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TECHNOLOGY REPORT

Generation of Mice Harboring a *Sox4* Conditional Null Allele

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Summary: Sox4 belongs to the family of Sry-related HMG box transcription factors, which specify cell fate and differentiation in many lineages. *Sox4* is widely expressed in the embryo and controls such processes as neuronal tissue, lymphocyte, heart, and bone development. *Sox4*-null mice die at embryonic day 14 from heart malformation. This early lethality has therefore limited studies on *Sox4* functions. We show here that we have generated mice harboring a *Sox4* conditional null allele ($Sox4^{fl+}$) by flanking the entire coding region with *loxP* sites. $Sox4^{fl+/fl+}$ mice are indistinguishable from wildtype mice and produce the wildtype Sox4 protein at a normal level. $Sox4^{fl+}$ is efficiently converted into a null allele ($Sox4^{fl-}$) by Cre recombinase in somatic and germ-line cells, and $Sox4^{fl-/fl-}$ embryos die from the same heart defects as $Sox4^{-/-}$ mice. This *Sox4* conditional null allele will thus be a valuable tool to further uncovering *Sox4* functions in various processes in vivo. genesis 45:776–780, 2007. © 2007 Wiley-Liss, Inc.

Key words: Sox4; mouse; gene targeting; Cre-loxP

Sox4 belongs to a family of 20 genes in mice and humans that encode transcription factors containing a highly conserved Sry-related HMG box (Sox) DNA-binding domain (Lefebvre *et al.*, 2007). Most Sox genes are expressed with a specific spatial and temporal pattern from development onto adulthood, and fulfill critical roles in determining cell fate and differentiation in discrete cell lineages. *Sox4* is most highly expressed in neuronal tissue in the embryo, but is also expressed in multiple sites, including heart, lungs and thymus (van de Wetering *et al.*, 1993). Heart malformation leads to death of $Sox4^{-/-}$ embryos around embryonic day 14 (E14; Schilham *et al.*, 1996). Interventricular and arterial outflow separation is incomplete, and semilunar valve development is impaired, resulting in blood regurgitation into the ventricles. The expression pattern of *Sox4*, however, strongly suggests that *Sox4* also has important roles in several developmental and adult physiological pathways that cannot be studied using $Sox4^{-/-}$ mice. Some of these roles have begun to be uncovered by using mouse chimera, transgenic mouse, ex vivo, and in vitro approaches. For instance, *Sox4* requirement in lymphocyte B expansion was shown by transplanting $Sox4^{-/-}$ embryo-derived hematopoietic stem cells into irradiated adult mice (Schilham *et al.*, 1997), and *Sox4* requirement for normal pancreatic islet development was shown by culturing $Sox4^{-/-}$ pancreatic explants (Wilson *et al.*, 2005). *Sox4* inactivation by RNA interference was used in primary osteoblasts and electroporated chick embryos to demonstrate *Sox4* implication in differentiation of osteoblasts (Nissen-Meyer et al., 2007) and neuronal cells (Bergsland *et al.*, 2006), respectively. Prolonged expression of *Sox4* in oligodendrocytes of transgenic mice was used to argue that *Sox4* might normally prevent premature differentiation of these cells (Potzner *et al.*, 2007). While these approaches are powerful, it is evident that the study of *Sox4* functions beyond E14 and in specific cell lineages would be greatly facilitated with use of a conditional knockout strategy in the mouse. Here, we describe the generation of mice harboring a *Sox4* conditional null allele. This allele was generated using the standard technologies of DNA homologous recombination into embryonic stem (ES) cells and the Cre-*loxP* and FLPe-*Frt* strategies (Cheah and Behringer, 2001; Kilby et al., 1993; Nagy, 2000).

