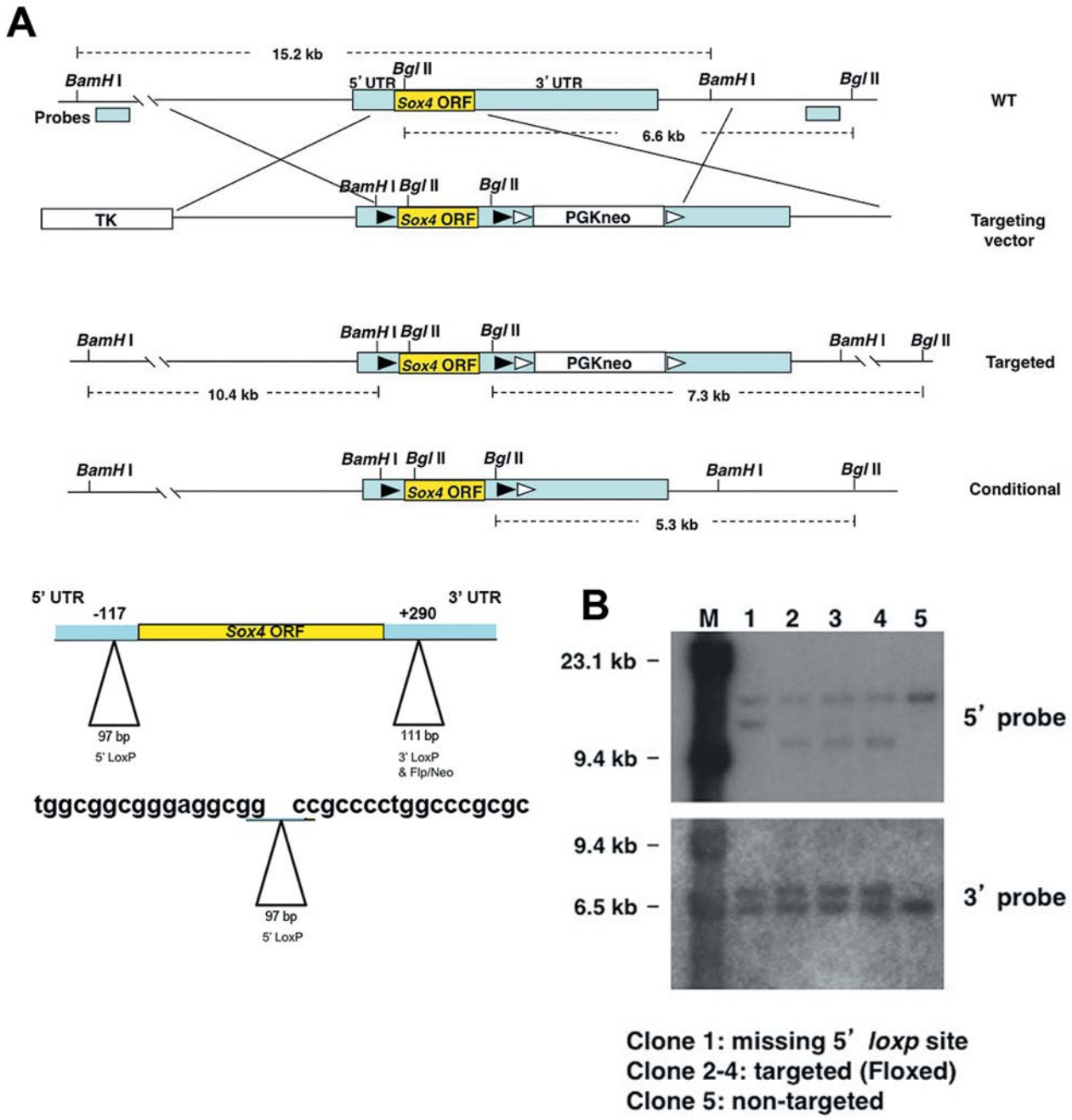

Figure 1. *(A) Targeting scheme for Sox4 gene with a schematic of the final Sox4 gene flanked by LoxP sites following removal of the Neo cassette. LoxP insertion sequences are indicated by triangles. The 5' LoxP site is located 117 bp upstream of the translation start ATG. The 3' LoxP site is located 290 bp downstream of the stop codon. (B) Representative Southern blot of ES clones using 5' and 3' external probes as indicated in part (A). Clones 2-4 show the expected size for proper integration of the Sox4 floxed allele.*

