The role of Tbx2 in the development of the atrioventriculair canal and conduction system of the heart. `Making the beat go on and on`
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Citation for published version (APA):
Aanhaanen, W. T. J. (2011). The role of Tbx2 in the development of the atrioventriculair canal and conduction system of the heart. `Making the beat go on and on`
Chapter 4

Generation of mice with a conditional null allele for Tbx2

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Chapter 4

Abstract

The T-box transcription factor *Tbx2* plays important roles in patterning and development, and has been implicated in cell-cycle regulation and cancer. Conventional disruption of *Tbx2* results in abnormalities of the heart, limbs, eye and other structures and early fetal lethality. To gain insight into the role of *Tbx2* in different tissues and at different stages of development, we have generated a conditional null allele of *Tbx2* by flanking exon 2 with *loxP* sites (*Tbx2*fl2). Homozygous *Tbx2*fl2 mice are viable and fertile, indicating that the *Tbx2*fl2 allele is a fully functional *Tbx2* allele. Cre-mediated recombination, using a ubiquitously active *CMV-Cre* line, results in deletion of exon 2 and loss of protein expression. Embryos homozygous for the recombined allele (*Tbx2∆2*) show the same heart and limb defects as conventional *Tbx2*-deficient embryos. This *Tbx2* conditional null allele will be a valuable tool to uncover tissue-specific roles of *Tbx2* in development and disease.
Methods

Transgenic mice
Bacterial artificial chromosome (BAC) RP23-462012 carrying the Thx2 gene was obtained via Clone Finder. Via several cloning steps with restriction fragments of this BAC clone, we generated both homologous targeting arms. The 5’ homologous arm of 4.4 kb, is a SacII-NheI fragment from 3.6 kb upstream of the Thx2 transcription start site to halfway intron 1. The 3’ homologous arm of 6.7 kb starts at this NheI site and ends at the NotI site in exon 7. A loxP site was cloned into the Sall site in intron 2 of the 3’ homologous arm. This 3’ arm with the additional loxP site was cloned into the NotI site of the pKOII conditional targeting vector (kindly provided by Dr. Ronald DePinho, Boston, USA),1 which furthermore contains a diphtheria toxin A cassette, one loxP site and an Frt-PGK-Neo-Frt sequence. The 4.4 kb 5’ arm was ligated into the Hpal site of pKOII to generate the complete Thx2 conditional targeting construct with floxed exon 2 allele (Figure 1A). After linearizing with Scal, the targeting construct was electroporated into V6.5 (F1 (C57Bl/6 x 129/Sv)) embryonic stem (ES) cells (kindly provided by Dr. Rudolf Jaenisch, USA), and neomycin resistant colonies were screened by PCR and Southern blot. 4 targeted ES cell clones were identified out of 96 screened ES cell clones (4% targeting frequency). On the basis of the colony morphology and karyotype analysis, two clones were chosen to produce chimeras by injection into blastocysts from FVB/N females. Only one clone gave rise to a total of 10 male and one female chimera, which had high coat color chimerism. The chimeric males were bred to FVB females and offspring was screened by Southern blot and PCR genotyping to confirm germline transmission of the conditional Thx2 null allele (Thx2fl2−Neo). To remove the Neo cassette, offspring which inherited the targeted allele were crossed with FLPe-deleter mice,2,3 which causes recombination of the Frt sites. To verify whether the loxP sites are functional, heterozygous mice for the mutated allele were bred with CMV-Cre mice4 to remove the floxed exon 2. Successful removal of the Neo cassette and exon 2 was assessed by PCR (Figure 1). All the transgenic mice have been maintained on an FVB background.