Sox4 is a 4.9 kb one-exon gene, with a 0.7 kb 5′ untranslated region, 1.4 kb coding region, and 2.8 kb 3′ untranslated region (Fig. 1A) (Schilham *et al.*, 1993). Based on this structure, we generated a *Sox4* conditional null allele by inserting *loxP* sites into the 5′ and 3′ untranslated regions. A $Sox4^{flneo}$ targeting vector was made with 129SvEv genomic DNA. The *loxP* sites were placed in the same orientation, such that Cre-mediated recombination would result in deletion of the coding region and would thereby produce a *Sox4* null allele ($Sox4^{fl-}$) (Fig. 1B). A neomycin resistance (neo^r) cassette was introduced directly upstream of the 3′ *loxP* site for positive selection of ES cells (Meyers *et al.*, 1998). This cassette was flanked with *frt* sites such that its deletion through FLPe-mediated recombination

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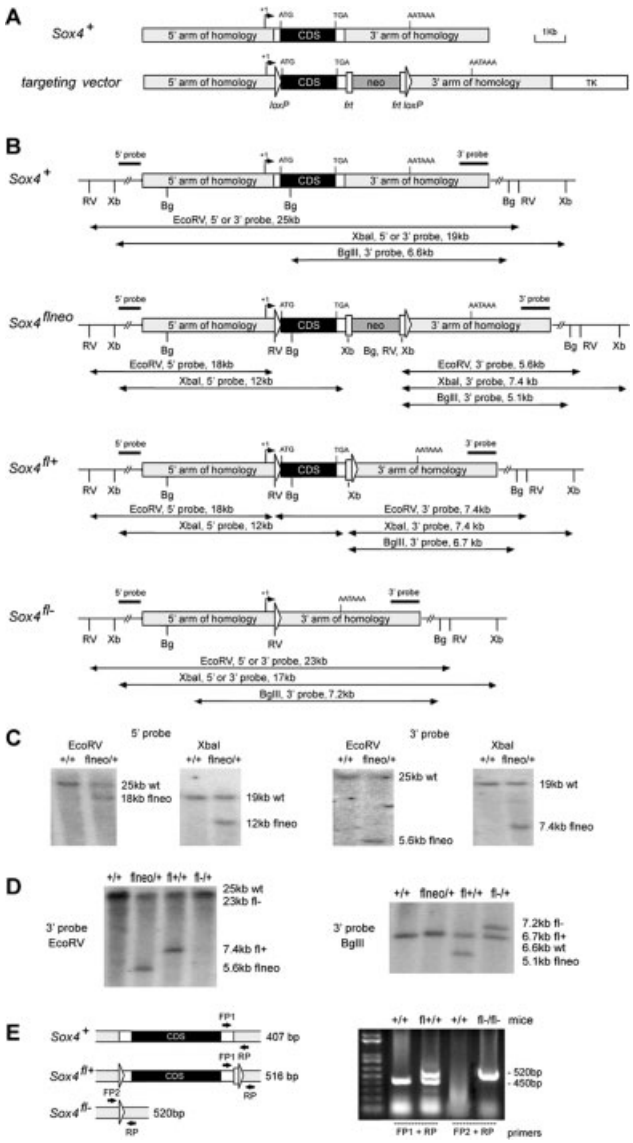

FIG. 1. Design and generation of the *Sox4* conditional null allele. **A:** Schematic of the mouse *Sox4* gene and *Sox4*^{flneo} targeting vector. CDS stands for coding sequence. The start of transcription (+1) and polyadenylation signals (AATAAA) are indicated, as well as the start (ATG) and termination (TGA) codons. The *loxP* and *Frt* sites are drawn larger than they actually are relative to the other featured DNA sequences. **B:** Schematic of the different *Sox4* alleles and Southern strategy. DNA fragments revealed in Southern analysis are shown as double arrows, with their size and the restriction enzymes used to generate them indicated. The 5′ and 3′ external probes are shown as black boxes above their genomic location. RV, *Eco*RV restriction site; Xb, *Xba*I site; Bg, *Bgl*II site. **C:** Southern blots of genomic DNA from *Sox4*$^{+/+}$ and *Sox4*$^{flneo/+}$ ES cell clones. Hybridization signals are seen at the level of expected fragments. **D:** Southern blots of genomic DNA from *Sox4*$^{+/+}$, *Sox4*$^{flneo/+}$, *Sox4*$^{fl+/+}$, and *Sox4*$^{fl-/+}$ mice digested with either *Eco*RV or *Bgl*II and hybridized with the 3′ probe, as indicated. The size and the allele origin of hybridization fragments are shown. wt, wild-type. **E:** PCR strategy to genotype mice carrying *Sox4*$^{+}$, *Sox4*$^{fl+}$, and *Sox4*$^{fl-}$ alleles. The position and orientation of three primers, FP1, FP2, and RP, are shown with arrowheads. The picture shows PCR products obtained using DNA from mice carrying the *Sox4* alleles indicated on top of the lanes and the primer sets indicated at the bottom.