line to C57bl/6 wild-type animals and then intercrossed the resultant offspring with the same results. As demonstrated in Table I we have consistently observed a highly statistically significant reduction in the number of homozygous $Sox4^{flox/flox}$ mice relative to expected ratios ($p<0.001$, Table I) from heterozygous matings. When intercrossing heterozygous $Sox4^{flox/wt}$ mice to each other, instead of obtaining 25% mice with homozygous flox/flox alleles, we obtained only 11%. Those homozygous animals that did survive demonstrated generally smaller size and stunted

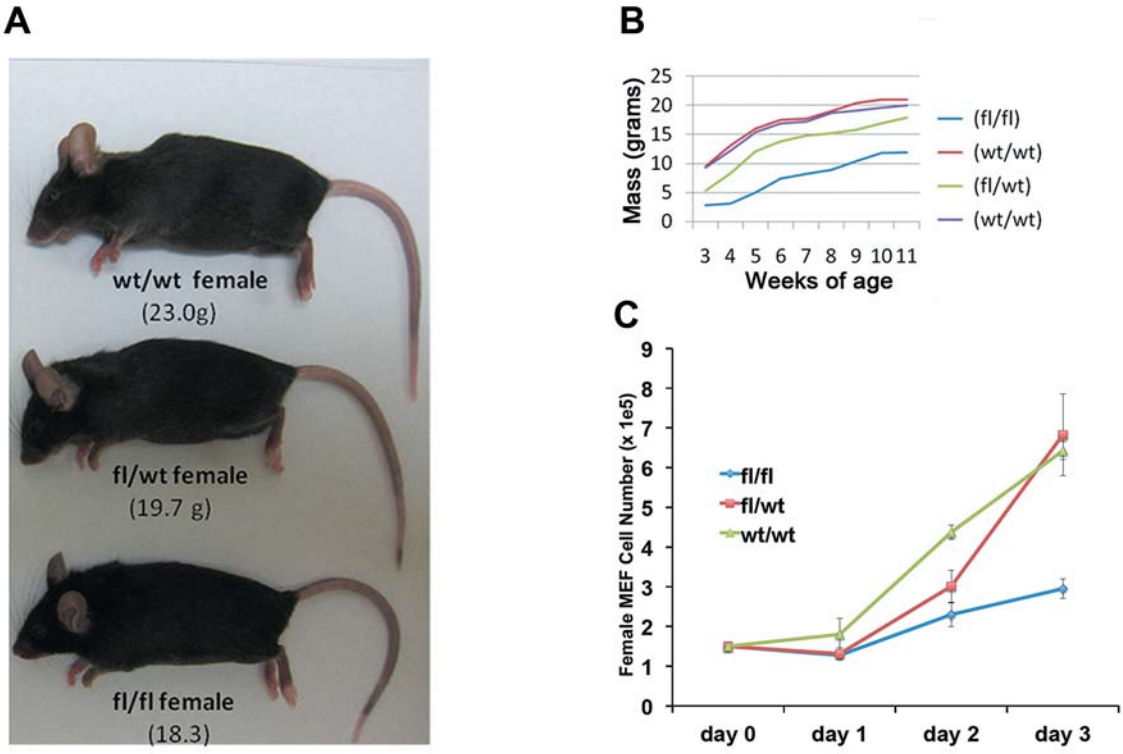

Figure 2. *Reduced growth rate of Sox4$^{fl/fl}$ mice. (A) Photograph of representative litter of SOX4 WT, heterozygous, and homozygous mice of both genders. Note reduced size of Sox4$^{fl/fl}$ mice. (B) Growth curve of females from a representative litter. (C) Growth curves of MEF cell lines derived from female embryos of Sox4$^{fl/wt}$ matings. Female-derived lines exhibit a significantly reduced growth rate. Average cell number are shown±SEM. Differences in cell numbers were significant (p<0.01) by two-tailed unpaired student t-test.*

