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Generation and characterisation of conditional transgenic mouse lines expressing Cre under the regulation of the mouse *Hoxb7* promoter fragment

Master's thesis in Developmental Biology

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Tartu 2005

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INTRODUCTION

Characterisation and creation of mutant mice has a history of more than a century. The common laboratory mouse, *Mus musculus*, is the most widely used model system in studies on the genetics of human diseases. Mouse has several advantages over other model organisms. First, mouse and human have 98% of genes in common. Further, during the past few decades, precise genetic and physical maps have been created for both organisms, which have ultimately resulted in the complete nucleotide sequences of the two genomes. In addition, numerous methods have been developed for manipulating the mouse genome (see Hardouin and Nagy, 2000 for an excellent review). Establishment of the mouse embryonic stem (ES) cell cultures was one of the first and most important achievements, and an essential prerequisite for many novel techniques in the gene technology field. These pluripotent stem cells were first derived from the undifferentiated inner cell mass of preimplantational mouse embryos, blastocysts, at 1981 by two independent research groups (Evans and Kaufmann, 1981; Martin, 1981). Next, it was shown that these cells can be reintroduced to blastocysts where they contribute to all embryonic tissues, including gonads, and hence can give rise to an ES cell-derived organism (Bradley et al, 1984). Finally, homologous recombination technique between endogenous genomic DNA and exogenous vector DNA in mammalian cells was invented by Smithies et al (1985), that enabled to introduce desired genetic modifications into the cell. These three achievements pioneered and created the basis for the targeted mutagenesis techniques used routinely nowadays (reviewed by Müller, 1999; Hardouin and Nagy, 2000, Lewandoski, 2001).

CONVENTIONAL MUTAGENESIS

Gene targeting technology in mouse

Gene targeting is generally defined as the introduction of modifications into the certain location in the mouse genome by homologous recombination (Torres and Kühn, 1998). All the modifications are performed in pluripotent embryonic stem (ES) cell culture, where it is possible to select for homologous recombinant cells by various selection markers included into targeting vector. ES cells can differentiate into any cell type of the embryo, so when desired modification is achieved, the modified cells are introduced to blastocyst to create a chimeric founder animal for transgenic mouse line.

There are many methods developed for mutation insertion: one-step gene inactivation, two-step techniques 'hit and run', double replacement, coelectroporation, and several recombinase-based systems. The latter technique is most widely used for conditionl gene modification, and in this paper I will further concentrate on this. All of the mentioned gene targeting techniques utilize (at some point) either replacement or insertion type vectors which are introduced to ES cells. This kind of vectors always include two in total 4-10 kb length regions of DNA homologous to the genomic locus to be targeted, and selection markers. To increase the efficiency of homologous recombination, the vector construction should base on clone from the same genomic background (mouse strain) where from are the final recipient ES cells derived. Still, the probability of recombination depends on the genomic locus where the recombination must take place, the chromatin structure in the locus and thus the accessibility of DNA. Positive and negative selection markers can be utilized for screening the homologous recombinant ES cell clones. Positive selection markers enable to enrich for stably transfected ES cell clones, but additionally, they can act as mutagens. Most used positive selection marker is the bacterial aminoglycoside phosphotransferase (neo) gene which is selected for with G418. Also there are expression cassets available that provide the resistence against

hygromycin, puromycin or histidinol (Santerre et al, 1984; Ramirez-Solis et al, 1995; von Melchner et al,1992). As *hprt*-defective ES cells are available, it is also possible to use *hprt* gene as a positive selection marker and screening in HAT medium for these cells. The herpes virus thymidine kinase (HSV *tk*) gene included at the distal end of homology arm serves as a negative selection marker against ES clones that have integrated the targeting vector randomly. Nucleoside analogs gancyclovir or FIAU that are conversed to toxic compounds by tk, are used for selection (Colbere-Garapin et al, 1980; Schwartz et al, 1991). The region between the homologous arms in the insertion type targeting vector also includes mutated DNA to be inserted into the genome.

The most robust way for targeted knockout is one step gene inactivation. In this case the positive selection marker inside or between the homologous arms of the targeting vector will replace functionally important fragments, e.g the promoter and the first exon of the gene to be inactivated. The technique is anyhow unsuitable for introducing mutations into the targeted gene, because the marker remains in the genome and may affect the regulation and splicing of the gene. Moreover, it can even interfere with the function of adjacent genes or with the genes on the opposite strand. To overcome these problems, two step gene inactivation methods have been developed.

’hit and run’

’Hit and run’ technique, and also double replacement and coelectroporation, are techniques that enable to modify the desired locus without any marker remainig in the genome (illustrated in fig. 1).

’Hit and run’ technique requires two subsequent events:

1. ’hit’ – complete integration of the targeting vector via homologous recombination leads to the partially duplicated target locus interrupted by plasmid sequences and selection markers.
2. ’run’ – elimination of the duplication either by intrachromosomal recombination or unequal sister chromatid exchange. This event occurs spontaneously, but at a low rate, also it can restore the wildtype

allele. The ES clones must be screened by PCR or Southern blot to confirm the incorporation of desired mutation.

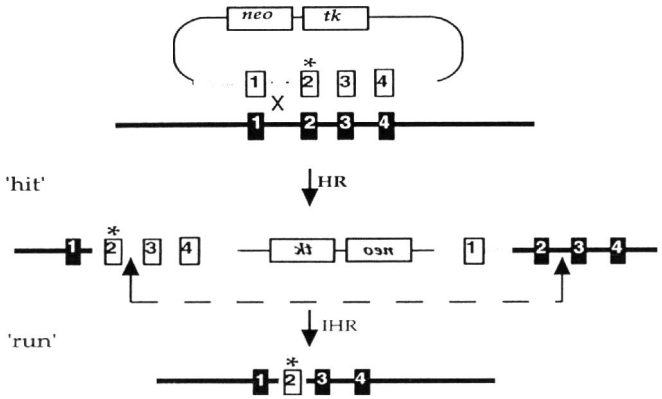

Figure 1. 'Hit and run' gene targeting technique. Figure from Torres and Kühn, 1998
HR – homologous recombination; IHR – intrachromosomal homologous recombination.

'Double replacement' and coelectroporation

'Double replacement' and coelectroporation (illustrated in Fig. 2) are gene targeting techniques which, unlike 'hit and run', use different targeting vectors in both sequential targeting events. The first vector used in 'double replacement' includes selection markers which are inserted into the target locus, second vector consists the modified DNA which repalces the selection markers after the next homologous recombination. The coelectroporation technique utilizes a vector harboring a nonselectable mutation together with an independent vector containing a selection marker, and both random integration and homologous recombination. The ES cell clones that have randomly integrated the selection marker must be screened for the homologous incorporation of the desired mutation. This techique is not very effective compared with the aforementioned techniques, but it might be the only alternative when chosen target locus does not tolerate the incorporation of a selection marker. These methods are useful in applications where several different mutations are introduced into the same locus, this can be achieved by changing only the second replacement vector.

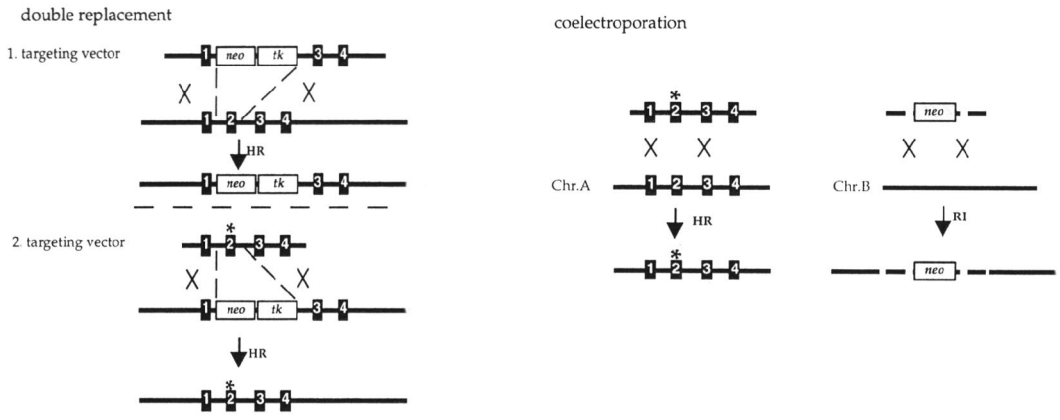

Figure 2. 'Double replacement' and coelectroporation gene targeting techniques. Figure from Torres and Kühn, 1998
HR – homologous recombination; RI – random integration.

Recombinase-based systems

These systems provide great versatility and can be used both for conventional and conditional gene targeting. The latter has become an absolute necessity together with realizing that the same genes play different roles in diverse periods in development as well as in diverse locations in the organism. These studies have been aided by the rapid advances in gene technology and improvements in techniques used for studying gene functions in the organism. The most widely used recombinase system is the Cre/*loxP* system. 38 kDa site specific P1 bacteriophage recombinase Cre (GenBank accession No X03453) recognizes and mediates recombination between 34 bp *loxP* sequences (**l**ocus **o**f crossover(**x**) in **P**). Because of the optimal temperature for the enzyme's activity is 37 °C, and there is no need for cofactors, the Cre recombinase is extremely useful and effective tool for performing modifications within animals. The *loxP* sequence (Fig. 3) consists of two 13 bp inverted repeats interrupted by 8 bp nonpalindromic sequence determining the orientation of overall sequence. These sequences are introduced into desired locations in the genome, using the ES cells and replacement vector. Depending on the orientation of the loxP sites participating in the recombination, the outcome can be different (Fig. 4).

5′-ATAACTTCGTATA-GCATACAT-TATACGAAGTTAT-3′
3′-TATTGAAGCATAT-CGTATGTA-ATATGCTTCAATA-5′

Figure 3. Recognition site for Cre (*loxP*) consistst of two 13 bp inverted repeats and a linker region between them.

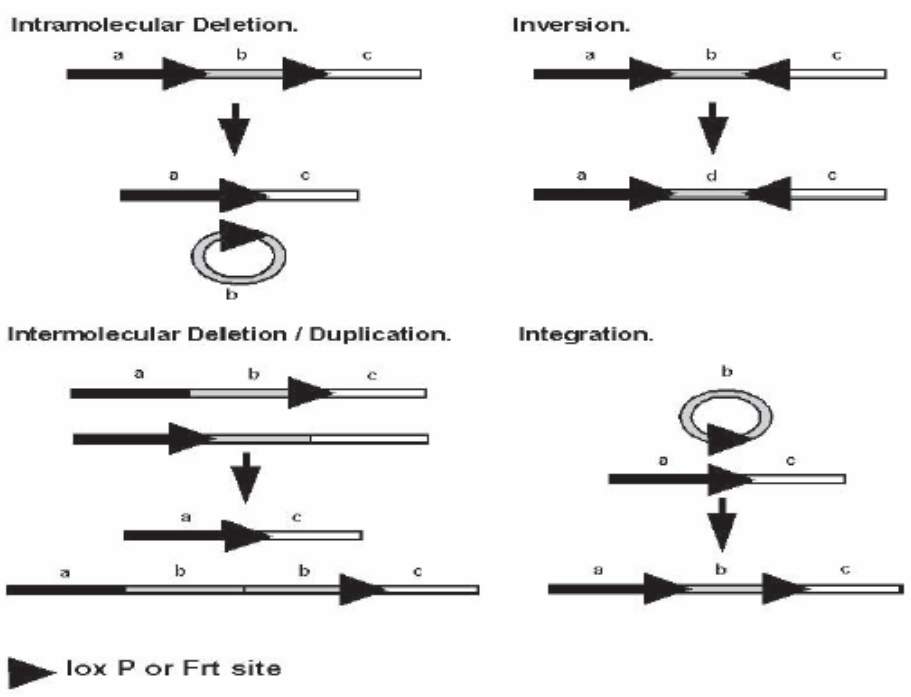

Figure 4. Cre- and Flp-mediated recombination can be used for deleting or inverting of DNA fragments *in cis* or exchanging of DNA fragments (reciprocal translocations) *in trans*. Figure from Ryding et al, 2001.

In desingning the replacement vector, it is important to place the *loxP* sites in such positions, that the site remaining in the genome after Cre-mediated recombination would not be interfering with the gene function, for example, would remain in an intron. Most frequently the Cre/*loxP* technique is used for the elimination of the DNA sequences between the two loxP sites (*loxP* sites in same orietation). The Cre-mediated recombination in cells which include *loxP* sites can be happening via transfection of cells

with Cre-expressing vectors, followed by the selection of recombined cells (these that miss selection markers) with negative selection; another possibility is the crossings of floxed mouse line derived from modified ES cells and a mouse line which expresses *Cre* in germ line cells.

Alternative recombinase system used quite widely is Flp/*FRT* system, where Flp is a *FRT* site specific recombinase derived from *Saccharomyces cerevisiae*. *FRT* primary sequence is different from *loxP* but their secondary structures are alike. These systems are thus very similar, however the Cre/*loxP* system has been proven more effective in the mammalian cell. Still, it has been shown that Flp-mediated recombination efficiency is 30-78 %, depending on cell type, and it takes place in different chromosomal locations. Flp recombinase has been successfully used in ES and EC cells for elimination of markers flanked by *FRT* sites as well as in transgenic mice. Flp-expressing mice showed no developmental or behavioral defects showing that Flp is not toxic or otherwise hazardous to the organism and the probability of recombination between random genomic sequences is low, so the system can be used in most cell types (Dymecki, 1996).

CONDITIONAL MUTAGENESIS

Development regulator genes often have distinct roles in separate time points and expression domains. The conventional inactivation of such gene can (and often will) lead to the embryonic lethal phenotype, reflecting only the gene's earliest function. Thus, in order to access the core of the biologiacl processes in development, there is a great need for conditional gene modifications, that would enable to turn genes on and/or off at the discretion of the researcher. A reliable conditional system must meet quite a number of requirements, so it is not very easy to create one. The ideal system for gene inactivation/activation must be highly specific for the target gene, the modification as such must not affect the metabolism of the cell and no other processes exept the one to be targeted.

There are two most used methods for conditional mutagenesis in mouse: transcriptional transactivation and Cre/*loxP* system (reviewed by Lewandosky, 2001). Both of these are

binary transgenic systems, in which the gene expression is controlled by the interaction of two components: the ’effecor’ transgene’s product is interacting on the ’target’ transgene, either by triggering its expression transcriptionally (transcriptional transactivation), or by rearranging of the target gene modified for that purpose (Cre/*loxP* strategy).

Transcriptional transactivation

The great advantage of transcriptional transactivation technique over DNA recombination based techniques is the possible reversibility of mutation – transactivation occurs only when the effecor is present, and, if inducible system is used, the original situation can be restored at desired time. Furthermore, if the system is carefully developed, then it is possible to control the level of transgene activation with the concentration of inducer. The main disadvatage with this technique is that position effects often occur, because many genomic locuses suppress the normal functioning of transgene. To avoid position effects, the transgene should be flanked with insulator sites which prevent the interactions between *cis*-regulator elements. The second alternative is to insert the transgene into some well known genomic locus, where low basal and high inducible gene expression is previously described and secured (Lewandoski, 2001).

