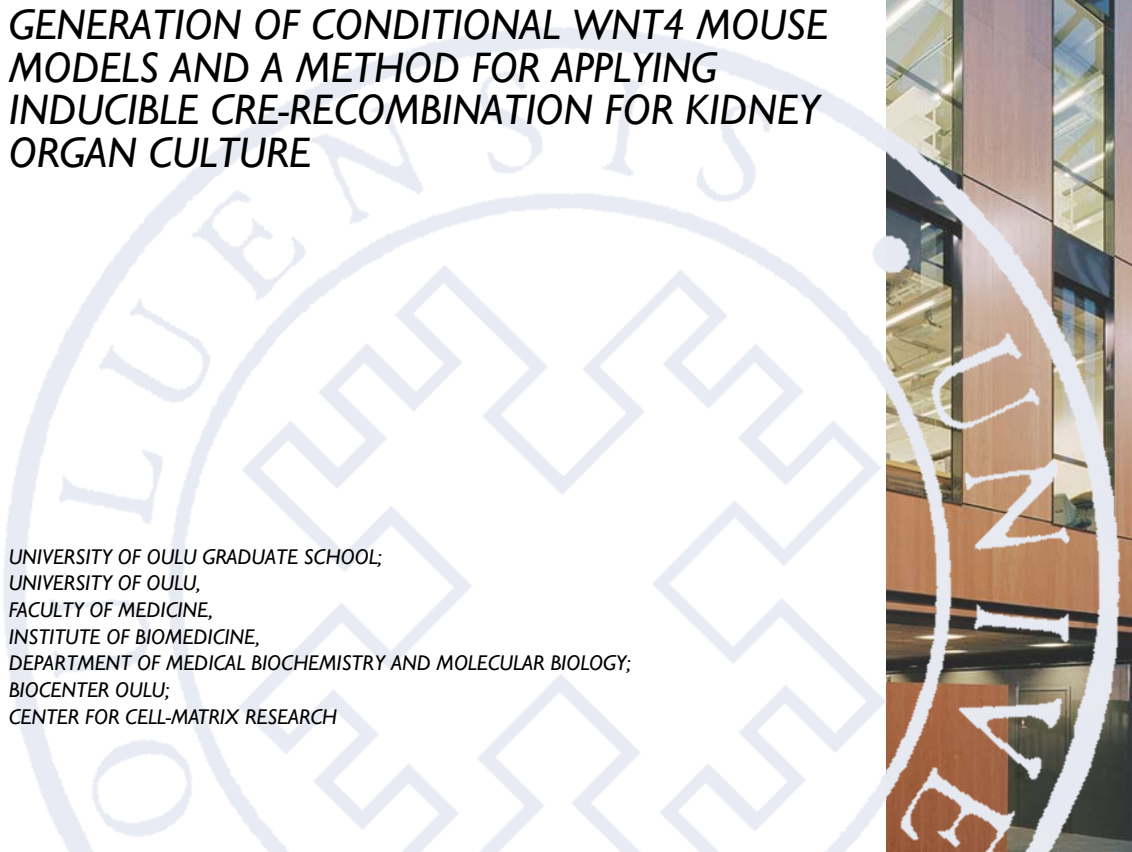


Tiina Jokela

ANALYSES OF KIDNEY
ORGANOGENESIS THROUGH
IN VITRO AND *IN VIVO*
APPROACHES

GENERATION OF CONDITIONAL WNT4 MOUSE
MODELS AND A METHOD FOR APPLYING
INDUCIBLE CRE-RECOMBINATION FOR KIDNEY
ORGAN CULTURE

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF MEDICINE,
INSTITUTE OF BIOMEDICINE,
DEPARTMENT OF MEDICAL BIOCHEMISTRY AND MOLECULAR BIOLOGY;
BIOCENTER OULU;
CENTER FOR CELL-MATRIX RESEARCH



ACTA UNIVERSITATIS OULUENSIS
D Medica 1208

TIINA JOKELA

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ORGANOGENESIS THROUGH *IN VITRO*
AND *IN VIVO* APPROACHES**

Generation of conditional *Wnt4* mouse models and a method for applying inducible Cre-recombination for kidney organ culture

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Health and Biosciences of the University of Oulu for public defence in the Auditorium of Kastelli Research Centre (Aapistie 1), on 17 May 2013, at 12 noon

UNIVERSITY OF OULU, OULU 2013

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Acta Univ. Oul. D 1208, 2013

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ISBN 978-952-62-0154-2 (Paperback)
ISBN 978-952-62-0155-9 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2013

Jokela, Tiina, Analyses of kidney organogenesis through *in vitro* and *in vivo* approaches. Generation of conditional *Wnt4* mouse models and a method for applying inducible Cre-recombination for kidney organ culture

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Acta Univ. Oul. D 1208, 2013

Oulu, Finland

Abstract

In mice, gene targeting has become a useful tool for resolving the functions of proteins and for creating new animal models. Cre/loxP technology has been used broadly for generation of genetically modified mice. The Cre recombinase recognizes a specific DNA sequence, called loxP, and removes any DNA fragment between two loxP-sites. The activity of the Cre recombinase can be controlled spatially and temporally with cell- or tissue-specific promoters and synthetic inducing agents, such as tamoxifen or tetracycline.