would convert the newly generated *Sox4* mutant allele (*Sox4*^{flneo}) into a conditional null allele (*Sox4*$^{fl+}$). A thymidine kinase cassette (*TK*) was introduced 3′ of the construct for negative selection (Cheah and Behringer, 2001). We also introduced an *Eco*RV site directly upstream of the 5′ *loxP* site to facilitate identification of ES cell clones harboring this *loxP* site. A Southern strategy was designed to identify ES cell clones and mice carrying the *Sox4* wild-type allele (*Sox4*$^{+}$) and correctly recombined *Sox4*^{flneo}, *Sox4*$^{fl+}$, and *Sox4*$^{fl-}$ alleles (Fig. 1B).

Following electroporation of mouse 129 ES cells with the targeting vector and clonal selection, Southern analysis revealed that seven ES cell clones carried a correct recombination of a *Sox4* wild-type allele into a *Sox4*^{flneo} allele (Fig. 1C). Two clones were used to generate mouse chimeras. These chimeras gave progeny in which the *Sox4*^{flneo} allele could be correctly recombined into *Sox4*$^{fl+}$ and *Sox4*$^{fl-}$ alleles (see later). Only one line was maintained and is described.

The *Sox4*$^{fl+}$ allele was generated by breeding chimeras with *hACTB-FLPe* mice, which express FLPe ubiquitously under the control of human β-actin gene sequences (Rodriguez *et al.*, 2000). *Sox4*$^{fl+/+}$*hACTB-FLPe* progeny was crossed with wildtype mice, and *Sox4*$^{fl+/+}$ progeny that did not carry the FLPe transgene was retained (Fig. 1D). To test the *Sox4*$^{fl-}$ allele, chimeras were bred with *PrmCre* mice, which express *Cre* in the male germ line (O'Gorman *et al.*, 1997). *Sox4*$^{flneo/+}$ *PrmCre* males were crossed with wildtype females, and *Sox4*$^{fl-/+}$ progeny that did not carry the *PrmCre* transgene was retained (Fig. 1D). PCR strategies were designed for routine genotyping of mice carrying the *Sox4*$^{+}$, *Sox4*$^{fl+}$, and *Sox4*$^{fl-}$ alleles (Fig. 1E).

The *Sox4*$^{fl+}$ allele differs from the *Sox4* wild-type allele by the presence of *loxP* sites in the 5′ and 3′ untranslated regions and an *frt* site upstream of the 3′ *loxP* site. It was thus expected that the *Sox4*$^{fl+}$ transcript encode a fully functional Sox4 protein. It was possible, however, that RNA stability and translation be affected by the *loxP* and *frt* site insertions. We therefore assessed the level of *Sox4* RNA and protein present in *Sox4*$^{fl+/+}$ and *Sox4*$^{fl+/fl+}$ embryos compared to *Sox4*$^{+/+}$ littermates generated by crossing *Sox4*$^{fl+/+}$ males and females. Northern blot with total RNA from E12.5 whole embryos showed similar levels of *Sox4* RNA in *Sox4*$^{+/+}$, *Sox4*$^{fl+/+}$, and *Sox4*$^{fl+/fl+}$ embryo littermates (Fig. 2A). The Sox4 protein was analyzed by western blot with an anti-Sox4 mouse monoclonal antibody. The Sox4 protein level was found to be similar in nuclear extracts from the thymus of *Sox4*$^{+/+}$, *Sox4*$^{fl+/+}$, and *Sox4*$^{fl+/fl+}$ newborn mice (Fig. 2B). As expected from these results, *Sox4*$^{fl+/fl+}$ mice looked externally normal, behaved normally, and had a normal life expectancy (data not shown). The *Sox4*$^{fl+}$ allele is thus a fully functional *Sox4* allele.