The first effectors used in binary transactivation systems were viral proteins, from which the VP1 domain of HSV (Herpes Simplex virus) has proven to be the most effective and is used nowadays. This protein triggers expression of genes containing VP1 response element in their regulatory regions. The main disadvantage of that system is the lethality of VP1 to the preimplatation embryos, so it can not be used in early developmental studies. In early studies also heat shock, heavy metals and steroid hormones have been used for inducing gene expression, but all these systems show the same disadvantages, and more. For example, they are dependent on the endogenous transcription factors, often the promoters are leaky, so that the basal expression of the transgene is high even in the absence of an activator. The inducers are often toxic to the organism or unspecific to the target gene, causing unsteady induction and/or pleiotrophic effects. Later the inducible systems derived from bacteria or viruses, instead of mammals, have developed and

proven to be very effective in mouse models – *E. coli* tet-operon based tetR system and yeast derived GAL4/UAS system (Fischer et al, 1988). The latter technique is especially applicable in *Drosophila* (different applications reviewed by Duffy, 2002)

TetR based systems

TetR based transactivation system was first described and developed by M. Gossen and H. Bujard (Gossen et al, 1992). The effector is a fusion of sequences that encode the VP16 transactivation domain and the *Escherichia coli* tetracycline repressor (TetR) protein, which recognizes and binds both tetracycline and the 19-bp operator sequences (*tetO*) of the tet operon placed in the target transgene; with such binding the fusion protein triggers transcription. The responder sequence upstrem of the target transgene usually contains the CMV (human cytomegalovirus) early promoter downstream of tetracycline response element: TRE – 7-12 *tetO* tandem repeats. Inducers typically are tetracycline (tet) or its analogues, to which tetR shows remarkable affinity. The binding constant of TetR for example to tetracycline is $K_a[tet{\bullet}Mg]^+ \approx 10^9\ M^{-1}$. The tet analogues, doxycycline (dox; $K_a[dox{\bullet}Mg]^+ \approx 10^{10}\ M^{-1}$) and anhydrotetracycline (atc; $K_a[atc{\bullet}Mg]^+ \approx 10^{11}\ M^{-1}$), are used in preference to tetracycline itself due to higher tTA binding affinities and lower toxicities (Gossen and Bujard, 1993; Berens and Hillen, 2003). Dox is also able to cross the placenta and it is harmless to pregnancy, thus it can be effectively utilized for gene activation/inactivation in embryonic studies (Shin et al, 1999).

Taken together, TetR system provides specific and effective gene expression regulation *in vivo*, together with high level transgene expression, which simplifies studies on the phenotype. All the components of the system are procaryotic, which lowens the probability of pleiotrophic effects nearly to zero in mouse or other higher model organisms (Shin, 2000).

There is two variations of the tetR based sytem: 'tet-on' and 'tet-off' (illustrated in Fig. 5)

1. 'tet-off'

Transactivator (tTA) binds to *tetO* sequences and activates the transcription through the VP1 domain. In the presence of the inducer (tet or its analogue) tTA can not bind *tetO*

and the target gene is inactivated. Thus, in tet-off system the target gene is inactivated in the presence of the inducer.

2. ’tet-on’

Transactivator reverse tTA – rtTA contains modifications in four amino acids, so that it only binds *tetO* in the presence of inducer. Thus, the situation is opposite of the ’tet-off’ system - the target gene is activated in the presence of the inducer (Fig. 5).

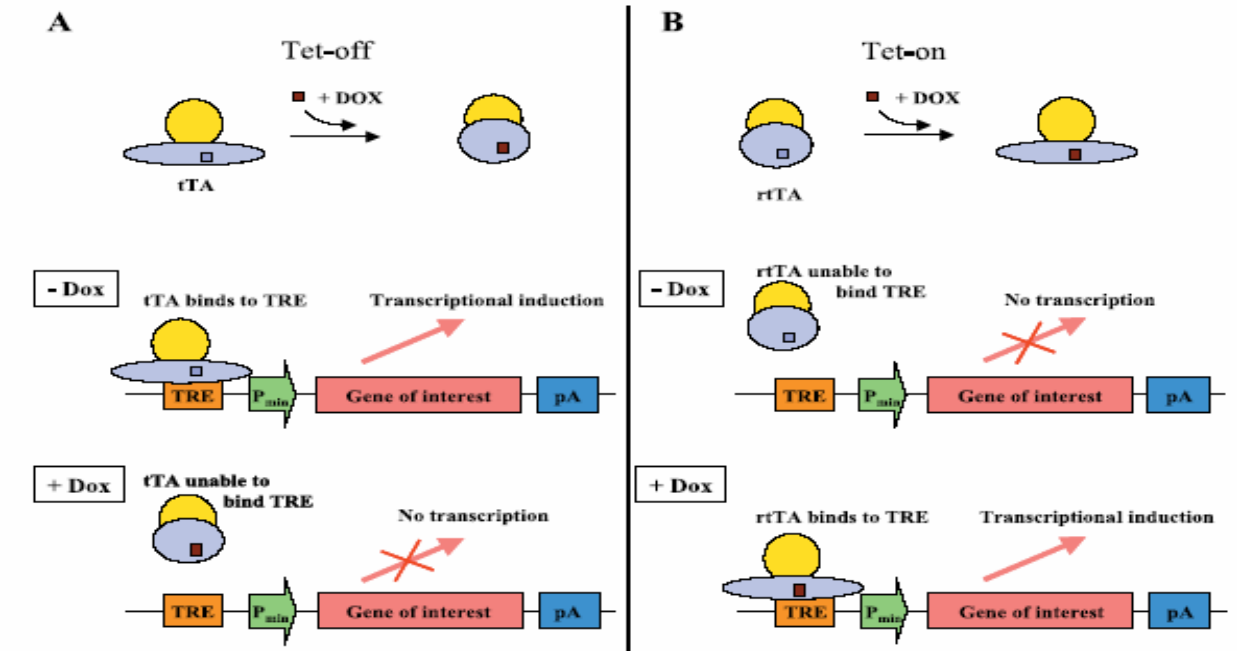

Figure 5. Tetracycline dependent transactivation systems. (A) tet-off system; (B) tet-on system. Figure from Bockamp et al, 2002.

TetR based systems can be improved by adding different transactivation domains besides VP16 or by including trans-repression domains. Changes in codon usage or amino acid modifications can improve the TA affinity to dox even 100-fold, thus facilitating the regulation of gene expression in brain and other tissues where the diffusion of chemical agents like dox is restricted (Urlinger et al, 2000). Variating the DNA binding specificity, different transgenes can be transactivated using different dox concentrations. By flanking the *tetO* sequences with two promoters, it is also possible to simultaneously control two

transgenes (Baron et al, 1995). When one of these genes is a reporter, for example a fluorescent protein, it is possible to register the exact time of the second transgene activation.

Cre/loxP strategy

This strategy of conditional gene gene targeting of endogenous genes consists of flanking a target gene or gene segment with *loxP* sites in ES cells by classical gene targeting and deleting of a selection marker by transient transfection with a Cre encoding plasmid. Subsequently, the mutation is transmitted into the germline. Conditional targeting of a floxed gene or gene segments can be achieved by crossing into a mutant animal from which Cre recombinase is expressed in a cell type specific or inducible manner (Rajewski et al, 1996) . Cre/*loxP* system is nowadays the most used technique for conditional mutagenesis in mouse, first because it is relatively simple and effective, secondly, it provides great range of possibilities appliable in different experiment schemes.

The first tissue-specific cre-mediated deletion in mouse was described in 1992. Mice carrying the SV40 large tumor-antigen separated from lens-specific *α-cristallin* promoter by floxed stop-sequence were crossed with mice expressing *Cre* under the *α-cristallin* promoter, resulting in tumor-antigen expression and subsequent eye tumor development in the double transgenic progeny (Lakso et al, 1992). First tissue specific gene knockout was done in mouse thymocytes, where part of the DNA polymerase β gene was deleted using Cre under the control of *lck* promoter (Gu et al, 1994). In the latter case, the efficiency of the deletion was determined to reach up to 99% specifically in thymocytes.

Conditional mutagenesis utilizing Cre/*loxP* is a three-step experiment (Fig. 6):

1.establishment of a floxed mouse line, where the genomic location under interest includes *loxP* sites;

2. establishment of a cre-transgenic mouse line, where Cre expression is temporally and/or spatially restricted to the time point or tissue of interest.

3. crosses between the mice created in previous steps and studies of the progeny.

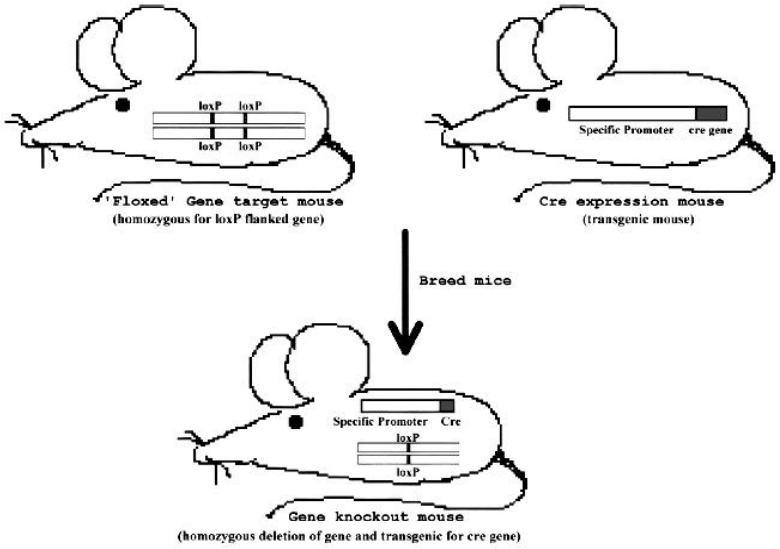

Figure 6. Conditional mutagenesis in mouse using Cre/*loxP* system. Figure from Stricklett et al, 1999.

There are several methods for producing mouse line carrying a transgene in its genome, depending on the specifics of the planned experiment and requirements on the transgene expression. If a transgene needs to be inserted into a predetermined place in the genome, the integration is proceeded in mouse ES cells, which are then used in blastocyst micronjection or morula aggregation plate to produce chimeric founders (applications reviewed by Prelle et al, 2002). When the transgene exact location is not very important, for example, in overexpression experiment, or in expressing alien genes in the organism, the mice can be produced by microinjecting the gene expression construct into fertilized oocytes, where the transgene integrates randomly into the genome.

Floxed mouse line

The *loxP* sites are introduced into the genomic locus in ES cells, and ES clones that harbor a *loxP* flanked allele suitable for conditional gene targeting, are used to create transgenic mice via blastocyst micronjection or morula aggregation. Those mice can be crossed with conditional Cre transgenic mice for gene inactivation or modification in specific cell types. There are three different basic strategies for conditional mutagenesis, called flox-and-delete, flox-and-replace and flox-and-invert. Flox-and-delete strategy can

be used for conditional gene inactivation, and is especially useful if the conventional mutant of the gene is lethal. The flox-and-replace and flox-and-invert strategies are designed for the conditional replacement of a gene fragment against a modified fragment, giving, for example, data about the gene's functionally important domains. Somewhat different gene targeting vectors are designed for introducing loxP sites into the genome, according to the selected strategy.

Flox-and-delete

This is a strategy that enables to conditionally fully inactivate a gene's function.

Flox-and-delete targeting vector has to contain three *loxP* sites in same orientation, two sites flanking a selection marker gene and within one arm of homology, third, isolated site at a distance up to 10 kb. It is possible to generate genomic deletions of different size within the locus, by using more than one isolated *loxP* sites. The selection marker(s) enables to select the ES clones that have integrated the vector. The locations of the floxed selection marker and the isolated *loxP* site in the genome must be carefully selected to ensure that gene is not disturbed in any way by the two *loxP* sites before the Cre mediated modification, but is efficiently changed after that. For gene inactivation, the *loxP* sites can be flanking one or several exons such that a frameshift will be obtained after recombination, or the promoter region and first exon. Anyhow, the distance between the *loxP* sites must also be considered, since the efficiency of Cre mediated recombination in vivo may decrease with the increasig distance between its recognition sites.

In homologous recombinant ES cells, transient Cre expression leads to three different recombination products (Fig. 7).

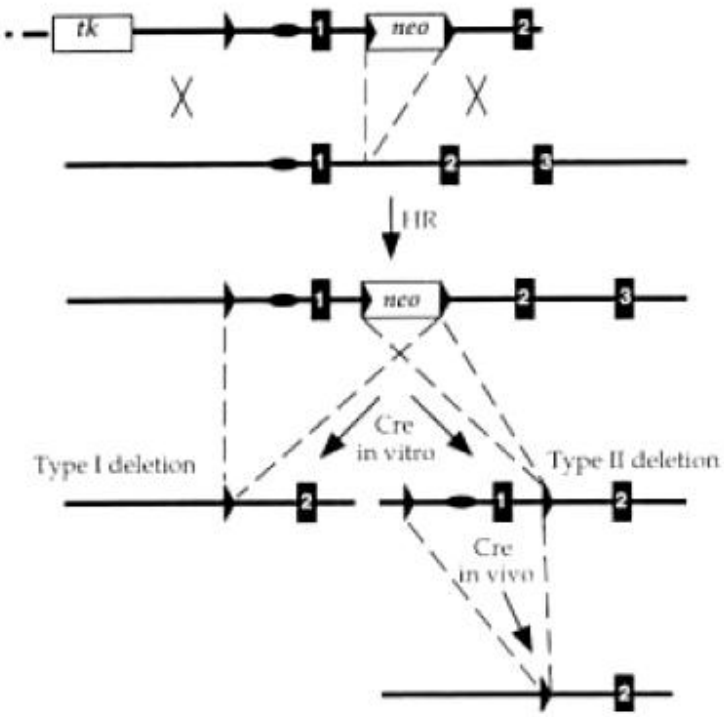

Figure 7. Flox-and-delete strategy for conditional gene modification in ES cells. Figure from Torres and Kühn, 1998. HR – homologous recombination; *neo*, *tk* – selection markers.

Type I deleton is a result of a recombination between outer *loxP* -s, which inactivates or modifies the gene irretrievably. Cells that go through this kind of mutation, can be used for generating conventional mouse line with the mutation.

Type II deletion is a result of a recombination between the *loxP* -s flanking the selection marker. The cells recombined this way, are truly the material for a mouse line applicable in conditional mutagenesis in vivo. The recombination creates a floxed version of the gene, leaving two *loxP* -s in the genome: one in the place where the selection marker was removed from, and the isolated *loxP*.

Type III deletion is a result of a recombination between inner *loxP* flanking the selection marker and the isolated *loxP*. This deletion has no practical use and the cells with that mutation are lost during selection for the cells that have lost the positive marker gene, for example, in the selection with G418, when the targeting vector has *neo* as a marker.

ES clones with type I or type II deletion provide the opportunity to also target the second allele with the same vector used in the first step, since they have lost the selection marker. Second targeting event will lead to hemi- or homozygous mutant ES cells, that sometimes are favored in generation of transgenic animals.

Flox-and-replace

This is a strategy designed for replacing a gene fragment against a modified fragment in vivo to allow the synthesis of a modified product in specific cell types. The modified fragment is introduced to the ES cells within a flox-and-replace vector that contains three *loxP* -s in the same orientation, two sites flanking a selection marker, and an isolated site in the arm of homology, placed distal to the DNA fragment which will replace the wildtype DNA after Cre-mediated recombination in a transgenic mouse. As in the flox-and-delete strategy, three recombination products are possible after transient Cre expression in ES cells harboring the vector (Fig. 8).