Table I. *Breeding results Sox4 fl/wt x Sox4 fl/wt. Observed and expected genotypes of surviving pups from Sox4-flox breeding. The reduction in surviving fl/fl mice was highly significant by Chi-squared test (p<0.0001). The increase in male fl/fl mice relative to female fl/fl mice was also significant by Yates' corrected Chi-square (p=0.0371).*

Adults	fl/fl	fl/wt	wt/wt	Total
Observed	23 (11%)	113 (54%)	72 (35%)	208 (100%)
Expected	52 (25%)	104 (50%)	52 (25%)	208 (100%)
	Fl/fl Males	Fl/fl Females	Fl/fl Total	
Observed	17 (74%)	6 (26%)	23 (100%)	
Expected	11.5 (50%)	11.5 (50%)	23 (100%)	

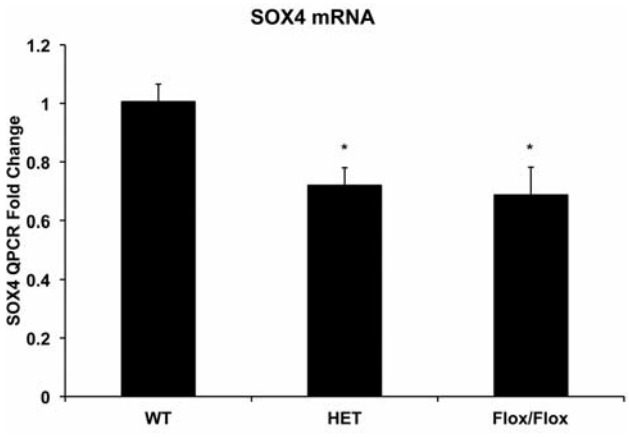

Figure 3. *QPCR analysis of Sox4 expression in MEF-derived RNA from Sox4$^{fl/fl}$ mice. RNA was prepared from MEFs generated from two WT, three heterozygous, and three homozygous embryos and analyzed by QPCR for Sox4 transcript levels, normalized to levels from a wild-type embryo. Message levels were reduced in both heterozygous (p=0.003) and homozygous animals (p=0.012), although levels varied, consistent with the partially penetrant nature of the phenotype. Statistical significance was calculated by one-sided student's t-test.*

growth (Figure 2) particularly in the females, and many died perinatally. In addition, mouse embryonic fibroblast (MEF) cell lines demonstrated strikingly reduced proliferation for *Sox4$^{flox/flox}$* lines derived from female embryos (Figure 2C). These data suggested a partially-penetrant phenotype for expression of Sox4 even in the absence of any Cre