Genotyping
Genomic DNA was isolated from ES cell clones and mouse tails, and analyzed by Southern blot analysis. Probes were made by genomic PCR using the following primer pairs: 5’fw (5’-ACCCAGTGTGTACCAAGAGCGA-3’) and 5’rv (5’-CAGCCTGTCCCGTCCACTT-3’) to obtain a 504 bp 5’ external probe; 3’fw (5’-TCCGCTTTACCCGCCACC-3’) and 3’rv (5’-AGGGCTCGCTGGCTCTCTAG-3’) to obtain a 344 bp 3’ external probe. Mice carrying the Thx2+ allele (223 bp) and the Thx2fl2+ allele (263 bp) were genotyped in a single PCR reaction using a forward primer fw2 (5’-GGGAGCATTAGTTGAACACC-3’) and a reverse primer rv1 (5’-CTTGACCTCGTGTTCTAG-3’). Mice carrying the Thx2+ allele (223 bp) and the Thx2∆2 allele (189 bp) were genotyped in a single PCR reaction using 3 primers: the targeting construct specific forward primer fw1 (5’-CGGTACCTCTAGAGATCTTC-3’)
together with fw2 and rv1 as mentioned above (Figure 1). Identical cycling conditions were used for fw2/rv1 and fw1/fw2/rv1 PCR reactions, using Hot Start DNA polymerase (Solis Biodyne); 95ºC for 15 min, 40 cycles at 95ºC for 45 s, 57.5ºC for 45 s, 72ºC for 1 min, and one additional cycle at 72ºC for 5 min. By increasing the elongation time to 4 min in combination with La Taq polymerase (TaKaRa), the fw1 and rv1 primer are also useful to show recombination over the Frt and loxP sites.

Figure 1. Generation of the Tbx2 conditional null allele (Tbx2\textsuperscript{fl2}). (A) The targeting region consists of a 5' arm with a sequence homologous to Tbx2 of 4.4 kb and a 3' arm of 6.7 kb homology containing an additional LoxP site. A DTA chain was included as a negative selection marker. Recombination of the Frt sites (open triangles) flanking the PGK-Neo results in the floxed exon 2 Tbx2 allele (Tbx2\textsuperscript{fl2}). Recombination of the LoxP sites (grey triangles) flanking exon 2 results in the Tbx2 null allele (Tbx2\textsuperscript{\Delta2}). X = XmnI; B = BamHI. (B) Southern blot analysis of mouse tail genomic DNA. A 3' external probe was used after a BamHI digest, to distinguish a 9.0 kb wild-type band from a 7.9 kb band after correct integration (Tbx2\textsuperscript{fl2-Neo}). A 5' external probe was used after XmnI digest to distinguish a 13.0 kb wild-type band from a 9.4 kb band after correct integration (Tbx2\textsuperscript{fl2-Neo}). (C) PCR used to assess successful FLPe and Cre recombination. The targeting construct-specific fw1 primer and the Tbx2-specific rv1 primer generate a 3190 bp fragment after the targeting event (Tbx2\textsuperscript{fl2-Neo}). FLPe mediated removal of the PGK-Neo cassette results in a 1513 bp fragment (Tbx2\textsuperscript{fl2}). After Cre mediated removal of exon 2, a 189 bp fragment (Tbx2\textsuperscript{\Delta2}) is amplified. (D) PCR genotyping of mice carrying Tbx2 wild-type (223 bp), Tbx2\textsuperscript{fl2} (263 bp) and Tbx2\textsuperscript{\Delta2} (189 bp) alleles.
In situ hybridization and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde, embedded in paraplast and sectioned at 12 μm for immunohistochemistry and in situ hybridization. Non radioactive in situ hybridization on sections were performed as described. The probes used were a 2 kbp Tbx2 cDNA fragment containing 43 bp of exon 2 and exon 3-7, and a Cx40 cDNA fragment. For immunohistochemistry, the paraffin sections were pressure-cooked for 3 minutes in Antigen unmasking solution (H-3300, Vector Laboratories Inc) after deparaffinization and rehydration. The sections were processed according to the TSA tetramethylrhodamine system protocol (NEL702001KT, Perkin Elmer LAS). The primary antibody used was a Tbx2 monoclonal (1:250, kindly provided by Colin Goding, Oxford, UK). The secondary antibody used was Alexa 488 goat anti-mouse (1:250). Nuclei were stained using Sytox Orange nucleic acid stain (1:30,000; Molecular Probes).