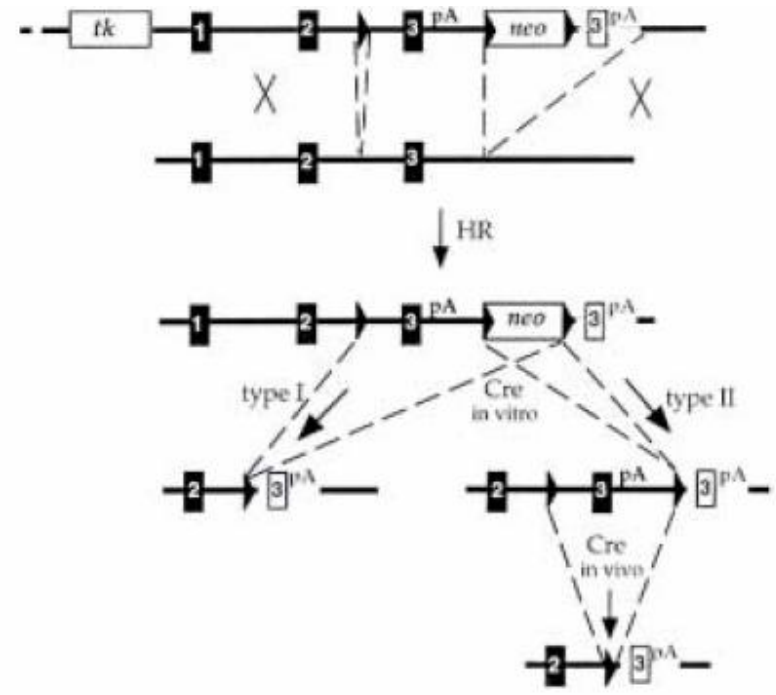

Figure 8. Flox-and-replace strategy for conditional gene modification in ES cells. Figure from Torres and Kühn, 1998.

Type I deletion yields ES cells that have already the modified fragment in the genome.
Type II deletion will lose only the selection marker and will yield in ES cells that have a wildtype gene with a *loxP* in an intron and a second *loxP* outside of the gene sequence, followed by the altered DNA sequence. Using flox-and-replace strategy, it is important to prevent any readthrough transcription of the altered fragment before modification, which can result, for example, from alternative splicing. To avoid this, the transcriptional stop sites can be included either upstrem or downstream of the *loxP* placed outside of the wildtype gene.

Flox-and-invert

In this experiment strategy, the modification of a gene is achieved by the Cre mediated inversion of the genomic sequence flanked by *loxP* -s in the opposite orientation. The advantages of this strategy is the reversibility of the modification, and the impossibility of readthrough transcription due to the configuration of the targeted locus, which will include the altered DNA in reverse orientation.

A vector for replacement of an exon in the target gene (Fig. 9) combines a floxed selection marker and a new, incoming exon together with a splice donor and acceptor sites into a single heterologous region which is inserted up- or downstream of the exon to be conditionally modified. The splice donor and acceptor sites will serve for the splicing out of the new exon from the primary transcript. The *loxP* site following the exon to be replaced must be in opposite orientation to the sites flanking the selection marker.

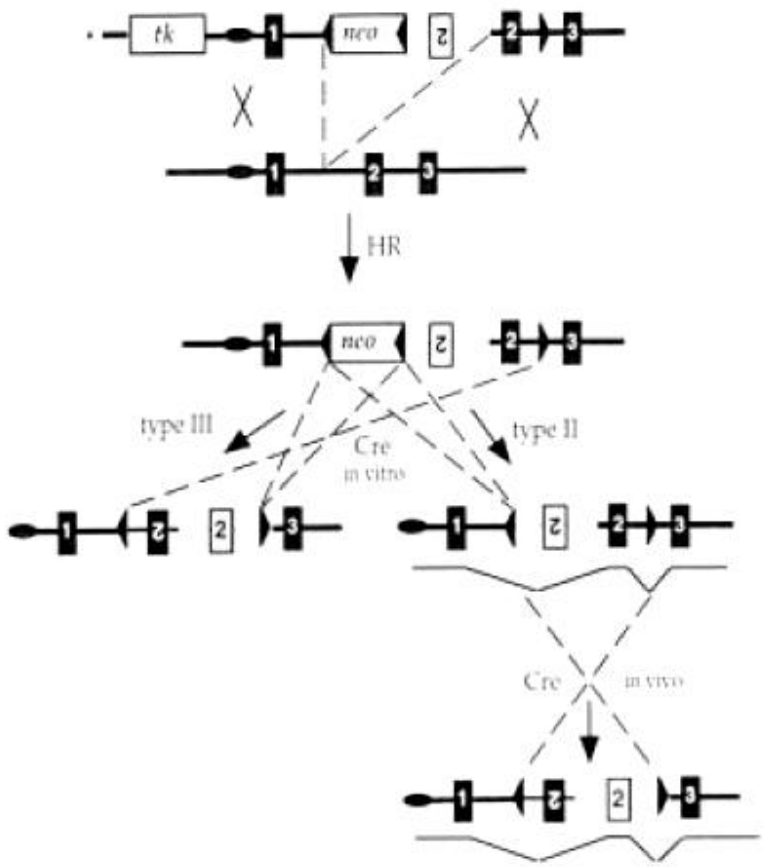

Figure 9. Flox-and-invert strategy for conditional gene modification in ES cells. Figure from Torres and Kühn, 1998.

Transient Cre expression in homologous recombinant ES cells gives two products valuable for further conditional experiment: type II recombination results in the original orientation of the modified locus, and type III recombination results in the opposite orientation locus. Transgenic mice derived from type II clones initially express the wildtype gene product, and can be crossed with Cre transgenic strain to conditionally replace it with an altered product. Animals derived from type III cloned express the modified product, which can be conditionally reversed to wildtype product.

In mice carrying a flox-and-invert construct, stable gene modification requires crossing with a line that harbor transient Cre expression in desired cell types, since Cre mediated inversion reaction is reversible.

Cre transgenic mouse line

Usually Cre transgenic mice are produced by microinjection, as this method is quicker and more straihghtforward than the targeted mutagenesis: no need for complex targeting vector construction and subsequent ES clone selection. The injected construct resembles typical cDNA expression construct, containing cell type specific promoter-conjugated Cre coding sequence and polyadenylation signal. Optionally, Cre sequence can be followed or preceded by splicing donor/acceptor sites, where usually intron-exon border regions from rabbit β-globin or human growth hormone gene are exploited. Promoter is selected, considering the aims of given experiment. Suitable promoter/enhancer combinations for constitutive cell type specific expression can be found from the genome, for example the regulator elements of tissue specific transcription factors can be used for triggering Cre expression in that tissue. After pronuclear microinjection a number of founder animals however have to be tested, because the level and pattern of transgene expression may vary greatly, depending on its copy number and and integration site. Furthermore, the efficiency of recombination also depends on the target gene chromosomal position. Hence, several transgenic founders with one construct should be tested for recombination efficiency to find the optimal for the planned experiment. For that, the founder is crossed with an indicator strain carrying a floxed gene segment and the recombination efficiency is measured in the double transgenic progeny, either using traditional methods for identifying DNA modification like Southern blot or PCR, or checking the gene expression in RNA or protein level by Northern blot, *in situ* hybridization or immunohistochemistry, but the most straightforward approach would be using a Cre reporter construct where a reporter gene (for example *lacZ*) is located downstrem from the floxed region and is activated after the recombination event. Fairly many reporter strains designed for this purpose are commercially available (Soriano, 1999; Srinivas et al, 2001). More details about different reporter lines is given further on, see pages 26-27.

Alternatively to random integration, the Cre transgene can be brought under the control of an endogenous promoter by homologous recombination in ES cells, replacing an endogenous gene with *Cre*. By this strategy, *Cre* expression becomes optimally regulated

since all control elements are in their natural genomic positions, thus avoiding frequent positional effects on transgene expression accompanying with randomly integrated transgene. However, gene targeting is much more laborious and time consuming, plus, it involves possible effects from the generation of a nonfunctional allele of the gene used for targeted *Cre* expression. The gene must be maintained in a heterozygous state and its expression level is reduced. Still, when the expression pattern of the particular gene is suitable, this may be the only realistic approach. Cre coding sequence is introduced to the genome using a replacement type targeting vector. Selection marker is placed downstream of *Cre*, and it is removed in ES cells in order to minimize the disturbance of the targeted locus. The selection marker should be flanked with *FRT* sites and removed with Flp, since the remaining *loxP* after the removal of the marker might influence future genomic rearrangements after the crossings of the Cre line and a floxed line. Alternatively, mutant *loxP* sites can be used (Torres and Kühn, 1999; Lewandoski, 2001).
In the first example of targeted insertion *Cre* was placed under the control of *CD19* promoter (Rickert et al, 1997). The *CD19* locus was used since the gene is exclusively expressed in B lymphocytes, the expression is activated very early in development and maintains steady during the differentiation.
Presently, fairly many Cre lines are constructed and available, allowing conditional gene targeting in specific or many cell types, either constitutively or upon induction. So, before planning a conditional gene modification, it is reasonable to check the Andras Nagy database in URL http://www.mshri.on.ca/nagy/cre.htm, or the database composed by Soriano lab (URL http://tbase.jax.org/), a more recent database. Cre transgenic lines are widely used in inactivation experiments, to a lesser extent for cell lineage analyses and for conditional gene activation or overexpression.

Gene inactivation using conditional Cre transgenic mice

Conditional inactivation of an endogenous gene enables to circumvent an early lethal phenotype and allows the biological questions to be addressed with exquisite accuracy. In such studies, Cre transgenic mouse line is crossed with a mouse in which an essential region (e. g. promoter, translation initiation codon region, or the regions coding important

protein domains) of a gene of interest is floxed, so that the gene's function is completely lost in the cells/tissue where Cre was active. The success of a conditional knockout experiment is determined by the presence and functionality of the regulator elements controlling Cre expression, as well as the maximum recombination efficiency achievable in the tissue by the concrete promoter. Unfortunately the recombination efficiency does usually not reach 100 %, especially when an inducible construct is used.

As an example, the 5.5-kb *α-myosin heavy-chain* (*α-MHC*) promoter has been used extensively to produce expression throughout the entire myocardium in newborn as well as in adult animals (Agah et al, 1997; Abel et al, 1999). Transgenic mice expressing a tamoxifen-inducible Cre recombinase protein under the control of the *α-MHC* promoter showed significant Cre expression also in the embryonic heart (Sohal et al, 2001). The myocyte specific recombination frequency (over 70 %) was measured by *lacZ* staining in double hetrerozygous animals from the *α-MHC-Cre* and *R26R* crossings. Importantly, in this work no alterations in cardiac development were shown due to the presence of the Cre fusion protein, thus the transgenic mice generated represent a reliable genetic tool for conditional experiments *in vivo*.

Mouse pancreas β-cell and hepatocyte specific expression of Cre has been achieved using transgenes that contain either rat *insulin* or *albumin* promoter/enhancer sequences, respectively (Postic et al, 1998). Recombination was registered in 82 % of pancreas β-cells in mice harboring a pancreas specific *insulin-Cre* construct and in 80 % of liver cells of mice harboring liver specific *albumin-Cre* construct. The ability of the transgenes to promote recombination in transgenic mice was assessed by crossing Cre-mice with *CATZ* mice, a line bearing a Cre-inducible lacZ reporter gene downstream of a floxed chloramphennicol acetyltransferase (CAT) gene as a 'stop' sequence (Araki et al, 1995).

And as another example, the chondrocyte specific deletion of floxed genes is achieved in a mouse line expressing Cre recombinase under the control of the mouse type II collagen gene regulatory regions (Sakai et al, 2001). Northern and in situ hybridization analyses were used to demonstrate the expression of the transgene specifically in cartilaginous tissues in this mouse line. Specific Cre activity was demonstrated by crossings to *R26R* reporter.

Kidney specific Cre expression

The mouse *Hoxb7* (earlier referred to as *Hox 2-3*) promoter is described by J.Deschamps and her colleagues to mediate *lacZ* expression in the mesonephric Wolffian duct-derived epithelium of the meso- and metanephric kidney and associating ducts throughout the mouse development, beginning from the time these structures first appear (Kress et al. 1990). Using *in situ* hybridization, the *Hoxb7* mRNA transcripts are first detected in the allantois primordium at E7.5, one day later, at E8.5 in embryonic endoderm and mesoderm. In E9.5 and E10.5 embryos Hoxb7 expression is observed in the CNS from a rostral boundary in the upper spinal cord to the caudal end, also in the peripheral nervous system, mesoderm and in the hindgut epithelium. During subsequent development the initially broad expression pattern becomes restricted to structures in the urogenital system, being completely lost from the adjacent neuroectoderm, somites and lateral plate (Vogels et al, 1990). The mouse *Hoxb7* promoter has been used to achieve kidney specific gene expression in several independent studies (Yu et al, 2002; Srinivas et al, 1999).

The mouse *Ksp-cadherin* promoter has also been successfully exploited in kidney epithelium specific transgene expression. Ksp-cadherin is a kidney specific member of cadherin superfamily that is expressed in the basolateral membranes of all cell types in both the tubular nephron and the collecting system, starting from E14.5, maintaining until adult age, as detected by immunolocalization (Thomson et al, 1999). It has been shown that Ksp-cadherin gene promoter can mediate Cre transgene expression beginning from E10.5, specifically in developing nephrons, ureteric bud, mesonephric tubules and Wolffian duct (Shao et al, 2002b). Same group has also shown GFP expression under the control of 1341-bp fragment of the *Ksp-cadherin* 5' flanking region, which was specific to tubular epithelial cells of meso- and metanephros, ureteric bud and gonadal ducts (Shao et al, 2002a).

Gene activation

In the case of gene activation, Cre removes a floxed 'stop' fragment that is put into the genome such that it inhibits gene's transcription. The 'stop' fragment can contain

polyadenylation sites, misleading translation initiation codon and/or splicing donor- or acceptor sequences. Sometimes *neo* selection marker gene acts as a 'stop' fragment. Alternatively to removal of a 'stop' fragment, the Cre mediated recombination can restore the correct reading frame interfered by floxing.

Cell lineage analysis

In cell lineage analysis, the Cre reporter lines (see below) have been proven extremely useful. Activation of a detectable protein enables to register and track cells that have had Cre activity in them or in their precursors, thus detect the origin of a certain cell lineage. For example, the lineages derived from neural crest cells have been detected this way (Chai et al, 2000). In this work, Cre recombinase was expressed under the control of the *Wnt1* promoter, resulting in *Wnt1* transgene expression in the migrating neural crest cells that are derived from the dorsal CNS.

Reporter lines

A reporter line indicates Cre activity by activating the expression of a detectable reporter gene product. Important requirement to these lines is that the reporter gene must be potentially activated in every cell in the organism. This can be achieved in different ways. First, reporter gene separated from its promoter by a floxed 'stop' fragment is introduced to *Rosa26* locus, which guarantees permanent and ubiquitous gene expression. In *R26R* (Soriano, 1999) reporter mice, the *'stop'/β-galactosidase* construct is introduced to *Rosa26* locus. The *β-galactosidase* expression is activated in cells upon Cre activity and maintains also in the descendants of this cell. Similarly, other reporter genes have been used in *Rosa26* locus. Alternative to *lacZ*, which expression can not be easily detected in living tissue, the variants of green fluorescent protein (GFP) – ECFP and EYFP (**e**nhanced **c**yan and **e**nhanced **y**ellow **f**luorescent **p**rotein) reporter strains are useful for monitoring the expression of *Cre* and tracing the lineage of these cells in cultured embryos or organs (Srinivas et al, 2001). The non-overlapping emission spectra of EYFP and ECFP allow also effective double labelling studies in living tissues.