In this thesis, we employed tamoxifen-induced Cre recombination *in vitro*. Cre-ERTM mice were crossed to *ROSA26LacZ* reporters and Cre-recombination induced by 4OH-TM was monitored by LacZ staining. 0.5 μ M 4OH-TM treatment was competent for tamoxifen-induced Cre-mediated activation of *LacZ* both in kidney cultures and in experimentally induced kidney mesenchymes.

Wnt4 is a secreted signaling molecule, which is necessary for the development of several organs including kidney, ovary, adrenal gland, mammary and pituitary glands. *Wnt4* is crucial for kidney development and conventional *Wnt4*^{-/-} mice die soon after birth, likely due to renal failure.

In this thesis, two different *Wnt4* alleles, *Wnt4*^{EGFPCre} and floxed *Wnt4*, were generated and analyzed to learn more about the *Wnt4* functions and to apply these mouse lines to renal functional genomics. In the *Wnt4*^{EGFPCre}, the EGFPCre fusion *cDNA* was targeted into exon I of the *Wnt4* locus. EGFP-derived fluorescence was observed in the pretubular aggregates from E12.5 embryonic kidney onwards. Further characterization by crossing with the floxed *ROSA26LacZ* and yellow fluorescent protein (YFP) reporter lines demonstrated that in addition to the kidney, reporter expression was observed in the gonad, spinal cord, lung and the adrenal gland. The expression pattern of the Cre activity recapitulates well the known pattern of the *Wnt4* gene. Time-lapse analysis in organ culture settings showed that the *Wnt4* expressing cells contributed to the nephrons, some cells near the stalk of the developing ureter, as well as a number of positive supposed medullary stromal cells.

In the conditional *Wnt4* knock-out, loxP sites were placed to flank exons 3 to 5. The *Wnt4* gene was specifically inactivated with *CAGCre* and *Wnt4*^{EGFPCre} lines. In both of these crosses deletion of *Wnt4* gene function led to impaired kidney development.

In conclusion, we identified the culture conditions that can be used for the tamoxifen-induced conditional mutagenesis in tissue cultures. In addition, the created *Wnt4* mouse lines serve as new useful tools for addressing the roles of *Wnt4* function in diverse tissues and in different stages of development.

Keywords: conditional mutagenesis, Cre/ loxP, EFGP, gene targeting, kidney, LacZ, time-lapse analysis, *Wnt4*, YFP

Jokela, Tiina, Munuaisen elinkehityksen tutkiminen käyttäen *in vitro* ja *in vivo* menetelmiä. Konditionaalisten *Wnt4* hiirilinjojen tuottaminen ja indusoituvan Cre-rekombinaation hyödyntäminen munuaisviljelmissä

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Lääketieteellinen biokemia ja molekyylibiologia; Biocenter Oulu; Center for Cell-Matrix Research, PL 5000, 90014 Oulun yliopisto
Acta Univ. Oul. D 1208, 2013

Tiivistelmä

Hiirillä geenikohdennuksesta on muodostunut hyödyllinen väline proteiinien tehtävien selvittämisessä ja uusien eläinmallien luomisessa. Cre/loxP -tekniikkaa on käytetty laajasti muuntogeenisten hiirien tuottamisessa. Cre-rekombinaasi tunnistaa spesifisen DNA-jakson, niin kutsutun loxP:n, ja poistaa kaikki DNA-jaksot kahden loxP-sekvenssin väliltä. Cre-rekombinaasin aktiivisuutta voidaan säädellä paikallisesti ja ajallisesti solu- tai kudosspesifisillä promoottoreilla ja synteettisillä indusoivilla kemikaaleilla, kuten tamoksifeenillä tai tetrasykliinillä.

Tässä väitöskirjassa hyödynsimme tamoksifeenin aiheuttamaa Cre-rekombinaatiota *in vitro* -kudosviljelmissä. Cre-ERTM-hiirilinja risteytettiin ROSA26LacZ-reportterilinjan kanssa, ja 4-hydroksitamoksifeenin indusoima Cre-rekombinaasin aktiivisuutta monitoroitiin LacZ-värjäyksellä. 0.5 µM:n 4OH-TM konsentraatiolla LacZ-reportterigeeni saatiin aktivoitua tehokkaasti Cre-rekombinaasin avulla sekä munuaisviljelmissä että munuaismesenkymiviljelmissä.