Figure 2. Analysis of the Tbx2\(^{\Delta 2}\) allele (A) The external phenotype of E13.5 embryo heterozygous and homozygous for the Tbx2 null allele (Tbx2\(^{\Delta 2}\)) is highly similar, except for the hind limb. (B) A closer view of the limbs shows the duplication of digit IV at both hind limbs of Tbx2\(^{\Delta 2/\Delta 2}\) embryos.
Results and discussion

Tbx2 belongs to the T-box family of transcription factors, which is involved in the development of diverse organs and tissues. Previous studies showed that homozygous disruption of Tbx2 causes heart, limb and eye abnormalities, while mice heterozygous for a mutation in this gene appear to be normal. In the heart, Tbx2 is expressed in the myocardium, endocardial cushions, second heart field and neural crest, but its tissue-specific functions in cardiogenesis is unknown. Tbx2 has also been implicated in cell-cycle regulation and cancer. Tbx2 can suppress senescence through a mechanism involving the repression of cyclin-dependent kinase inhibitors, and Tbx2 was found to be deregulated in melanoma, pancreatic and breast cancers.

Tbx2 expression overlaps with that of Tbx3, which is structurally and functionally related to Tbx2, suggesting that functional redundancy has prevented a full appreciation of the role of these two genes during development. Heterozygous mutations in TBX3 cause ulnar-mammary syndrome in human, and mice lacking Tbx3 show defects in development of the heart, limbs, mammary glands and other tissues, and die during embryogenesis. Double heterozygous mice for Tbx2 and Tbx3 (Tbx2+/−;Tbx3+/−) are not viable after birth (our unpublished observations), which makes it unfeasible to study embryos lacking both Tbx2 and Tbx3.

To study the role of Tbx2 in specific tissues, during later stages of development, in cancer, or in the context of (heterozygous) loss of Tbx3, we generated and characterized a Tbx2 conditional null allele using the Cre-loxP and FLP-Frt systems. The strategy for targeting the Tbx2 locus is shown in Figure 1. Exon 2, which encodes residues 132-221 of the highly conserved DNA binding domain (T-box) of the gene, was flanked with loxP sites. Deletion of exon 2 causes loss of the domain essential for DNA binding and protein interactions and introduces a frame shift resulting in 2 stop codons in exon 3 and early termination of translation.

After the targeting event, neomycin resistant clones were identified by Southern blot analysis and PCR. Male chimeras obtained from correctly targeted ES cells were crossed with FVB females and offspring was screened by Southern blot and PCR, to confirm germline transmission of the conditional Tbx2 null allele (Tbx2 fl2-Neo). To remove the Neo cassette, offspring which inherited the targeted allele were crossed with FLPe-deleter mice. Successful removal of the Neo cassette was assessed by PCR (Figure 1).

The Tbx2 fl2 allele has loxP sites in intron 1 and 2 and an Frt site downstream of the loxP site in intron 1. To investigate whether these sites affect the locus, double Tbx2 fl2 heterozygous crosses were performed to obtain mice homozygous for the floxed Tbx2 allele (Tbx2 fl2/fl2) (Table 1). Offspring was obtained in expected Mendelian ratio, and no defects were observed among the different genotypes. These data indicate that the floxed Tbx2 allele is a functional Tbx2 allele.