Secondly, the reporter gene can be exposed to ES cells followed by screening for a broad expression pattern. This method was used in creating *Z/AP* mouse line showing constant expression of *lacZ* in all cells, which switches to alkaline phosphatase (*AP*) expression upon Cre recombinase activity (Lobe et al, 1999). This line enables to clearly separate the cells where Cre is active from the cells where it is not.
The availability of different Cre reporter strains is valuable because of the advantages of different reporter genes as well as the efficiency of Cre-mediated excision may be dependent on the target locus.

Deleter- and balancer lines

Mouse lines where Cre is expressed in the early embryo before germ line differentiation are called deleter lines. When a deleter and floxed mouse lines are crossed, the progeny will have a mutation in most tissues. This strategy can be used as an alternative to transient Cre mediated recombination step in ES cells (removal of a selection marker, or gene inactivation), thus reducing the time of ES cell cultivation on a dish, which often tends to lead to differentiation and reduces the ES cells' potential to contribute in all the tissues in the embryo, especially in the germ cell population. The transfer of first recombination step into transgenic animals is possible, when targeting vector is introduced to ES cells containing Cre transgene specific for male germ line. Recombinase expression in male germ line means that the marker will be excised in at least some of the progeny of these ES cell chimeras. Thus, only two generations of mice would give a full mutant for a conditional allele, instead of elaborate and time-consumic crossing of chimeras. A germ-line specific Cre transgene can also be used to deliver recombined target transgenes to the early embryo (O'Gorman et al, 1997).
Mosaic mice, which contain a certain proportion of mutant cells in all organs, sometimes allow to circumvent lethality, analyze the potential of mutant cells to contribute to different cell lineages and to identify cell lineages for whose development a given target gene is critical. Betz et al (1996) describe such balancer line generated using a rat *nestin* promoter and its second enhancer mediating partial deletion of floxed alleles before E10.5 in all organs, including germ line cells.

Inducible Cre expression

In Cre/*loxP* system, the time of the gene modification can be regulated, using inducible Cre recombinase. The first inducible Cre mouse line used the *Mx1* promoter, which can be activated by injecting mice with interferon α or interferon β (Kühn et al, 1995).

To date, two inducible systems have been successfully used in transgenic mice: tetracycline dependent system and the Cre/*loxP* recombinase system with inducible *Cre*. The latter system provides also a possibility to obtain both temporal and spatial control over a gene expression simultaneously.

A reliable inducible system must result in low or no basal gene activity when 'off' and high levels and rapid gene activation after turning 'on'. Often the reversibility of the system is also desired. These requirements are met by a binary system where Cre expression is transcriptionally or posttranslationally controlled by the interaction of two components. Inducible cell type specific transcriptional control is enabled by specific transactivating proteins that regulate the transcription from an artificial minimal promoter in the presence of specific inductor molecules, for example ecdysone or tetracycline (No et al, 1996). Using Cre placed under such artificial promoter, its transcription and recombination of floxed genome segment can be triggered with adding the inducer molecules. The posttranslational control over Cre activity is achieved in systems using cell type specifically expressed Cre fusion proteins that contain domains dependent on an inducer. Typically, these fusion proteins contain the original Cre recombinase and a specific ligand-binding domain (LBD) of some steroid hormone receptor. In the absence of an inducer, the LBD is bound to heat shock (hs) proteins that inactivate the recombinase. LBD recognizing proteins replace the hs proteins and the recombinase becomes activated. To avoid activation of the engineered Cre recombinases by endogenous ligands *in vivo*, Cre has been fused with mutant progesteron and estrogen receptor LBDs for use in transgenic mice (inducible systems reviewed by Jaisser, 2000 and Lewandoski, 2000).

Cre/loxP system combined with TetR system

Combining Cre/*loxP* with TetR system, *Cre* is brought under the control of *tetO*-conjugated promoter. Tissue specifically expressed transactivator (tTA or rtTA) binds to *tetO*, but for this activity needs an inducer – most commonly doxycycline (dox) is used. Both tTA and rtTA system has been utilized in Cre mediated inducible reporter gene expression. Using classical tTA sytem it has been shown that site specific genomic recombination can be induced *in vivo* (St-Onge et al, 1996). Still, in these first experiments, the Cre background activity was observed in at least half cases, supposedly because of insufficient or fluctuating concentrations of the administred inducer during the experiment. Combining *Cre* with rtTA system, more stringent regulation can be achieved. Tet-on system or rtTA has been thus used in numerous successful applications and is recommended to be preferred over tTA system for gene inactivation studies because of its kinetics, but the system still encounters some considerable limitations *in vivo*. First, the full activation of an rtTA-dependent promoter is achieved only at the dox concentration of 1-2 µg/ml in the tissue. In some tissues this concentration is hard to obtain. Secondly, the commonly used rtTA has some residual activity in absence of dox, which in some cases can be observed as background activity. Another thing that might hinder the application of the system, is the reduced stability of both rtTA mRNA and protein in certain cell types (Urlinger *et al,* 2000). The stability and dox specificity has been improved in mutant rtTA proteins, which provide up to 10-fold higher affinity to dox, compared to the original wt rtTA, are shown to be more stable in eucaryotic cells and have no (or undetectable) background activity in the absence of dox (Urlinger et al, 2000). From the mutant rtTAs created in this work, the rtTA2-M2 is now most used reverse transactivator in tetracycline dependent system construction. Since the tet-dependent gene inactivation system consists of quite a lot of components – Cre gene and inducible tet-conjugated promoter as well as tissue specifically expressed transactivator, it is useful to introduce them to the genome as a united system, avoiding the producing and crossing of many transgenic mouse lines, and long optimizations. To improve the tetracycline-regulated Cre/*loxP* system the universal transgenic system has been established that combines two layers of regulation, provided by cell type specific promoter and doxycycline (Utomo et al, 1999). In the described system a cell type

specific promoter controls the expression of rtTA, which is activated by doxycycline administration, followed by the induction of Cre expression by active rtTA, and Cre-mediated deletion of floxed DNA fragment. Using single bidirectional dox-dependent promoter triggering both Cre and rtTA expression after dox administration, an auto-inducible Cre-expressing deleter line has been produced (Holzenberger et al, 2000). Still, it should be noted that in this line the full Cre activation is achieved after 3 weeks of dox treatment and only postnatally.

Activation of Cre with synthetic steroids

Fusion proteins between Cre and the mutant ligand-binding domains (LBD) of nuclear receptors, such as receptors for glucocorticoids, estrogens, or progesterone have been generated. In the case of a mutated domain, the endogenous ligands cannot bind to Cre recombinase; only can the synthetic ligands like RU486, tamoxifen or *Drosophila* hormone ecdysone. In the absence of an appropriate ligand, the Cre fusion protein is associated to hs proteins and inactivated.

Progesterone LBD

Mutant human progesterone (PR) receptor LBD-Cre fusion protein has been tested in vivo (Kellendonk et al, 1996) and shown not to respond any endogenous progesterone. The protein is activated by RU486, synthetic progesterone analog, which is rapidly and widely distributed after oral or intravenous administration. Still, the system has considerable drawbacks as the PR LBD appears to inhibit recombinase activity when placed close to Cre active domains, at the same time as the proximity of these domains improves the stringency of the regulation. Furthermore, RU486 is toxic to animals, inducing the abortion of the pregnancy. In the study of Kellendonk et al (1996), it was anyhow shown that the effective doses of RU486 used induced no teratogenic or mutagenic effects.

PR LBD-Cre fusion proteins generally provide mosaic recombination which efficiency strongly depends on the tissue, and often the background Cre activity is a problem. Therefore, the mutant proteins with improved sensitivity to the inducer have been

developed. The response to low doses of inducer has been significantly improved by elongating the PR LBD C-terminally, deleting the first 18 amino acids from Cre, and mutating the splice donor sites within the sequence. The optimal fusion protein modified this way provided >200-fold inducibility (Wunderlich et al, 2001), in comparison the unmodified PR LBD-Cre was maximally induced 40-fold (Kellendonk et al, 1996).

Estrogen LBD

Successful induction of Cre mediated recombination in mouse B lymphocytes was described by Schwenk et al (1998), with the efficiency up to 80%. In this work the expression of Cre recombinase fused to mutated estrogen (ER) receptor LBD was limited to B lymphocytes by use of tissue specific elements in its promoter.

Mutant ER LBDs have been used that bind the synthetic analogues of estrogen, tamoxifen (TM) and 4-hydroxytamoxifen (4-OHT). Still, studies on the functionality of mutated ER LBD-Cre fusion proteins in cell culture showed 5-10 % basal Cre activity, possibly due to a cleavage of the linker between the Cre and LBD by intracellular proteases. The system could be improved, using fusion protein where ER LBD was added to the both termini of Cre (Zhang et al, 1996).

Danielian et al (1998) demonstrated that tamoxifen-inducible recombination can be used to effectively modify gene function in the mouse embryo. A tamoxifen-inducible form of Cre recombinase was used to modify gene activity *in utero*. Using the enhancer of the *Wnt1* gene to restrict the expression of Cre to the embryonic neural tube, it was shown that a single injection of tamoxifen into pregnant mice induced Cre-mediated recombination within the embryonic central nervous system, thereby activating expression of a reporter gene. Induction was ligand dependent, rapid and efficient, but the recombination could be induced only after E9.5; probably the chorio-allantoic placental connection established around E9.0 increases the availability of ligand to the embryo. Although the induction is tightly controlled and rapid, the problem with this system is the high concentration of inducer needed. In fact, the optimal dose determined in this work, is the minimal dose that has no negative effect to the pregnancy. The Cre-ER LBD system can be improved with mutagenesis, in fact, different mutant fusion proteins (Cre-

ER^T, Cre-ER^{T2}) showing up to 10 times hihger 4-OHT sensitivity have been developed and tested *in vivo* (Indra et al, 1999).

The usage of drug-induced fusion proteins can be complicated in tissues with limited access, for example in brain and nervous system. For tamoxifen, it has anyhow been demonstrated that it can pass the blood-brain barrier, thus the ER LBD-Cre transgenic mice can be used for conditional mutagenesis in nervous system (Weber et al, 2001).

Virus-mediated Cre transfer into the organism

Injection of Cre expression vector provides exact control over the time of recombination and with a short time, very high concentration of recombinase is achieved. Adenoviruses and herpes simplex virus (HSV1) are most used in *Cre* transmission. This method is suitable in adult animals because the transmission of the virus vectors into the uterus is complicated.

The Cre recombinase under regulation of the HSV *tk* promoter carried by recombinant adenovirus vector has been shown to trigger recombination in most tissues, and in some cases the recombined gene locus is maintained for an extended period. The recombination level varies between tissues, being highest in liver and spleen, and only a trace level in brain in the case of intravenous administration of virus vectors. Still, infecting specific brain regions with the vectors, the recombination is achieved (Wang et al, 1996). The efficiency of recombination mediated by adenoviral transmitted Cre has been measured in various tissues and proved to be highest in hepatocytes: about 80 % of cells recombined after infusion of 10^9 p.f.u adeno/Cre viruses (Akagi et al, 1997).

With this technique, the main problems are the possible host-virus interactions, cytopathological effects and possible cell death. Adenovirus could trigger either humoral or cellular immunoresponse, thus destroying the virus vectors and infected cells. Different infection efficiency in different cell types restricts the number of tissues the strategy can be used. The recombination in the place of infection is usually mosaic; only part of the cell types might be infected. Still, all these disadvantages can turn out to be useful, depending on a certain experiment strategy and requirements.

Generally, the greatest problem with the conditional system is the recombination efficiency, which in most cases remains below 100%. Hence, at the current moment a perfect conditional system for studying cell-autonomous effects (where all the cell have to be genetically mutant in order to show the mutant phenotype) has not been established. However, in majority of reported studies the recombination efficiency has been high enough to adequately estimate the cell non-autonomous effects of the gene. In this case only a part of cells in the tissue have to carry a mutation in order to express the phenotype (Lewandoski, 2001). Less important disadvantages are the irreversibility of the modifications or 'all-or-nothing' principle – gene silencing cannot be observed dynamically.

MATERIAL AND METHODS

Cre expression constructs

Mouse *Hoxb7* promoter sequences –1316 to +8 bp from translation initiation site were extracted from the plasmid *pGENBlue* (provided by J. Deschamps, Utrecht). In cloning of the kidney specific *Cre* expression construct *Hoxb7-cre*, *Litmus29* vector (New England BioLabs Inc.) was used to connect the promoter sequence with the Cre coding sequences derived from the plasmid *pNLS-cre-SV40pA* (provided by K. Rajewski). In cloning of the conditional Cre expression constuct *Hoxb7-core*, the promoter sequences were inserted into *Eco*RI promoter cloning site in *Core Construct* vector (provided by W. H. Lee, San Antonio, Texas, described in Utomo et al, 1999, Fig. 1A). E.Coli strain DH5α was used in cloning procedures. DNA fragments for oocyte microinjections were extracted from plasmids using endogenous restrictases (Finnzyme). Fragments were separated from plasmid sequences by agarose-gel electrophoresis and purified using QIAQuick Gel extraction kit.

Transgenic mice

GTRosa26 lacZ reporter strain

GTRosa26 lacZ reporter mice were purchased from the Jackson Laboratory, stock no 003310.

Original transgenic mouse lines

Transgenic mice were generated by microinjection of Cre expression constructs *Hoxb7-cre* or *Hoxb7-core* into the male pronucleus of fertilized oocytes. Oocytes were left to develop overnight and the developed blastocysts were transferred to pseudo-pregnant foster mice from the line C57/bl6. The offspring was analysed by PCR and Southern blot. Founders were crossed with wildtype C57/bl6 animals to establish transgenic mouse lines.

Genotyping of mice

PCR

Genotyping was performed using DNA lysate from mouse tail biopsies, or the lysate from the yolk sacs of the embryos. The tissue was incubated overnight at 56 ˚C in the ~0.3 mg/ml proteinase K solution in buffer (80 mM Tris•HCl, 20 mM $(NH_4)_2SO_4$), then heated 20 min at 98 ˚C to inactivate proteinase K.

The primers used for amplification of *Cre* sequence were *creF1* (TTCGCAAGAACCTGATGGAC) and *creR1* (GAACCTGGTCGAAATCAGTGC).

These primers yield in 369 bp amplification product in mutant.

The PCR reaction for amplifying *Cre* sequence (94ºC 30", 60 ºC 30", 72ºC 40", 33 cycles) contained: 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.1% Tween20, 2.5 mM $MgCl_2$, 0.1 mM dNTP, 0.17 μM each primer, 1 U Taq DNA polymerase (FIREPol) in 20 μl reaction mix and 10-50 ng DNA.

For genotyping *Rosa26* mice we used the following primers and PCR protocol suggested by Phil Soriano (URL http://www.fhcrc.org/labs/soriano/protocols/pcrgen.html):

R26F2 (AAAGTCGCTCTGGTTGTTAT),

R1295 (GCGAAGAGTTTGTCCTCAACC) and

R523 (GGAGCGGGAGAAATGGATATG).

These primers yield in ca 250 bp product on mutant *Rosa26* allele and 500 bp product on the normal allele.