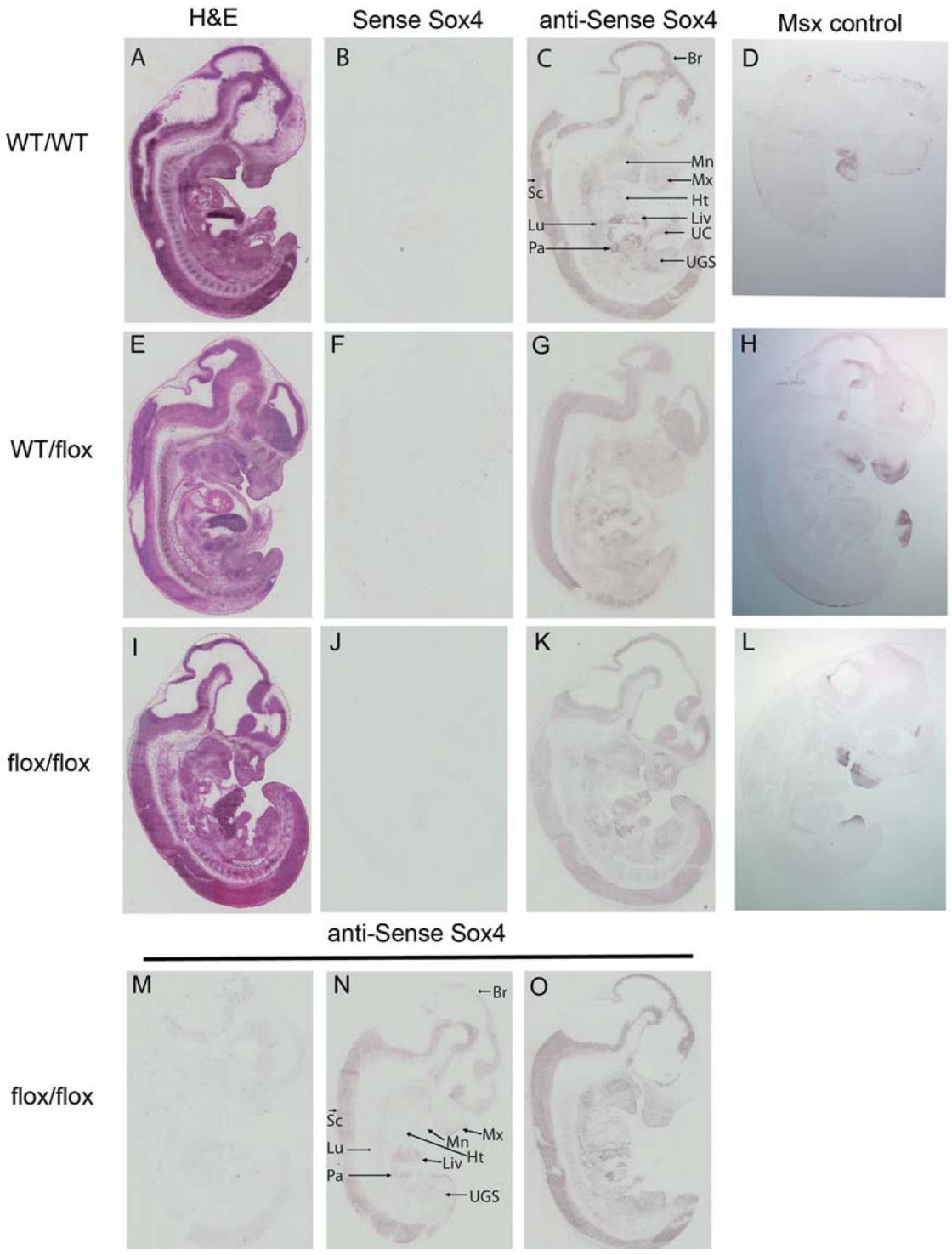

Figure 4. *Expression of Sox4 in mouse embryos. H.E. staining and in situ hybridization were performed on sagittal sections of E12.5 mouse embryos. No significant morphology changes were observed on H.E.-stained sections. Hybridization to sense Sox4 probe was performed as a negative control. Hybridization to Msx was performed as a positive control. In wild-type embryos (C), Sox4 was strongly expressed in the developing brain (Br) and spinal cord (Sc). Sox4 was also detected with lower intensity in the maxillary region (Mx), mandibular region (Mn), heart (Ht), liver (Liv), lung (Lu), pancreas (Pa), urogenital sinus (UGS), and umbilical cord (UC). A similar pattern was observed in fl/wt embryos (G). The expression of Sox4 varied in fl/fl embryos. Some had normal expression (K, O), one had globally reduced expression (M), and another had mildly reduced expression (N) in the brain, spinal cord, liver and pancreas with significantly reduced expression in the maxillary region, mandible, heart, lungs, and urogenital sinus. The reduced expression was specific to Sox4, as Msx1 was equally expressed in all embryos (Figure 5).*