The *Sox4*$^{fl-}$ allele differs from the wild-type allele by having the whole coding region and flanking untranslated regions replaced with a *loxP* site. To verify that *Sox4*$^{fl-}$ is a *Sox4* null allele, we generated E12.5 *Sox4*$^{+/fl-}$

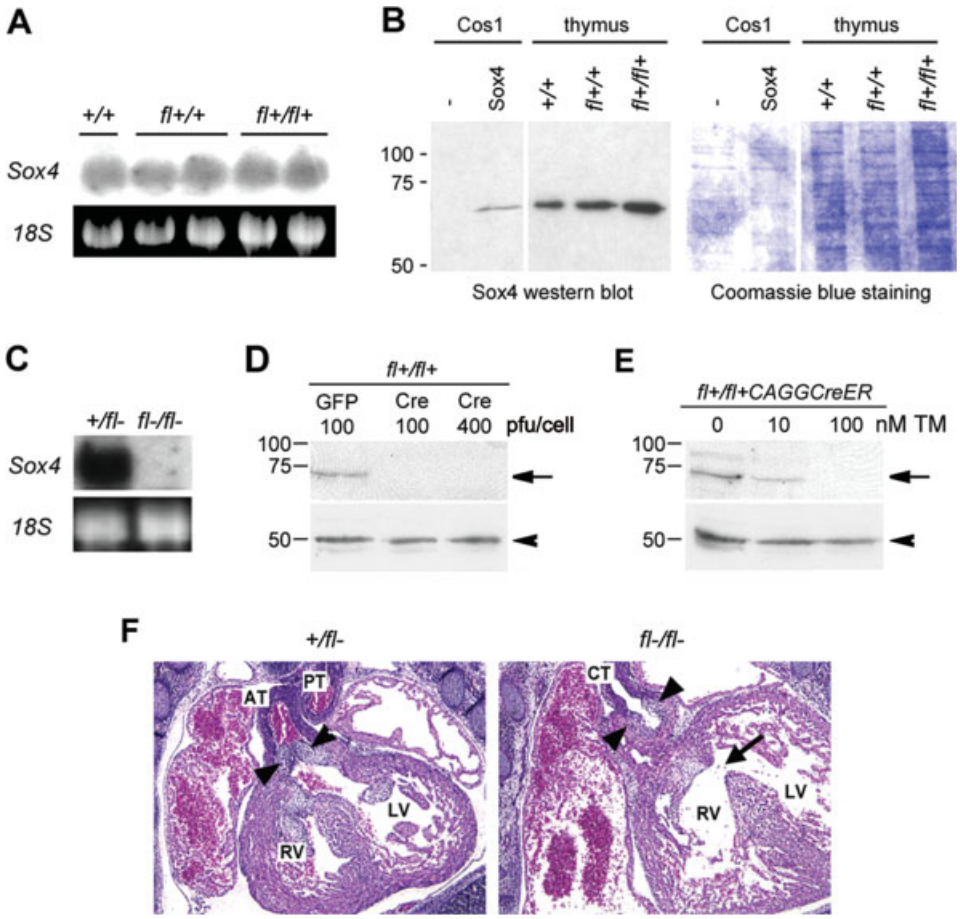

FIG. 2. Analysis of *Sox4* conditional null mice. **A:** Northern blot with total RNA from E12.5 whole embryos. One $Sox4^{+/+}$, two $Sox4^{fl+/+}$, and two $Sox4^{fl+/fl+}$ littermates were processed together. The top panel shows the hybridization signal obtained with a *Sox4* RNA probe. The bottom panel is a picture of 18S RNA stained with ethidium bromide to demonstrate equal loading of RNA in all samples. **B:** Detection of the Sox4 protein present in COS1 and thymus nuclear protein extracts. COS1 cells were transfected with an empty (–) or a Sox4 expression plasmid (Sox4). The thymus extracts were prepared from $Sox4^{+/+}$, $Sox4^{fl+/+}$, and $Sox4^{fl+/fl+}$ newborn littermates. The left panels show a western blot hybridized with a mouse Sox4 monoclonal antibody (14D9). The right panels show a twin gel stained with Coomassie blue as a control for protein loading. Note that Sox4 runs with an apparent Mr (69k) higher than expected (45k) and that the $Sox4^{fl+/fl+}$ sample contained a slightly higher amount of both Sox4 and other proteins than the $Sox4^{+/+}$ and $Sox4^{fl+/+}$ control samples. **C:** Northern blot of total RNA from E12.5 $Sox4^{+/fl-}$ and $Sox4^{fl-/fl-}$ littermates hybridized with a Sox4 probe. **D:** Western blot analysis of Sox4 protein in $Sox4^{fl+/fl+}$ primary osteoblasts infected with 100 or 400 pfu/cell Cre- or GFP-expressing adenovirus, as indicated. The Sox4 protein (arrow) was detected using Sox4 rabbit polyclonal antibodies. A nonspecific protein (arrowhead) was also detected by these antibodies and is shown as a protein loading control. **E**: Western blot analysis of Sox4 protein in $Sox4^{fl+/fl+}$*CAGG-CreER* primary osteoblasts treated with 0, 10, or 100 nM 4-hydroxy-tamoxifen, as indicated. **F:** Frontal sections through the heart of $Sox4^{+/fl-}$ and $Sox4^{fl-/fl-}$ E13.5 littermates stained with hematoxylin and eosin, showing defective interventricular septation (arrow), semilunar valve hypoplasia (arrowheads), and arterial trunk fusion (CT) in the $Sox4^{fl-/fl-}$ embryo. RV, right ventricle; LR, left ventricle; AT, aortic trunk; PT, pulmonary trunk; CT, common arterial trunk.