The PCR reaction for amplifying *Rosa26* sequence (94ºC 30", 58 ºC 30", 72ºC 60", 35 cycles) contained: 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM $(NH_4)_2SO_4$, 0.1% Tween20, 2.5 mM $MgCl_2$, 0.15 mM dNTP, 0.2 μM *R26F2* primer, 0.125 μM *R523* primer, 0.075 μM *R1295* primer, 1 U Taq DNA polymerase (FIREPol) and 10-50 ng DNA.

Southern blot

Southern blot and radioactive hybridization procedures were performed following the protocols from The Short Protocols in Molecular Biology (3rd edition, Ausubel et al,

1995). The DNA for Southern blot was salted out from ca 10 mg mouse tail biopsies. Tissue was lysed overnight at 55 ˚C in 10 mM Tris (pH 7.5), 400 mM NaCl, 100 mM EDTA, 0.6% SDS containing 15 µg/ml proteinase K. 80 µl of 6M (saturated) NaCl was added to the suspension, vortexed for 10 sec, and spinned for 5 min at 14000 rpm in a microfuge. After adding 600 µl 100% EtOH to supernatant, white DNA precipitate formed, which was pulled out from solution with a glass rod, washed in 70% EtOH, and dissolved in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA).

DNA was digested overnight and precipitated with ethanol, DNA fragments were separated in agarose gel electrophoresis (in 0.8% agarose/TBE gel), denaturated in 0.5 M NaOH, 1.5 M NaCl solution for 45 min, and transferred overnight to HybondN^+ by using alkaline transfer method. Filter was neutralized in 0.5 M Tris pH 7.5, 1.5M NaCl solution for 15 min, and DNA was crosslinked using the standard program in UV-Stratalinker.

^{32}P-labelled hybridization probes against *Cre* and *Hoxb7* sequences were synthesized from fragments extracted from plasmid *HB7-Cre-L29*. Radioactive hybridization signal was detected with PhospoImager after overnight exposure of the filter.

Doxycycline administration to mice

Doxycycline was administred to pregnant mice from the crossings of *Hoxb7-core* transgenic mice with the *GTRosa26* reporter line. For intraperitoneal injections doxycycline (80 mg per 1 g body weight) was dissolved in 1xPBS at a stock concentration of 10 mg/ml. The doxycycline was administred to pregnant mice daily beginning from 6.5 dpc, and embryos were dissected at 10.5 dpc or at 18.5 dpc.

Histochemistry

Dissected embryos or kidneys were fixed in 4% paraformaldehyde in phosphate-buffered saline (4% PFA/PBS) for maximum 1 hour and rinsed three times in 0.1M phosphate buffer (pH 7.3) containing 2mM $MgCl_2$. The β-galactosidase activity was visualized by overnight incubation in rinse buffer containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6$ and 1 mg/ml X-gal (5-Bromo-4-chloro-3-indolyl-ß-D-galactoside). Stained embryos and

kidneys were postfixed overnight, and stored in 70% EtOH or embedded into paraffin and sectioned.

Embryos were embedded into gelatin/albumin for microvibratome sectioning or into TissueTek O.C.T. compound (Sakura Finetek) for cryosectioning. Free floating 50 μm vibratome sections were fixed for 3 min in 4% PFA/PBS, also 15 μm cryosections on glass slides. The β-galactosidase activity was visualized as described for embryos. Stained vibratome and cryosections were postfixed in 4% PFA/PBS for 10 min and mounted. Paraffin and cryosections were counterstained with eosin or fast red nuclear stain, dehydrated and mounted with Depex mounting medium (Gurr®, BDH Lab supplies). Vibratome sections were mounted with Aquamount medium (DAKO).

Tyrosine hydroxylase (TH) antibody stainings were performed on lacZ stained cryosections. Tissue was blocked in 10% goat serum in dilutant (2% bovine serum albumin in PBS, 0.2% TritonX). Sections were incubated in anti-TH antibody (1:500, Chemicon AB152) in dilutant overnight at 4 ºC followed by 4 h incubation in horseradish peroxidase conjugated anti-rabbit IgG secondary antibody (1:500, Chemicon AP132P) in dilutant. For peroxidase development, sections were placed in 50 mM Tris-HCl (pH 7.5) containing 25 mg/ml diaminobenzide, 0.005% H_2O_2 and 1% ammonium nickel(II)sulphate. After color development sections were counterstained with fast red nuclear stain, dehydrated and mounted with Depex.

Microscopy

Embryos and kidneys were photographed under Olympus binocular light microscope using CCD camera. Sections were viewed and photographed under Olympus AX70 microscope connected to Olympus DP70 camera. The photos were edited with programs SensiCam and compiled using Adobe Photoshop.

RESULTS

Cre expression vectors

For cloning the *HB7-cre* construct I used the *Litmus 29* plasmid vector, utilizing convenient restriction sites in its MCS. The mouse *Hoxb7* promoter sequences were extracted from the plasmid *pGENblue* with *Sma*I ja *Eco*RI, and then ligated into the same enzymes' restriction sites in *Litmus 29* MCS, resulting in the construct *HB7-L29*. *Cre* sequence linked to a polyadenylation signal was extracted from plasmid *pNLS-cre-SV40pA* using restrictases *Pst*I ja *Hin*dIII and cloned into the same sites in *HB7-L29* (cloning scheme in Fig. 10A) Resulting construct *HB7-cre-L29* (Fig. 10B) was verified by restriction (Fig. 10C) and PCR analysis. The 2.8 kb DNA fragment containing only *Hoxb7* promoter followed by *Cre* coding sequence and polyadenylation signal (further on referred as *HB7-cre*, Fig. 10B) was isolated for oocyte microinjection as follows. The *HB7-cre-L29* was digested with *Pvu*II ja *Hin*dIII and the *HB7-cre* fragment was separated from plasmid sequences by gel electroforesis.

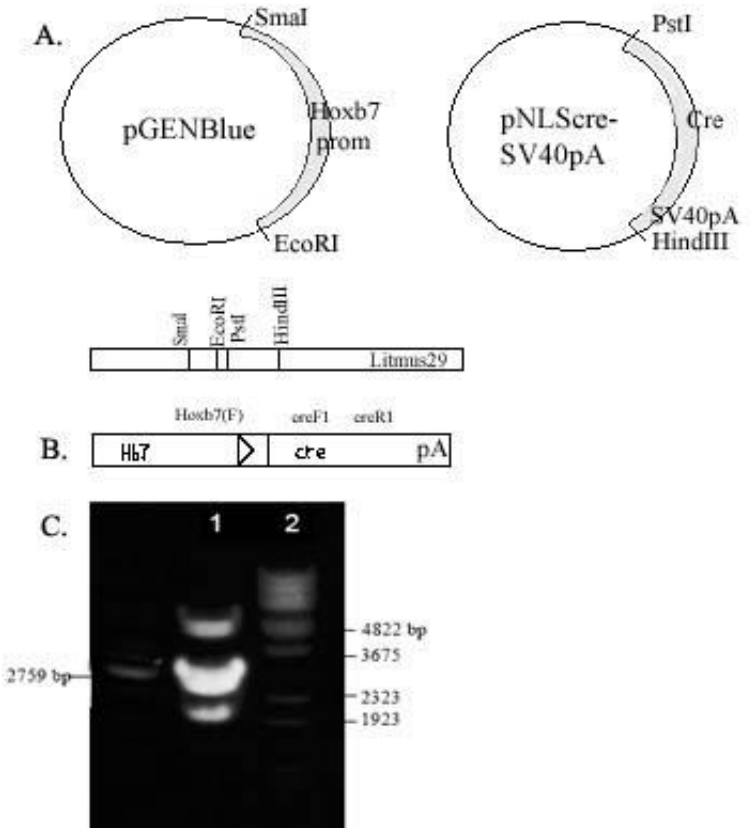

Figure 10. (A) Vectors and restriction enzymes used in cloning of the *Hoxb7-cre* Cre expression construct. (B) *Hoxb7-cre* Cre expression construct used in the generation of transgenic mice via oocyte microinjection. (C) Extraction of the *Hoxb7-cre* fragment from the plasmid sequences. 1 – plasmid restricted with *Pvu*II, *Sma*I and *Hin*d III; 2 – marker λDNA/*Eco*91I.

Cloning of the *Hoxb7-core* construct was based on the *Core Construct* plasmid which utilizes reverse tetracycline dependent transactivator together with *Cre* under the control of human CMV promoter linked to *tetO* sequences (cloning scheme at Fig. 11A). The *Sma*I/*Eco*RI extracted *Hoxb7* promoter fragment was ligated to the *Eco*RI site in front of the rtTA gene; the 5' end of the promoter was ligated after Klenow reaction blunting the remaining *Eco*RI site in the vector. The construct was checked by restriction analyses (Fig. 11B). The final 9 kb *Hoxb7-core* construct (Fig. 11C) for microinjection was

separated from the plasmid by *Sca*I digestion and purified as previously described for *HB7-cre*.

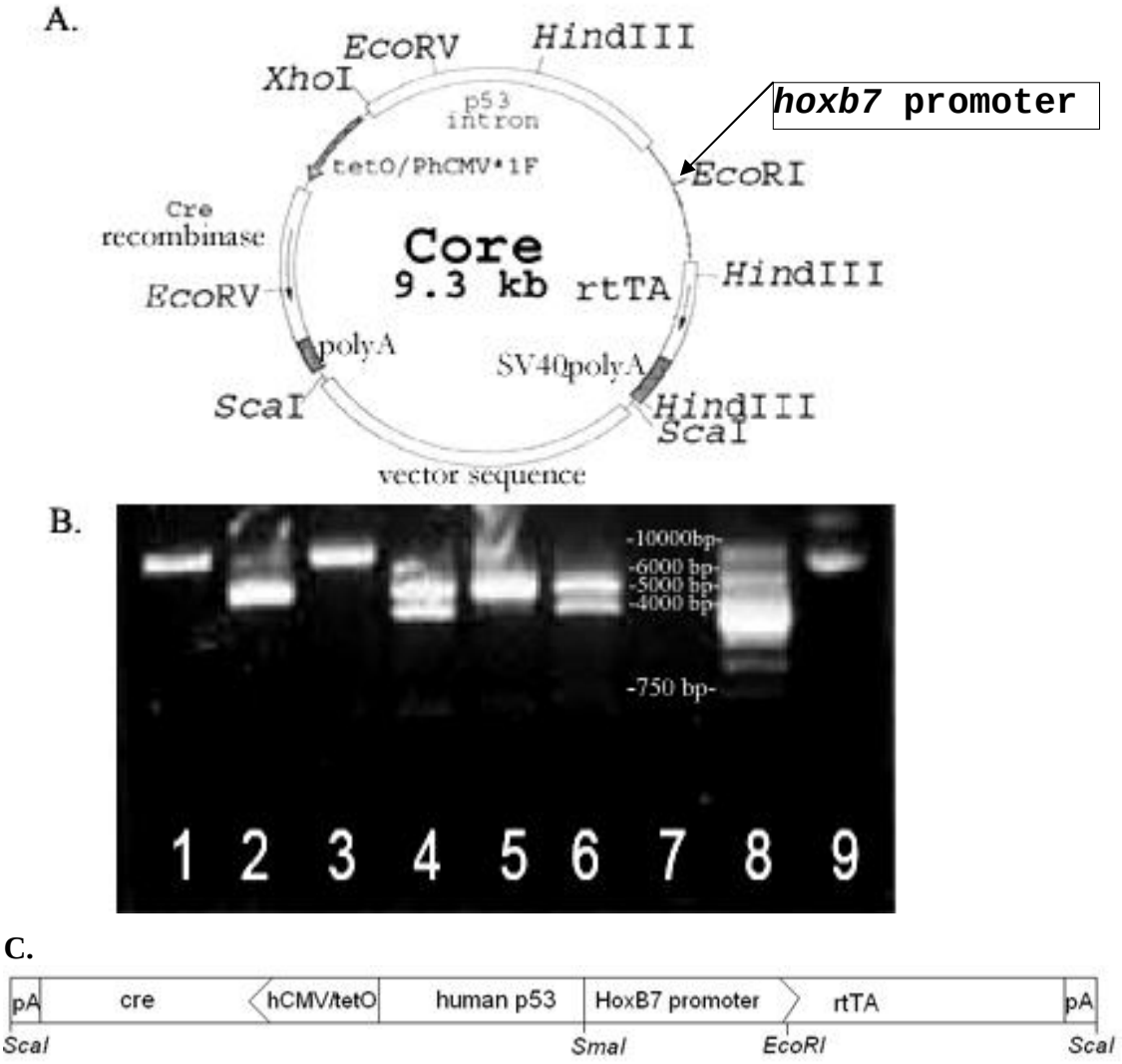

Figure 11. (A) Cloning of the *Hoxb7-core* Cre expression construct. (B) Restriction analysis to determine the orientation of the inserted *Hoxb7* promoter. The plasmid consisting the promoter in the right orientation at the rows 2 and 5. 1,2,3 – *Hoxb7-core construct* restricted with *Eco*RV; 4,5,6 – restriction with *Eco*RI/*Eco*RV; 8 – marker, 9 – *Core Construct* plasmid. (C) *Hoxb7-core* fragment used in oocyte microinjections.

Transgenic mice

Generated *Cre* expression constructs were injected into the male pronuclei of fertilized oocytes, resulting in the random integration of the transgenes into the genome. Procedures were performed by the qualified personnel in VISGENYX company.

Altogether 101 mice were born from these microinjections, 28 from the injection of *HB7-cre* and 73 for *Hoxb7-core*. The DNA samples from these mice were analysed first by PCR to identify the animals carrying *Cre* in their genome. Thus 7 founder animals were identified carrying *HB7-cre* and 8 carrying *Hoxb7-core*. These PCR-positive animals were further confirmed for the presence of Cre in the genome on Southern blot (Fig. 12). For Southern blot, the genomic DNA was digested either with *Pst*I (*HB7-cre* transgenic founders, hereafter referred as *Cre* mouse lines) or with *Eco*RI (*Hoxb7-core* transgenic founders, hereafter referred as *Core* mouse lines). These restriction enzymes have intrinsic recognition sites in the respective transgenes, enabling to estimate the mosaicism (the percentage of cells that have incorporated the transgene), or the transgene copy number in the genome. From *Cre* founders, two were transgenic with mosaic transgene incorporation.

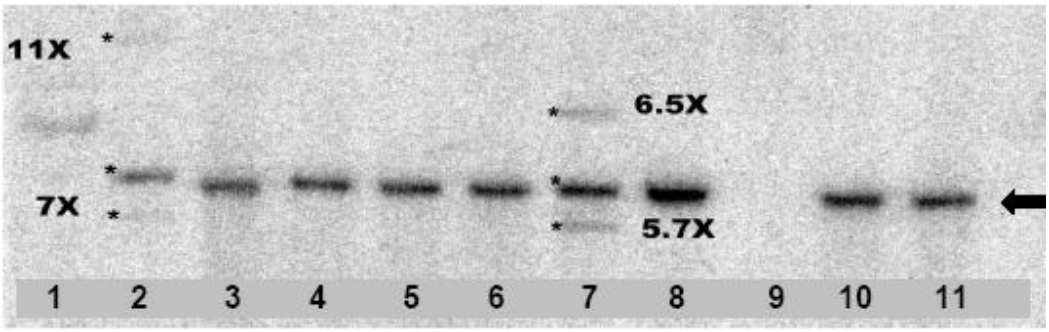

Figure 12. Southern blot with *Hoxb7* probe on Cre founders. Wildtype band indicated with the arrow. 2 – DNA from the founder *Cre3*, 7 – founder *Cre4*. The intensity of bands was quantified and the relative intensity of transgenic bands compared to the wildtype is expressed as the inverse figure (x).