Table II. *Observed and expected genotypes and sex data for embryos derived from timed matings and analyzed at day E12.5. Deviations of observed from expected values were not statistically significant.*

Embryos (E12.5)	fl/fl	fl/wt	wt/wt	Total
Observed	26 (22%)	59 (49%)	35 (29%)	120 (100%)
Expected	30 (25%)	60 (50%)	30 (25%)	120 (100%)

	Fl/fl Males	Fl/fl Females	Fl/fl Total
Observed	16 (62%)	10 (38%)	26 (100%)
Expected	13 (50%)	13 (50%)	26 (100%)

recombinase activity. These mice have been bred continuously since 2006, and have been outcrossed to other strains, or C57BL/6 controls for at least 4 generations, without any alteration in the ratios of flox/flox animals from heterozygous matings, as demonstrated in Table I. Since the reduced number of homozygous mice continues to migrate with the targeted *Sox4* allele regardless of the variations in genetic background, it would strongly suggest that the reduction in numbers of homozygous animals is due to the *Sox4* targeting event, and not to an unlinked aberrant targeting event or chromosomal rearrangement. This would also be reinforced by both the Southern blot data (Figure 1B) and the Sox4 allele sequencing data (data not shown).

Furthermore, examination of the gender of the surviving homozygous $Sox4^{flox/flox}$ animals determined that there was a significant (p=0.037) bias towards surviving male mice. To determine the range of time points during which lethality occurs, we performed timed matings of heterozygous $Sox4^{flox/wt}$ mice. We observed some rare instances of craniofacial defects in $Sox4^{flox/flox}$ embryos obtained at embryonic day 13.5 (E13.5) (data not shown) but these were sufficiently rare that they could not be definitively associated with the genotype. The number of $Sox4^{flox/flox}$ mice observed to expected ratios was reduced slightly, but not statistically significant at E13.5 (Table II). Moreover, the number of females was slightly reduced at embryonic days E12.5 to E13.5, but also not significantly. Thus, it appears that the bulk of the lethality occurs at later time points, either during late embryonic development or shortly after birth. We did observe evidence of post-natal lethality in the colony, some of which could be attributed to loss of $Sox4^{flox/flox}$ animals between birth and postnatal day 7. Whenever dead pups from this age range were identified they were $Sox4^{flox/flox}$ and predominantly female; however, in most cases no remaining pup carcass could be found for genotyping. This would be indicative of some post-natal lethality, rather than embryonic loss, but the extent of this loss could not be accurately assessed.

Figure 5. *Expression of Msx1 in mouse embryos. H.E. staining and in situ hybridization were performed as in Figure 4 and described in the Materials and Methods section. Msx1 expression was not significantly changed in $Sox4^{wt/wt}$, $Sox4^{flox/wt}$, or $Sox4^{flox/flox}$ embryos.*

Sox4 mRNA is variable, but reduced in Sox4^{flox} mice. To investigate whether *Sox4* mRNA levels were reduced in *Sox4$^{flox/flox}$* mice, we prepared RNA from mouse embryo fibroblasts (MEFs) derived from wild-type, heterozygous, and homozygous female mice. To quantitate any changes in Sox4 mRNA, we performed real-time QPCR on MEF-derived total RNA from *Sox4$^{wt/wt}$*, *Sox4$^{flox/wt}$*, and *Sox4$^{flox/flox}$* embryos. We observed variable but significantly decreased expression of Sox4 mRNA in both heterozygous and homozygous mice (Figure 3).