and $Sox4^{fl-/fl-}$ embryo littermates by crossing $Sox4^{+/fl-}$ males and females. A northern blot with a probe located in the *Sox4* coding region readily detected the presence of Sox4 RNA in $Sox4^{+/fl-}$ samples but failed to detect it in $Sox4^{fl-/fl-}$ embryos (Fig. 2C). Furthermore, a western blot showed that $Sox4^{fl+/fl+}$ primary osteoblasts lost the Sox4 protein within 48 h of infection with a Cre-expressing recombinant adenovirus (Fig. 2D). Similarly, $Sox4^{fl+/fl+}$ osteoblasts carrying the ubiquitously expressed *CAGG-CreER* transgene (Hayashi and Mcmahon, 2002) lost the Sox4 protein within 48 h of induction of Cre recombinase activity by 4-hydroxytamoxifen (Fig. 2E). These protein data thus indicate that $Sox4^{fl+}$ can be efficiently recombined into a $Sox4^{fl-}$ null allele by Cre recombinase in somatic cells. As expected from these results, $Sox4^{fl-/fl-}$ mice displayed generalized edema at E13.5 and died between E13.5 and E14.5 (not shown). Histology analysis of E13.5 embryo sections revealed incomplete interventricular septation of the heart and fusion of the proximal aorta and pulmonary outflow tracts (Fig. 2F), as described in $Sox4^{-/-}$ mice (Schilham *et al.*, 1996). Endocardial cushions formed at the level of the semilunar valves, but failed

to develop into functional valve flaps. Regurgitation of blood into the heart thus likely caused death of $Sox4^{fl-/fl-}$ embryos, as previously proposed for $Sox4^{-/-}$ embryos. These data thus demonstrate that $Sox4^{fl-}$ is a true *Sox4* null allele.

In conclusion, the *Sox4* allele that we have generated is a bona fide conditional null allele. We anticipate that this allele will constitute a very valuable tool to further uncover the in vivo functions and molecular roles of *Sox4* in the multiple developmental pathways where this gene is expressed.

MATERIALS AND METHODS

Gene Targeting and Generation of Mutant Mice

The $Sox4^{flneo}$ targeting vector was built in pBluescript $KS^{+/-}$ (Stratagene, La Jolla, CA). Mouse *Sox4* DNA segments were amplified by PCR of 129Sve mouse genomic DNA, cloned, and verified by sequencing. The *frt-neo^r-frt-loxP* neomycin resistance cassette (Meyers *et al.*, 1998) and *MC1tkpA* thymidine kinase cassette (Cheah and Behringer, 2001) were as described. The 5′ *loxP* site and adjacent *EcoR*V site were synthesized as an oligonucleotide. ES cell clones and mouse chimeras were generated by the Case University Transgenic Core. The targeting vector was electroporated into R1 mouse ES cells, and recombinant ES cell clones were amplified following selection with neomycin (G418) and gancyclovir. Mouse chimeras were generated by injection of mutant ES clones into C57BL/6J mouse blastocysts. *hACTB-FLPe* and *CAGG-CreER* mice were obtained from the Jackson Laboratories (Bar Harbor, ME), and *PrmCre* mice from O'Gorman. All mice were maintained on the 129SvEv × C57BL/6J hybrid genetic background.