The mosaicism in cre founders was determined, comparing the quantified intensity of wild type *Hoxb7* signal to that of the transgene's (Fig. 12). The mosaicism for *Cre3* founder was 10%, for *Cre4*, 20%.
All the founders for *Core* mouse lines appeared to be positive for the transgene and probably have incorporated tandemic copies of the transgene into the genome.

Two *Cre* and eight *Core* transgenic mouse lines were established on the C57/bl6 background. The progenies were genotyped against Cre sequence. In F1, the transgene was inherited to 30% of *Cre3* founder offspring and to 20% to *Cre4* offspring. Further matings resulted in normal mendelian distribution (50%). In case of *Core* founders, transgene was passed on to at least 50% F1 offspring. The inheritance efficiency in F1 for some *Core* lines reached up to 80-90%, suggesting multiple genomic transgene integration sites in these founders. In further matings, these integration sites separated and the transgene is passed on in normal mendelian distribution (50%).

Cre expression patterns in established transgenic lines were analysed via crossing these mice with *GTRosa26* LacZ reporter strain and visualizing Cre-triggered β-galactosidase activity in double transgenic offspring using enzymatic LacZ staining reaction (Figs. 13 - 19). Transgene expression patterns in all lines were first analysed in E10.5- E11.5 embryos; generated kidney specific lines *Core3* and *Core8* were further analysed at newborn stage (Figs. 17 and 18). Cre expression pattern in line *Core5* was described dynamically through development and in adult stage (Fig. 19), and the *Cre* expressing cells were also tested for colocalization with neuronal markers, double labelling with tyrosine hydroxylase as a dopaminergic neuron marker is shown (Fig. 19, I-K). However, the nature of these cre expressing cells remains yet to be elucidated as this project is still on progress.

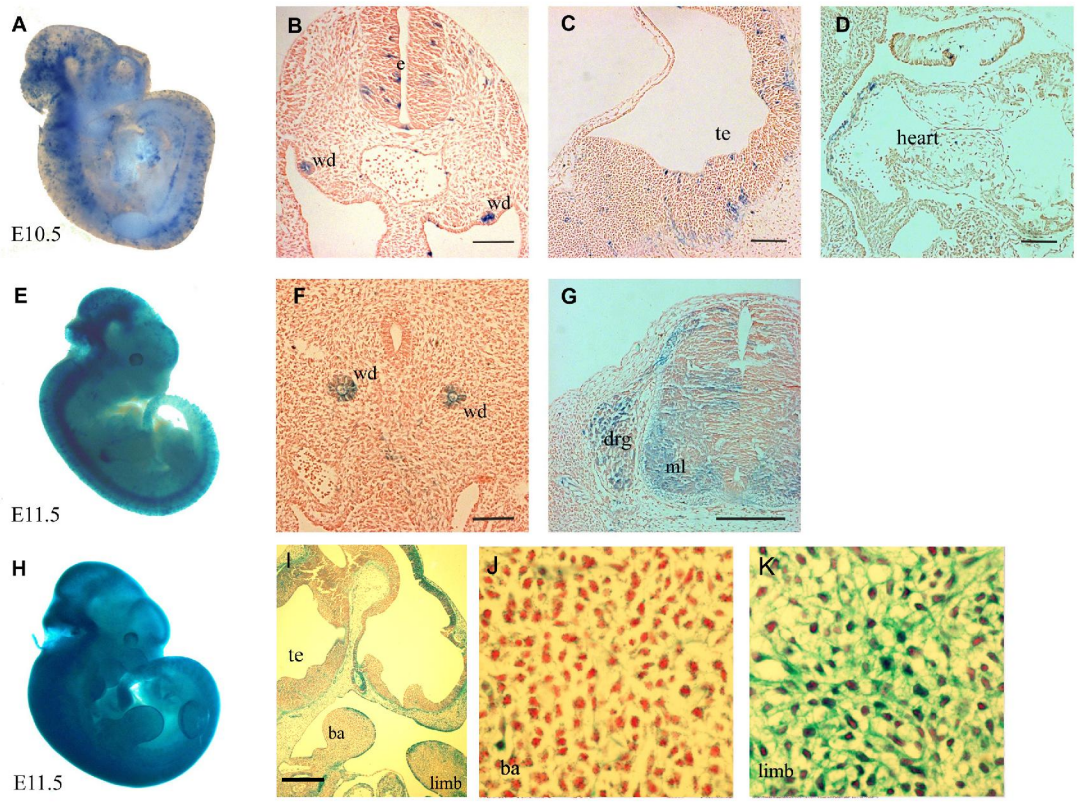

Figure 13. Cre-mediated recombination patterns in transgenic lines *Cre3* (A-G) and *Cre4* (H-K). Whole mount photos (A, E, H) and photos of paraffin sections of $cre^{+/-}/rosa^{+/-}$ embryos are presented, Cre-expressing cells are stained blue. Cre activity results in the transient recombination in the epidermis and wolffian ducts of *Cre3* mouse line, and pandemically in the line *Cre4*, panels J and K present 5x enlarged close-up images from branchial arch (ba) and limb from I.

e, ependyma; wd, Wolffian duct; te, telencephalon; ml, mantle layer cells; drg, dorsal root ganglion; ba, branchial arch. Scale bar: 100 μm.

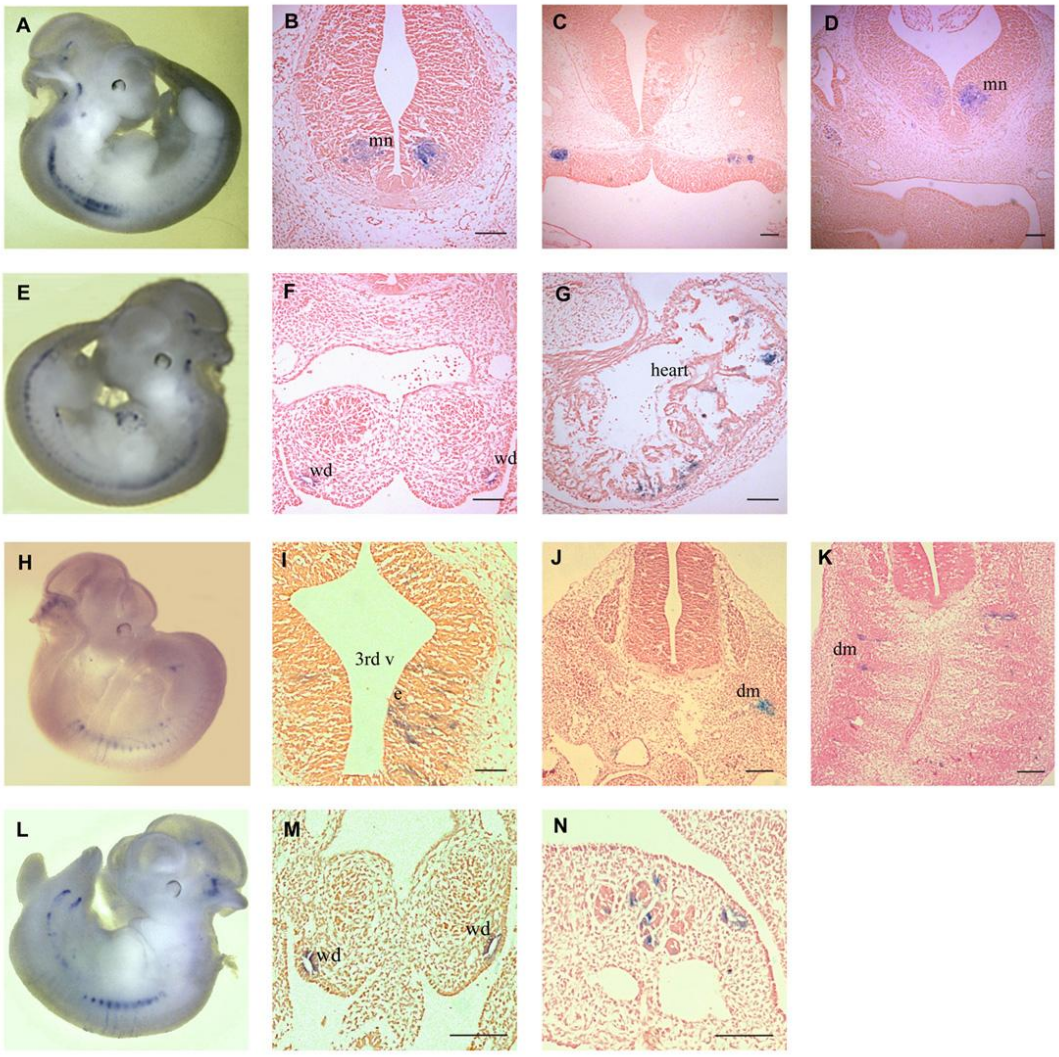

Figure 14. Whole mount photos (A, E, H, L) and photos of paraffin sections of transgenic embryos from two different mouse lines derived from *Core2* founder. Both mouse lines show high level Cre mediated recombination in Wolffian ducts, accompanied by recombination also in mesonephric tubulae in line *Core2-8*; in *Core2-5* line the additional recombined cell groups can be identified as motoneurons of neural tube and some cardiomyocytes.

wd, Wolffian duct; mn, motoneurons; dm, dermomyotome. Scale bar: 100 μm.

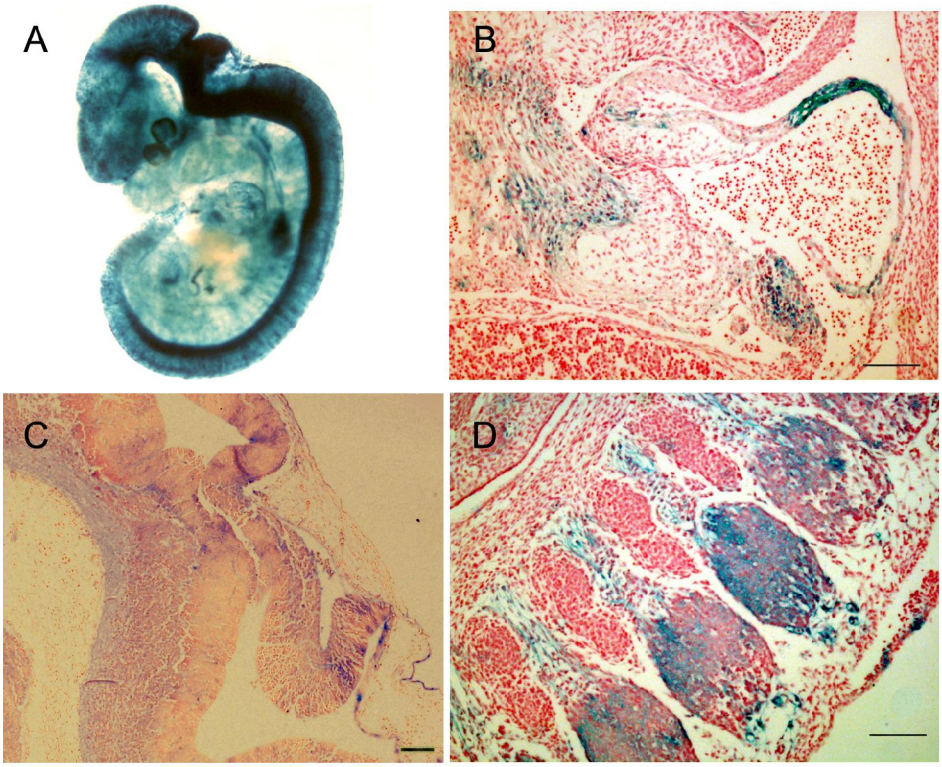

Figure 15. Cre-mediated recombination pattern in mouse line *Core1*. Whole mount photo (A) and photos of lacZ stained paraffin sections of E12.5 transgenic embryo. Cre recombination in the heart (B), developing midbrain and cerebellum (C) and dorsal root ganglia (D). Scale bar 100 μm.

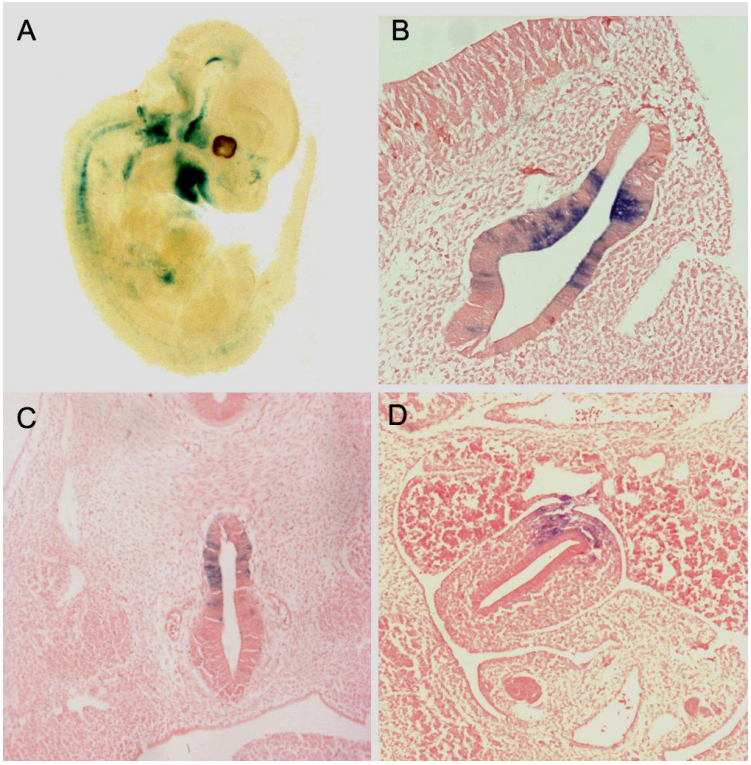

Figure 16. Cre-mediated recombination pattern in mouse line *Core7*. Whole mount photo (A) and photos of lacZ stained paraffin sections of E12.5 transgenic embryo. Cre recombination in the developing inner ear (B), region of neural tube (C) and heart (D).