To further investigate the tissue-specific changes in *Sox4* expression, we performed *in situ* hybridization against *Sox4* mRNA in wild-type, heterozygous, and homozygous *Sox4^{flox}* mouse embryos. Thirteen E12.5 mouse embryos, including four wild-type, four heterozygous and five homozygous, were analyzed for *Sox4* expression by ISH. The sections were stained with H.E. to determine the locations of corresponding *Sox4* signal. *Sox4* was highly expressed in the developing brain and spinal cord in the midsagittal plane of wild-type embryos. *Sox4* was also detected with lower intensity in the maxillary region, mandibular region (including the tongue), heart, lung, liver, pancreas and urogenital sinus. In heterozygous embryos, the *Sox4* expression pattern was indistinguishable from that of wild-type controls in all the embryos analyzed (Figure 4G). However, in the five homozygous *Sox4$^{flox/flox}$* embryos, we observed variable *Sox4* expression. Notably, two of the five animals (40%) demonstrated reduced Sox4 expression. One of the animals had significantly reduced expression throughout the embryo, (Figure 4M), whereas one other had noticeably reduced expression in the brain, spinal cord, liver and pancreas, and significantly reduced expression in other regions (Figure 4). The other three animals had normal levels of *Sox4* expression at this stage of development. The reduced expression was not due to RNA degradation, since *Msx1* mRNA was detected with expected patterns (22, 23) at equivalent levels in all embryos analyzed (Figure 5). These data demonstrate that the targeted insertions into the *Sox4* 5'UTR and/or 3'UTR cause partially penetrant disruption of *Sox4* expression to varying degrees, which results in increased embryonic and perinatal lethality and reduction in body mass for surviving animals.

Discussion

Herein we have described a strain of conditional knockout *LoxP*-flanked *Sox4* mice with partially penetrant developmental defects in the absence of *Cre* recombinase activity. We observed by QPCR and ISH that Sox4 mRNA is variably reduced in *Sox4$^{flox/flox}$* mouse embryos. One limitation of our study is that we analyzed the expression of *Sox4* by ISH in only a small number of embryos in the midsagittal plane, and at only one developmental time point. Interestingly, these variations and abnormalities were observed only in mutant embryos, again indicating strongly that the effects seen in this line are due to the mutation at the *Sox4* allele and are not due to some other background mutation. Nevertheless, the effects in homozygous and heterozygous animals were heterogeneous and only partially penetrant, which may account for some of the variability between the QPCR results (Figure 3) and what we observed in ISH (Figure 4).

Sox4 expression levels differ at different stages of development. The *Sox4* expression pattern of our ISH results at E12.5 is consistent with previously published studies (15, 24, 25). In addition, detection of *Sox4* expression in these organs also supports the hypothesis that abnormal *Sox4* levels may contribute to diseases, such as craniofacial abnormality (our finding), cardiac outflow abnormality (24) and tumors of brain, lung, liver, prostate, and bladder (26).

Homozygous *Sox4$^{flox/flox}$* mice survived at statistically significantly lower numbers than expected by Mendelian ratios. Moreover, female mice were more severely affected than male mice, suggesting an important sex-specific regulation of *Sox4* in the developing mouse brain, and perhaps in other tissues. The presence of *LoxP* sites in the 5' and 3' UTR of both alleles of *Sox4* resulted in variably reduced expression of *Sox4* in many tissues, but the phenotype was particularly striking in the developing central nervous system. The 3' UTR *LoxP* site did not disrupt any known or predicted microRNA binding sites, suggesting that the 5' UTR *LoxP* site likely mediates the reduced *Sox4* expression in this animal model. The identical *LoxP* sequence is inserted in a different location in the *Sox4* gene in the strain published by Penzo-Mendez *et al.* (14). Thus, there is no reason why the LoxP sequences *per se* would be responsible for the reduced Sox4 expression. Moreover, this same sequence has been used in hundreds of other transgenic knockout strains without effects on endogenous gene expression. We infer that the effects of *Sox4* expression are instead due to disruption of native genomic sequences in the *Sox4* UTR regions.