To assess whether the Tbx2 fl2 allele could be converted to the Tbx2Δ2 allele by Cre recombination, we crossed the Tbx2 fl2 mice to CMV-Cre transgenic mice, which causes
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Figure 3. In situ hybridization of sections of E13.5 embryos shows expression of Tbx2 and target gene Cx40 in the left side of the heart of an heterozygous Tbx2+/∆2 embryo (A, C) and a Tbx2∆2/∆2 embryo (B, D). In the left atrioventricular canal (avc) of heterozygous Tbx2+/∆2 embryos, expression of Tbx2 and Cx40 was complementary, as expected. The Tbx2 transcript lacking exon 2 is still detectable in the left avc of the Tbx2∆2/∆2 embryo (B, arrow), but Cx40 is ectopically expressed (D, arrow), indicating that the mutant protein does not repress Cx40 in the avc. left atrium, la; left ventricle, lv.

Figure 4. Immunohistochemistry detection of Tbx2 at stage E13.5 in Tbx2+/∆2 and Tbx2∆2/∆2 embryos. (A, B) The presence of protein in the bronchia (br) of heterozygous Tbx2+/∆2 embryos was not observed in the bronchia of homozygous littermates. (C) In situ hybridization of sections of a Tbx2−/−/∆2 littermate shows expression of Tbx2 in the bronchia. oe, oesophagus.
deletion of exon 2 (Figure 1C). To investigate whether the Tbx2\(^{\Delta2}\) allele is acting as a null allele, we performed Tbx2\(^{+/\Delta2}\) heterozygous intercrosses. 107 embryos were isolated between E11.5 and E13.5, which included 25 wild-type (23%), 66 heterozygous (62%) and 16 homozygous (15%) Tbx2\(^{\Delta2/\Delta2}\) embryos. 13 of the isolated homozygous mutants did not show cardiac contractions anymore. No viable embryos were obtained after E13.5. These findings are in agreement with reported embryonic lethality of homozygous mutants.\(^9,10\) However, the viability of the Tbx2 mutant phenotype depends on genetic background and is expected to increase on FVB or NMRI backgrounds.\(^9,10\) Homozygous Tbx2\(^{\Delta2/\Delta2}\) embryos had a duplication of digit IV of both hind limbs (Figure 2), in agreement with the defect in Tbx2-deficient embryos.\(^9,10\) This defect was not observed in any of the wild-type or Tbx2\(^{+/\Delta2}\) heterozygous littermates.

To further verify that the Tbx2\(^{\Delta2}\) allele functions as a null allele, embryos from double heterozygous (Tbx2\(^{+/\Delta2}\)) crosses were isolated and sectioned for histological analysis and in situ hybridization on serial sections to investigate heart abnormalities. By using a Tbx2 mRNA probe recognizing the part of the gene downstream of exon 2, we detected Tbx2 mRNA in all genotypes (Figure 3). In Tbx2\(^{\Delta2/\Delta2}\) embryos, Cx40 mRNA was detected in a pattern overlapping that of Tbx2 mRNA in the left atrioventricular canal, whereas this overlap was never observed in wild-type or Tbx2\(^{+/\Delta2}\) heterozygous littermates (Figure 3). Furthermore, the left atrioventricular canal tissue morphology of Tbx2\(^{\Delta2/\Delta2}\) embryos was affected. These findings agree with the defects observed in the constitutive Tbx2-deficient embryos.\(^9,10\) Immunohistochemistry using an antibody against Tbx2 showed that no protein was produced in the Tbx2\(^{\Delta2/\Delta2}\) mice, as expected (Figure 4).

Taken together, the results demonstrate that the Tbx2 allele we have generated is a functional conditional null allele. We expect that this allele will be a valuable tool to further uncover the multiple tissue specific functions of Tbx2 during development and disease.

**Acknowledgments**

We thank Dr. Rudolf Jaenisch, Whitehead Institute for Biomedical Research, USA, for V6.5 ES cells, Dr. Colin Goding, Oxford, UK, for Tbx2 antibody, and Dr. Ronald DePinho, Dana-Farber Cancer Institute, Boston, USA, for the pKOII construct.
Reference List