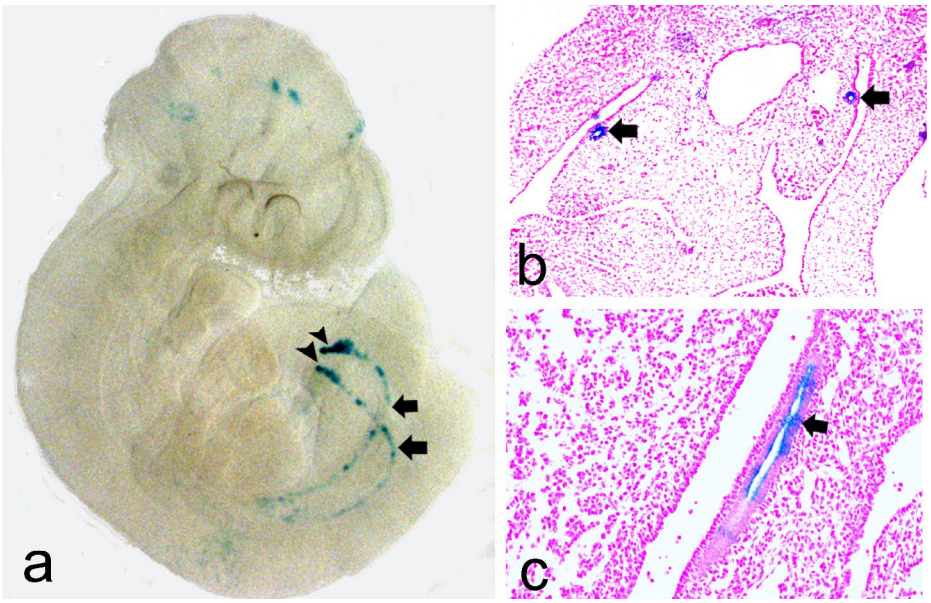

Figure 17. Kidney specific Cre recombinase expression in E10.5 embryo of mouse line *Core8*. a, lacZ staining on *core8*$^{+/-}$/*rosa*$^{+/-}$ whole mount embryo; b, transverse section and c, sagittal section showing wolffian duct specific Cre mediated recombination.
arrow, wolffian duct; arrowhead, ureteric bud

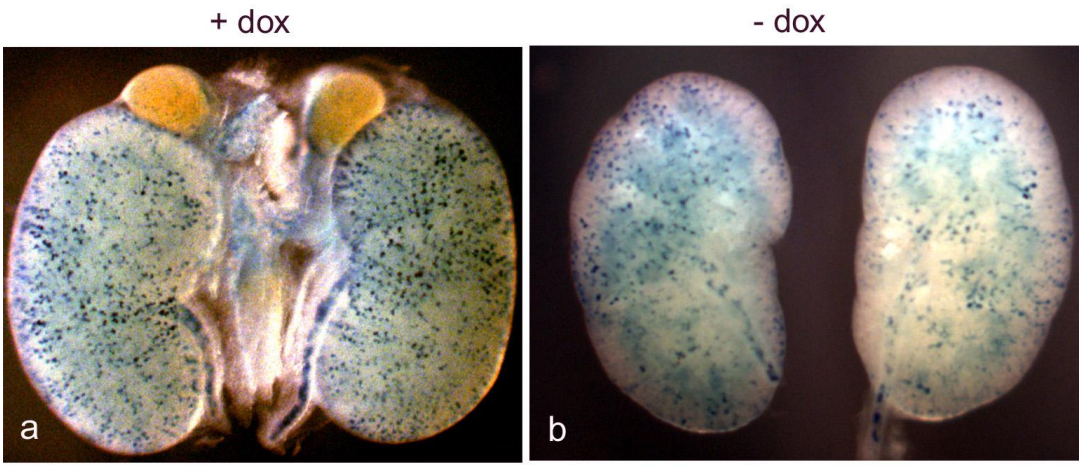

Figure 18. Cre mediated recombination pattern in kidneys from *Core8* newborn mice. Slight difference in recombination efficiency can be noticed between kidneys from dox-treated and untreated animals.

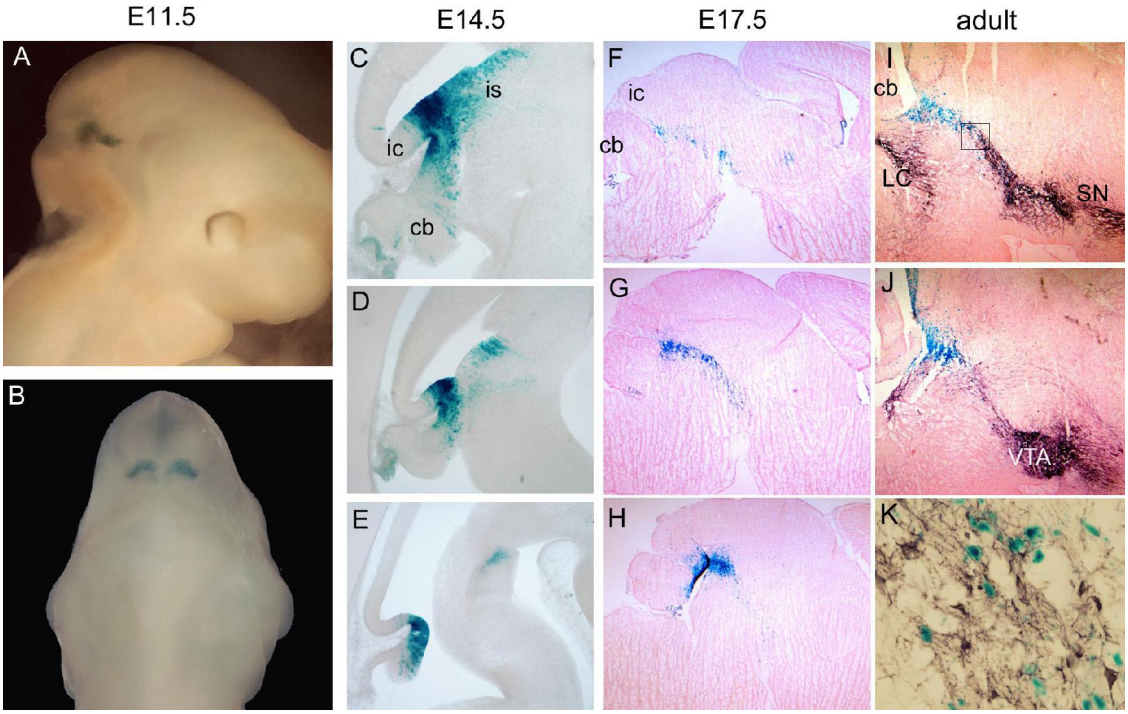

Figure 19. Cre recombination pattern in mouse line *Core5*. Recombination in the mid- and hindbrain boundary starts at 44 somites stage (approximately E10.75) and persists throughout life. A, B, E11.5 embryos; A, lateral view and B, posterior view. C-E, 50 µm sagittal vibratome sections from the head of a E14.5 *core5*$^{+/-}$*/rosa*$^{+/-}$ embryo; C shows lateralmost and E is the medial section. F-H, 15 µm cryosections from the brain of a E17.5 *core5*$^{+/-}$*/rosa*$^{+/-}$ embryo, shown from lateral to medial again, I-K, 25 µm cryosections from the brain of an adult *core5*$^{+/-}$*/rosa*$^{+/-}$ mouse, double staining with LacZ (blue) to localize Cre transgene expressing cells and anti-tyrosine hydroxylase antibody (black) that labels dopaminergic neurons. K, high magnification image from the area marked with square on I.

cb – cerebellum, ic – inferior colliculus, is – isthmus, LC – locus coeruleus, SN – substantia nigra, VTA – ventral tegmental area.

DISCUSSION

The aim of this work was to generate conditional *Cre* mouse line to provide a tool for studying developmentally important genes in kidney. For example, GATA-3, one of the crucial transcription factors in early kidney development, is one of those genes which conventional knockout results in embryonic lethal phenotype. *GATA-3*$^{-/-}$ mice die around E10.5 due to noradrenergic deficiency and simultaneus cardiac failure (Lim *et al*, 2000), and therefore further roles of this factor in development are largely unknown.

In this paper I described the generation of two conditional *Cre* expression constructs as well as subsequent establishment and analysis of transgenic mouse lines. To achieve kidney specific Cre expression, the *Hoxb7* promoter fragment extending from –1316 to +8 bp from translation initiation codon, which is described to induce gene expression in the mesonephric Wolffian duct-derived epithelium of the meso- and metanephric kidney and associating ducts throughout development, beginning from the time these structures first appear (Kress *et al*, 1990). This promoter fragment has been used for kidney specific gene expression several times (Yu *et al*, 2002; Srinivas *et al*, 1999).

Cre expression constructs were used in production of transgenic mouse lines via oocyte microinjection. This method results in random transgene integration into the host genome, and, compared to targeted transgene insertion in ES cells followed by transgenic line production via blastocyst microinjection, is considerably less time and material consuming. On the other hand, the random integration of transgene can result in its unexpected expression.

The mice born from microinjection were genotyped by PCR and Southern blot. The transgene integration efficiency as seen in Southern blots, was variable throughout the lines, being significantly lower in the case of *Hb7-cre* transgene, which resulted in two 10-20 % mosaic founders out of 28 animals born (7%), at the same time when *Hb7-core* transgenic founders (9% of born animals) were not mosaic and some had probably multiple integration sites. The mosaicism is quite typical for the F1 generation of transgenic mice produced by oocyte microinjection, as the transgene often does not integrate into the genome momentarily, but some time after the injection, when the oocyte has already cleaved. In such cases only part of the cells in the organism are

transgenic, the proportion of these modified cells is expressed as the mosaicism percentage. In the next generation completely transgenic animals are born, but in first crossing mosaicism causes lower inheritance of the transgene, as only part of the gametes are modified. The inheritance efficiencies of 20 % and 30 % observed in lines *Cre3* and *Cre4* are relatively good, considering the possibility that the transgene could not be inherited at all, if there is no germline contribution. The mosaicism still does not exclude the possibility that there are tandem repeats of transgene in the integration site. In *Hb7-Core* mouse lines, anyhow, no mosaicism could be detected. Instead, very intensive signals on Southern rather implicate full integration plus tandem repeats of trasgene in the genome. The inheritance of transgene was at least 50 %, in some lines reaching up to 80-90 %, suggesting multiple integration sites which are separated in next generations. Indeed, multiple gene expression pattern were seen in the progeny of the founder showing the highest transgene transfer efficiency.

Cre expression patterns in eight established mouse lines were described using LacZ reporter line *GTRosa26*. In the double transgenic progeny from these crossings, the Cre activity pattern is labelled by beta-galactosidase expression activated by Cre mediated recombination. The range of Cre expression patterns observed in this study (table 1) confirm expressively the major effect of the final genomic location on a randomly inserted transgene. The Cre expression was often observed in developing central and peripheral nervous system raising the suspection that the promoter fragment originally described to drive stringent transgene expression in Wolffian duct and its derivatives by Kress et al (1990), still might contain some nervous system specific elements, probably responsible for the early *Hoxb7* expression in the developing nervous system (Vogels et al, 1990). In this respect, the Ksp-cadherin promoter could prove to be more reliable in driving kidney specific *Cre*, because *Ksp-cadherin* itself is only expressed specifically in Wolffian duct ant its derivatives, starting from E14.5 (Thomson et al, 1999). The *Ksp-cadherin* promoter driven Cre has been shown to mediate recombination of floxed fragments beginning from E10.5 specifically in developing kidney (Shao et al, 2002a, 2002b).

In this work we also tested a tet-on/cre expression cassette described previously as a united inducible and tissue specific expression construct providing stringent control over

Cre expression (Utomo *et al*, 1999). This system should enable to trigger transgene expression with doxycycline. Anyhow, we have registered no considerable difference in Cre activity between the treated and untreated 10.5 dpc embryos and only a slight difference in 18.5 dpc prenatal kidneys. Although rtTA has been used in numerous successful applications and is recommended to be preferred over tTA system for gene inactivation studies because of its kinetics, the system still encounters some considerable limitations *in vivo*. First, the full activation of an rtTA-dependent promoter is achieved only at the Dox concentration of 1-2 µg/ml in the tissue. In some tissues this concentration is hard to obtain. Another thing that might hinder the application of the system, is the reduced stability of both rtTA mRNA and protein in certain cell types. Finally, the conventional rtTA has some residual activity in absence of Dox, which in some cases can be observed as background activity. Now there are enhanced rtTA proteins available (Urlinger *et al*, 2000, Das *et al*, 2004). Using these enhanced rtTA proteins, different tet-on based single cassette expression sytems have recently been generated and showed to be functional at mammalian cell lines (Bäckman *et al*, 2004). The residual affinity of rtTA to *tetO*-promoter and subsequent *Cre* expression is very likely the reason why we obtained no inducible Cre activity in analysed mouse lines, but still there remains a possibility that the low level *Cre* expression triggering efficient recombination in mutant *rosa26* locus in *GTRosa26* reporter line is not enough to do so in some other floxed lines.

The transgenic mouse lines established and described in this work show *Cre* expression first in Wolffian duct derivatives, but additionally in specific structures in the embryonic nervous system, heart and inner ear, being therefore potential tools in studies that require genetic alterations in these organ systems.

	CNS	dermomyotome	epidermis	Wolffian duct	myocard	inner ear
Cre3	xx		xx	x		
Cre4	**xx**	**xx**	**xx**	**xx**	**xx**	**xx**
Core1	xx		xx		x	
Core2-5	xx			xx	x	
Core2-8	x	xx		xx		
Core3		xx		xxx		
Core5	**x**					
Core7	x		x			xx
Core8				xxx		

Table 1. Cre expression domains in generated transgenic mouse lines.

SUMMARY

For many genes, conventional mutagenesis, which leads to complete loss of function, causes an early lethal phenotype, thus hampering subsequent analysis. Some genes have different roles during embryogenesis and adulthood and therefore it would be desirable to use an inducible system that gives complete control over timing of gene alteration. In the literature review, I overview the most common techniques of conditional (cell-type specific) mutagenesis that enable to investigate such genes and also to create models for somatically developed diseases. Modification of the individual gene restricted to a specific tissue and/or timepoint can be achieved using the site-specific Cre recombinase. This system requires generation of two lines of mice. In one of them, the gene or an essential segment of the gene of interest must be flanked with *loxP* sites. Modification of the floxed gene can be achieved, crossing the mouse with the second line which expresses *Cre* under tissue-specific or inducible promoter. The aim of the current work was to create a conditional transgenic mouse line that expresses *Cre* recombinase specifically in the kidney. Besides kidney specific Cre lines, also several other interesting Cre transgenic lines were established.

In the course of these studies, I cloned two conditional *Cre* expression constructs, using the mouse *Hoxb7* promoter to drive kidney-specific expression expression of *Cre*. The *Hoxb7* genomic fragment extending from 1316 bp upstream to 8 bp downstream of its translation start site has been described as a strong transcriptional activator in the Wolffian duct and its derivatives.

Both constructs were injected into fertilized oocytes. Mice born from the injections were genotyped using PCR and Southern blot. 11 mice out of 101 carried a transgene. The founders transfer the transgene with variable efficiency, 20-30 % in *HB7-cre* lines and 80-90 % in some of *HoxB7-core* lines.

Cre expression pattern was determined by lacZ staining on *cre+/-/rosa+/-* embryos, progeny from original transgenic mice crossings with GTRosa LacZ reporter line. In this work I have described two different patterns of *Cre* expression in *HB7-cre* mice, and six patterns in *Hoxb7-core* lines. These results indicate that the previously described mouse *Hoxb7* promoter fragment is applicable for triggering kidney specific transgene

expression, but highly affected by the genomic context, and is able to confer transgene expression besides the Wolffian duct derivatives, to specific structures in the embryonic nervous system, heart and inner ear. Generated lines may be useful tools in studies that require genetic alterations in these organ systems.

KOKKUVÕTE

Käesoleva töö kirjanduse ülevaates on kirjeldatud konditsionaalse ehk rakutüübi-spetsiifilise mutageneesi tehnikaid. Konditsionaalse mutageneesi rakendamine võimaldab erinevalt konventsionaalsetest tehnikatest uurida embrüonaaleas letaalseid mutatsioone, geene, millel on erinev funktsioon eri rakutüüpides ja/või eri arenguperioodidel ning konstrueerida mudeleid somaatiliselt omandatud haigustele. Konditsionaalset mutageneesi saab kasutada nii geenide inaktiveerimiseks kui ka aktiveerimiseks. Põhjalikumalt on tutvustatud Cre/*loxP* strateegiat, mis seisneb geeni või selle segmendi ümbritsemises *loxP* saitidega, misjärel loksitud geeni saab välja lülitada, ristates mutanti loomaga, kes kannab rakutüübispetsiifiliselt ekspresseeruvat või ka indutseeritavat Cre rekombinaasi.