Klf7 is expressed in the developing mouse brain in the telencephalic pallium and subpallium and regulates differentiation of neuroectodermal and mesodermal cell lineages (27). The *LoxP* site in the 5'UTR disrupts a Klf7 site (CCGCCCCTG) that is conserved in the human *SOX4* gene, suggesting that Klf7 may induce *Sox4* expression in the developing brain. The female-specific lethality suggests that Sry may partially or completely rescue the defects resulting from disruption of the *Klf7* site, since *Sry* is expressed in the male developing mouse brain (28). Alternatively, increased levels of progesterone receptor (*PR*) expression in male relative to female brains (29-31) could be responsible for the differing survival rates, since progestins can induce *Sox4* expression (32). *PR* expression is higher in the medial preoptic nucleus (MPN) of the male rat brain due to the intracellular conversion of testosterone into E2 in males (33). In fact, these mechanisms may be related, since transgenic

Sry expression in mice has been shown to induce *PR* expression in the anteroventral periventricular nucleus, the medial preoptic nucleus, and the ventromedial nucleus (34).

Sequence analysis of the previously published *Sox4*$^{flox/flox}$ strain (14) determined that the insertion site of the 5' UTR *LoxP* sequences differs by only two base pairs, and position-weight matrix redundancies make it uncertain whether there would be a difference in *Klf7* binding between the two strains. However, the strain with no effects on viability has only a 47-bp insert in the 5'UTR, whereas the strain described here has a 97-bp insert. Thus, it could be that the additional 50 bp of inserted DNA in the 5'UTR causes the phenotypes described here, either due to increased spacing between functional binding sites that affect their ability to activate transcription, or *via* introduction of novel binding sites that interfere with *Sox4* expression. Alternatively, we cannot rule-out an effect from the 3'UTR, since sequencing of the previously published strain determined that the 3'*LoxP* sites are at least 354 bp or more downstream of the *Sox4* ORF, whereas the insert for the strain described here has the 3'LoxP site only 291 bp downstream of the *Sox4* ORF.

The *Klf7* site disrupted by the 5' UTR LoxP site is also recognized by the zinc finger transcription factor *Sp4* as determined by UNIPROBE database analysis, which contains recognition motifs for many human and mouse transcription factors (18-20). *Sp4* is a member of the *Sp1* family of transcription factors that is highly expressed in the murine CNS during development (35). In *Sp4* null mice there is impaired post-natal development of the hippocampal dentate gyrus (36) with defective dendrite branching (37). Moreover, *Sp4* is a susceptibility gene for bipolar disorders, highlighting its importance in CNS function (38). Chromatin immunoprecipitation (ChIP) assays to test for direct chromatin binging of *Sp4* in MEFs derived from wild-type, heterozygous, and homozygous mutant mice did not provide clear evidence of *Sp4* binding to the Sox4 5'UTR (data not shown), but ChIP-grade antibodies to *Klf7* are not commercially available. Future studies will be needed to determine whether *Klf7* plays a critical role in regulation of Sox4 expression during development.

Conclusion

The presence of *LoxP* sites in the 5' and 3' UTR of both alleles of *Sox4* resulted in variably reduced expression of *Sox4* in many tissues, but the phenotype was particularly striking in the developing central nervous system. The 3' UTR *LoxP* site did not disrupt any known or predicted microRNA binding sites, suggesting that the 5' UTR *LoxP* site likely mediates the reduced Sox4 expression in this animal model. Moreover, female mice were more severely affected than male mice, suggesting an important sex-specific regulation of *Sox4* in the developing mouse brain, and, perhaps, in other tissues.

Conflicts of Interest

The Authors declare they have no competing interests.

Acknowledgements

This work was supported by grant R01CA106826 from the National Cancer Institute of the National Institutes of Health. Imaging of mouse embryos was conducted in the Integrated Cellular Imaging (ICI) Core of the Emory Winship Cancer Institute supported by NCI Cancer Center Support Grant P30CA138292. CDS was supported in part by NCI Training grant 5T32GM008490-22. The authors thank Soma Sannigrahi for assistance with PCR assays.

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Received April 9, 2014
Revised June 16, 2014
Accepted June 18, 2014