Genotyping

Genomic DNA from targeted ES clones and *Sox4* mutant mice was prepared and analyzed by Southern blotting following standard protocols. The 5′ external probe was a 904 bp *Bgl*II genomic fragment located upstream of the 5′ arm of homology. The 3′ probe was a 760 bp *Sac*I/*Bgl*II genomic fragment located in the 3′ end of the 3′ arm of homology. Mice carrying $Sox4^{+}$ and $Sox4^{fl+}$ alleles were genotyped in a single PCR reaction using a forward primer "FP1," 5′-GAA GGA GGC GGA GAG TAG ACG G-3′, and a reverse primer "RP," 5′-CAT AGC TCA ACA CAA ATG CCA ACG C-3′. Mice carrying the $Sox4^{fl-}$ allele were genotyped by PCR using a forward primer "FP2," 5′-TAG AGA CGA TGT CGC TTT CCT GAG-3′, and the RP reverse primer. PCR was carried out using Taq polymerase in a standard buffer supplemented with 2% DMSO for the $Sox4^{fl-}$ allele. A denaturation step at 94°C for 1.5 min was followed with 35 cycles at 94°C for 30 s, 65°C for 75 s, and 72°C for 90 s, and an extension step for 10 min at 72°C. Mice carrying *Flpe* and *Cre* transgenes were genotyped as previously described (O'Gorman et al., 1997; Rodriguez et al., 2000). PCR products were visualized with ethidium bromide following standard DNA electrophoresis in agarose gels. The $Sox4^{+}$ PCR product was 450 bp, the $Sox4^{fl+}$ PCR product 520 bp, and the *Sox4*$^-$ PCR product 520 bp.

Cell culture, Transfection, and Adenoviral Infection

Cos-1 cells were cultured and transfected with a mouse Sox4 expression plasmid as described (Lefebvre *et al.*, 1997), using FuGENE6 (Roche, Basel, Switzerland). Primary osteoblasts were prepared from newborn mouse calvaria as described (Ducy *et al.*, 1999). They were plated at the density of 120,000 cells per 10 cm^2 dishes, and were infected 6 h later with up to 400 pfu/cell Cre- or GFP-expressing recombinant adenovirus (Gene Transfer Vector Core, University of Iowa, Iowa City, Iowa). Alternatively, 4-hydroxy- tamoxifen (Sigma, St. Louis, MO) was added to the culture medium to a final concentration of up to 100 nM. Cells were further cultured for 48 h prior to protein analysis.

RNA and Protein Analysis

Total RNA was extracted from E12.5 whole embryos using TriZol (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. Northern blots were prepared according to a standard protocol and hybridized with a *Sox4* cDNA probe corresponding to a *Bgl*II/*Sma*I 634bp fragment of the coding sequence. Cos-1 nuclear extracts were obtained as described (Lefebvre *et al.*, 1997). Thymus extracts were obtained using the same procedure after homogenization of the organ in ice-cold PBS using a Potter homogenizer. Protein concentration was determined using the Dc protein assay kit (Bio-Rad, Hercules, CA). Osteoblasts were directly lysed in Laemmli SDS-PAGE buffer. SDS-PAGE, Western blot, and staining with Coomassie brilliant blue G-250 were carried out according to standard procedures. The 14D9 Sox4 monoclonal antibody was a gift from Dr. Hans Clevers. Custom-made Sox4 polyclonal antibodies (Sigma) were generated by immunizing rabbits against the peptide CGSAATAKPGEKGDK, which corresponds to the residues 142–155 in the mouse Sox4 protein. This peptide was selected based on predicted immunogenicity and absence of homology in other proteins.

Mouse Histology

E13.5 embryos were fixed in 4% formaldehyde in PBS, embedded in paraffin, and processed into 7 μm-thick sections. Sections were stained with Harris' hematoxylin and eosin Y following a standard procedure.

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