Oma töös kloneerisin ma konditsionaalsed *Cre* ekspressioonikonstruktid, kasutades neeruspetsiifilise ekspressiooni saavutamiseks fragmenti hiire *Hoxb7* promootorist, mis kirjanduse põhjal omab tugevat transkriptsioonilist aktiivsust Wolffi juhas ja selle derivaatides (Kress jt. 1990). Transgeesed hiired on loodud konstruktide süstimisega viljastatud munarakkudesse, mille tagajärjel transgeen integreerub juhuslikku kohta genoomis. Käesolevas töös kirjeldatud Cre espressioonimustrid etableeritud transgeensetes liinides tõendavad *Hoxb7* promootori võimet suunata neeruspetsiifilist transgeeni ekspressiooni, kuid viitavad ka sellele, et see ekspressioon sõltub tugevalt genoomsest kontekstist. Kahe neeruspetsiifilise Cre liini kõrval on rajatud ka mitmeid teisi huvipakkuva ruumiliselt piiratud Cre ekspressioonimustriga transgeenseid liine. Niisiis, peale neeru saab loodud hiiri kasutada näiteks veel aju, sisekõrva ning närvisüsteemiga seotud haiguste uurimisel.

ACKNOWLEDGEMENTS

I thank everybody who have bored to deal with me.
From the bottom of my heart.

There are lot of people who have helped me in my work and I am grateful to.
My supervisors, Illar Pata, my first supervisor, and second supervisor Jüri Kärner, head of the Institute of Zoology and Hydrobiology.
Sulev Kuuse, head of the TÜMRI mouse facility, has helped me with mouse experiments, as well as the personnel in the mouse facility, Sirje Kask and Sirje Habak.
Alar Karis, head of the Department of Integrative Zoology (until 2003), director of Visgenyx company, provided the chance to start the work in his lab.
Külli Haller and Külli Samuel from Visgenyx performed the oocyte microinjections.
Ph.D students Madis Jakobson and Kersti Lilleväli have advised me very kindly.
Ranno Rätsep was working with me, characterising some of the founders for *Core* mice.
Lab technician Mare Tamm instructed me in histology.

Profound characterization of *Core5* mouse line was done in the Helsinki University Institute of Biotechnology, where I am currently continuing this project under the supervision of Juha Partanen.

REFERENCES

Abel ED, Kaulbach HC, Tian R, Hopkins JC, Duffy J, Doetschman T, Minnemann T, Boers ME, Hadro E, Oberste-Berghaus C, Quist W, Lowell BB, Ingwall JS, Kahn BB. 1999 Cardiac hypertrophy with preserved contractile function after selective deletion of GLUT4 from the heart. *J Clin Invest* **104**: 1703-14.

Agah R, Frenkel PA, French BA, Michael LH, Overbeek PA, Schneider MD. 1997. Gene recombination in postmitotic cells. Targeted expression of Cre recombinase provokes cardiac-restricted, site-specific rearrangement in adult ventricular muscle in vivo. *J Clin Invest* **100:** 169-79.

Akagi K, Sandig V, Vooijs M, Van der Valk M, Giovannini M, Strauss M, Berns A. 1997. Cre-mediated somatic site-specific recombination in mice. *Nucleic Acids Res* **25**: 1766-73.

Annilo T, Klaassen T, Kurg A, Lilleväli H, Pata I, Rebane A, Sibul H, Teder M, Tõnisson N. *Molekulaarse biotehnoloogia praktikumi juhend*. Tartu, Tartu Ülikooli Kirjastus, 2000. 83 lk.

Araki K, Araki M, Miyazaki J, Vassalli P. 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. *Proc Natl Acad Sci U S A* **92**: 160-4.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. *Short protocols in molecular biology*. 3rd ed. John Wiley & Sons Inc, 1995.

Baron U, Freundlieb S, Gossen M, Bujard H. 1995. Co-regulation of two gene activities by tetracycline via a bidirectional promoter. *Nucleic Acids Res.* **23**: 3605-6.

Bäckman CM, Zhang Y, Hoffer BJ, Tomac AC. 2004. Tetracycline-inducible expression systems for the generation of transgenic animals: a comparison of various inducible systems carried in a single vector. *J Neurosci Methods* **139**: 257-62.

Betz UA, Vosshenrich CA, Rajewsky K, Muller W. 1996. Bypass of lethality with mosaic mice generated by Cre-loxP-mediated recombination. *Curr Biol* **6**: 1307-16.

Bradley A, Evans M, Kaufman MH, Robertson E. 1984. Formation of germ-line chimaeras from embryo-derived teratocarcinoma cell lines. *Nature* **309**: 255-6.

Bockamp E, Maringer M, Spangenberg C, Fees S, Fraser S, Eshkind L, Oesch F, Zabel B. 2002. Of mice and models: improved animal models for biomedical research. *Physiol Genomics* **11**: 115-32.

Chai Y, Jiang X, Ito Y, Bringas P Jr, Han J, Rowitch DH, Soriano P, McMahon AP, Sucov HM. 2000. Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis. *Development* **127**: 1671-9.

Colbere-Garapin F, Horodniceanu F, Kourilsky P, Garapin AC. 1980. Cloning of the herpes simplex virus type 1 thymidine kinase gene in E. coli K 12: a selective marker for gene transfer into animal cells. *Dev Biol Stand* **46**: 75-82.

Danielian PS, Muccino D, Rowitch DH, Michael SK, McMahon AP. 1998. Modification of gene activity in mouse embryos in utero by a tamoxifen-inducible form of Cre recombinase. *Curr Biol* **8**: 1323-6.

Das AT, Zhou X, Vink M, Klaver B, Verhoef K, Marzio G, Berkhout B. 2004. Viral evolution as a tool to improve the tetracycline-regulated gene expression system. *J Biol Chem*. **279**: 18776-82.

Duffy, JB. 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. *Genesis* **34**: 1-15.

Dymecki SM. 1996. Flp recombinase promotes site-specific DNA recombination in embryonic stem cells and transgenic mice. *Proc Natl Acad Sci U S A* **93**: 6191-6.

Evans MJ, Kaufman MH. 1981. Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**: 154-6.

Fischer JA, Giniger E, Maniatis T, Ptashne M. 1988. GAL4 activates transcription in Drosophila. *Nature* **332**: 853-6.

Gossen M, Bujard H. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A*. **89**: 5547-51.

Gossen M, Bujard H. 1993. Anhydrotetracycline, a novel effector for tetracycline controlled gene expression systems in eukaryotic cells. *Nucleic Acids Res.* **21**: 4411-2.

Gu H, Marth JD, Orban PC, Mossmann H, Rajewsky K. 1994. Deletion of a DNA polymerase beta gene segment in T cells using cell type-specific gene targeting. *Science* **265**: 103-6.

Hardouin SN, Nagy A. 2000. Mouse models for human disease. *Clin Genet* **57**: 237- 44.

Holzenberger M, Zaoui R, Leneuve P, Hamard G, Le Bouc Y. 2000. Ubiquitous postnatal LoxP recombination using a doxycycline auto-inducible Cre transgene (DAI-Cre). *Genesis* **26**: 157-9.

Indra AK, Warot X, Brocard J, Bornert JM, Xiao JH, Chambon P, Metzger D. 1999. Temporally-controlled site-specific mutagenesis in the basal layer of the epidermis: comparison of the recombinase activity of the tamoxifen-inducible Cre-ER(T) and Cre-ER(T2) recombinases. *Nucleic Acids Res* **27**: 4324-7.

Jaisser F. 2000. Inducible gene expression and gene modification in transgenic mice. *J Am Soc Nephrol* **11**: S95-S100.

Kellendonk C, Tronche F, Monaghan AP, Angrand PO, Stewart F, Schütz G. 1996. Regulation of Cre recombinase activity by the synthetic steroid RU 486. *Nucleic Acids Res* **24**: 1404-11.

Kress C, Vogels R, De Graaff W, Bonnerot C, Meijlink F, Nicolas JF, Deschamps J. 1990. Hox-2.3 upstream sequences mediate lacZ expression in intermediate mesoderm derivatives of transgenic mice. *Development* **109**: 775-86.

Kühn R, Schwenk F, Aguet M, Rajewsky K. 1995. Inducible gene targeting in mice. *Science* **269**: 1427-9.

Lakso M, Sauer B, Mosinger B Jr, Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. 1992. Targeted oncogene activation by site-specific recombination in transgenic mice. *Proc Natl Acad Sci U S A* **89**: 6232-6.

Lewandoski M. 2001. Conditional control of gene expression in the mouse. *Nat Rev Genet* **2**: 743-55.

Lim KC, Lakshmanan G, Crawford SE, Gu Y, Grosveld F, Engel JD. 2000. Gata3 loss leads to embryonic lethality due to noradrenaline deficiency of the sympathetic nervous system. *Nat Genet* **25**: 209-12.

Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A. 1999. Z/AP, a double reporter for cre-mediated recombination. *Dev Biol* **208**: 281-92.

Martin GR. 1981. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A* **78**: 7634-8.

von Melchner H, DeGregori JV, Rayburn H, Reddy S, Friedel C, Ruley HE. 1992. Selective disruption of genes expressed in totipotent embryonal stem cells. *Genes Dev* **6:** 919- 27.

Müller U. 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mech Dev* **82**: 3-21.

No D, Yao TP, Evans RM. 1996. Ecdysone-inducible gene expression in mammalian cells and transgenic mice. *Proc Natl Acad Sci U S A* **93**: 3346-51.

O'Gorman S, Dagenais NA, Qian M, Marchuk Y. 1997. Protamine-Cre recombinase transgenes efficiently recombine target sequences in the male germ line of mice, but not in embryonic stem cells. *Proc Natl Acad Sci U S A* **94**: 14602-7.

Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, Magnuson MA. 1998. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem* **274**: 305-15.

Prelle K, Zink N, Wolf E. 2002. Pluripotent stem cells--model of embryonic development, tool for gene targeting, and basis of cell therapy. *Anat Histol Embryol.* **31**: 169-86.

Ramirez-Solis R, Liu P, Bradley A. 1995 Chromosome engineering in mice. *Nature* **378**: 720-4.

Rickert RC, Roes J, Rajewsky K. 1997. B lymphocyte-specific, Cre-mediated mutagenesis in mice. *Nucleic Acids Res* **25**: 1317-8.

Ryding AD, Sharp MG, Mullins JJ. 2001. Conditional transgenic technologies. *J Endocrinol* **171:** 1-14.

Santerre RF, Allen NE, Hobbs JN Jr, Rao RN, Schmidt RJ. 1984. Expression of prokaryotic genes for hygromycin B and G418 resistance as dominant-selection markers in mouse L cells. *Gene* **30**: 147-56.

Sakai K, Hiripi L, Glumoff V, Brandau O, Eerola R, Vuorio E, Bosze Z, Fassler R, Aszodi A. 2001. Stage-and tissue-specific expression of a Col2a1-Cre fusion gene in transgenic mice. *Matrix Biol* **19**: 761-7.

Schwartz F, Maeda N, Smithies O, Hickey R, Edelmann W, Skoultchi A, Kucherlapati R. 1991. A dominant positive and negative selectable gene for use in mammalian cells. *Proc Natl Acad Sci U S A* **88**:10416-20.

Schwenk F, Kuhn R, Angrand PO, Rajewsky K, Stewart AF. 1998. Temporally and spatially regulated somatic mutagenesis in mice. *Nucleic Acids Res* **26**: 1427-32.

Shao X, Johnson JE, Richardson JA, Hiesberger T, Igarashi P. 2002b. A minimal Ksp-cadherin promoter linked to a green fluorescent protein reporter gene exhibits tissue-specific expression in the developing kidney and genitourinary tract. *J Am Soc Nephrol* **13**: 1824-36.

Shao X, Somlo S, Igarashi P. 2002a. Epithelial-specific Cre/lox recombination in the developing kidney and genitourinary tract. *J Am Soc Nephrol* **13**: 1837-46.

Shin MK, Levorse JM, Ingram RS, Tilghman SM. 1999. The temporal requirement for endothelin receptor-B signalling during neural crest development. *Nature* **402:** 496-501.

Shin MK. 2000. Controlling gene expression in mice with tetracycline: application in pigment cell research. *Pigment Cell Res* **13**: 326-31.

Smithies O, Gregg RG, Boggs SS, Koralewski MA, Kucherlapati RS. 1985. Insertion of DNA sequences into the human chromosomal beta-globin locus by homologous recombination. *Nature* **317**: 230-4.

Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, Penninger JM, Molkentin JD. 2001. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. *Circ Res*. **89:** 20-5.

Soriano P. 1999. Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* **21**: 70-1.

Srinivas S, Watanabe T, Lin CS, William CM, Tanabe Y, Jessell TM, Costantini F. 2001. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1**: 4.

Srinivas S, Wu Z, Chen CM, D'Agati V, Costantini F. 1999. Dominant effects of RET receptor misexpression and ligand-independent RET signaling on ureteric bud development. *Development* **126:** 1375-86.

St-Onge L, Furth PA, Gruss P. 1996. Temporal control of the Cre recombinase in transgenic mice by a tetracycline responsive promoter. *Nucleic Acids Res* **24**: 3875-7.

Stricklett PK, Nelson RD, Kohan DE. 1999. The Cre/loxP system and gene targeting in the kidney. *Am J Physiol***. 276**: F651-7.

Thomson RB, Aronson PS. 1999. Immunolocalization of Ksp-cadherin in the adult and developing rabbit kidney. *Am J Physiol* **277**: F146-56.

Torres MR, Kühn R. *Laboratory Protocols for Conditional Gene Targeting*. Oxford, Oxford University Press, 1999. 167 lk.

Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W. 2000. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* **97**: 7963-8.

Utomo AR, Nikitin AY, Lee WH. 1999. Temporal, spatial, and cell type-specific control of Cre-mediated DNA recombination in transgenic mice. *Nat Biotechnol* **17**: 1091-6.

Vogels R, de Graaff W, Deschamps J. 1990. Expression of the murine homeobox containing gene Hox-2.3 suggests multiple time-dependent and tissue-specific roles during development. *Development* **110**: 1159-68.

Wang Y, Krushel LA, Edelman GM. 1996. Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. *Proc Natl Acad Sci USA* **93**: 3932-6.

Weber P, Metzger D, Chambon P. 2001. Temporally controlled targeted somatic mutagenesis in the mouse brain. *Eur J Neurosci* **14**: 1777-83.

Wilkinson, D. 1992. In situ hybridization: a practical approach. In book *Wholemount in situ hybridization of vertebrate embryos*. Ed.by D. Wilkinson. IRL Press, Oxford. P. 75-83.

Wunderlich FT, Wildner H, Rajewsky K, Edenhofer F. 2001. New variants of inducible Cre recombinase: a novel mutant of Cre-PR fusion protein exhibits enhanced sensitivity and an expanded range of inducibility. *Nucleic Acids Res* **29**: E47.

Yu J, Carroll TJ, McMahon AP. 2002. Sonic hedgehog regulates proliferation and differentiation of mesenchymal cells in the mouse metanephric kidney. *Development* **129**: 5301-12.

Zhang Y, Riesterer C, Ayrall AM, Sablitzky F, Littlewood TD, Reth M. 1996. Inducible site-directed recombination in mouse embryonic stem cells. *Nucleic Acids Res* **24**: 543-8.