Wnt4 on erittyvä signaalintomolekyyli, jolla on keskeinen rooli useiden elinten, kuten munuaisen, munasarjan, lisämunuaisen, rintarauhasen ja aivolisäkkeen kehittämisessä. Wnt4-geenillä on ratkaisevan tärkeä rooli munuaisen kehityksessä, ja poistogeeninen Wnt4^{-/-}-hiiri kuolee pian syntymän jälkeen, todennäköisesti munuaisen vajaatoimintaan.

Tässä väitöskirjatyössä tuotettiin kaksi eri Wnt4 alleelia, Wnt4^{EGFP^{Cre}} ja konditionaalinen Wnt4. Nämä hiirilinjat analysoitiin, jotta saisimme lisää tietoa Wnt4-geenin toiminnasta ja pystyisimme soveltamaan kyseisiä hiirikantoja munuaisen toiminnan selvittämisessä. Wnt4^{EGFP^{Cre}}-alleelissa EGFP^{Cre}-fuusio -cDNA kohdennettiin osaksi endogeenisen Wnt4-geenin ykköseksonia. Vihreän fluoresoivan proteiinin (EGFP) aktiivisuus havaittiin varhaisen munuaisen kehityksen aikana. Wnt4^{EGFP^{Cre}}-alleelin lisäarakterisointi reportterilinjoilla (Rosa26LacZ ja Rosa26YFP) osoitti, että Wnt4-geenin ilmentyminen havaittiin munuaisen lisäksi sukuruuhassissa, selkäytimessä, keuhkoissa sekä lisämunuaisessa. Wnt4^{EGFP^{Cre}}-alleeli ilmentyi niissä kudoksissa, joissa endogeenisen Wnt4-geenin tiedetään olevan aktiivinen. Time-lapse -analyysin avulla osoitettiin, että Wnt4-geeniä ilmentävät solut muodostavat tiettyjä rakenteita munuaisen kehityksen aikana. Wnt4-geeni ilmentyi nefroneissa, kehittyvän virtsajohtimen soluissa sekä useissa medullaarisissa stroomasoluissa.

Konditionaalisissa (ehdollisissa) Wnt4 knock-out-hiirilinjassa loxP-sekvenssit sijoitettiin eksoneiden kolme sekä viisi ympärille. Wnt4-geenin toiminta inaktivoitiin CAGCre- ja Wnt4^{EGFP^{Cre}}-hiirilinjojen avulla. Näissä molemmissa tapauksissa Wnt4-geenin toiminnan poistaminen johti munuaisen kehityshäiriöön.

Yhteenvetona voimme todeta, että olemme tunnistaneet ne kasvatusolosuhteet, joita voidaan hyödyntää, kun halutaan aktivoida reportterigeenejä tai kehityksen kannalta tärkeitä geenejä tamoksifeenin aiheuttamaa Cre/loxP -rekombinaatiota hyväksikäyttäen kudosviljelmissä. Samoja olosuhteita ja menetelmää käyttäen voidaan myös poistaa jonkun kehityksen kannalta tärkeän geenin toiminta ja tutkia sitä kudosviljelmässä. Tuotetut Wnt4-hiirikannat ovat lisäksi uusia hyödyllisiä työkaluja, kun halutaan tutkia Wnt4-geenin toimintaa erilaisissa kudoksissa ja eri kehitysvaiheiden aikana.

Asiasanat: Cre/loxP, EGFP, ehdollinen mutageneesi, geenikohdennus, LacZ, munuainen, time-lapse analyysi, Wnt4, YFP

Isälleni

“Annan toisten elää enkä kulje surun suolla, silmistäni en mä anna unelmien kuolla,
silloin kun on päivä viimeinen, olkoon purjeeni valkoinen.“

-R.Reiman

Acknowledgements

This work was carried out at Biocenter Oulu, the Department of Medical Biochemistry and Molecular Biology of the Institute of Biomedicine.

I wish to express my sincere gratitude to my supervisor, Professor Seppo Vainio, for the opportunity to do my thesis in his research group, and for introducing me to the interesting world of developmental biology.

Docent Satu Kuure and docent Petra Sipilä are acknowledged for their careful reading and valuable commenting on the thesis. I also wish to thank Anna Vuolteenaho for the language revision.

I warmly thank Professor Taina Pihlajaniemi, Professor Johanna Myllyharju, Professor Peppi Karppinen, Professor Kari Kivirikko and all the other group leaders for creating a stimulating atmosphere and providing excellent research environment and equipments in the Department of Medical Biochemistry and Molecular Biology, Biocenter Oulu. The chair of my thesis committee Professor Robert Winqvist as well as other members of the follow-up group deserves special compliments for fruitful discussions and encouraging advices during these years.

I wish to thank my co-authors. I would also like to thank other members, previous and present, of the SV-group. Johanna Kekolahti-Liias, Hannele Härknam, Aila Holappa, Paula Haipus and Jaana Kujala are acknowledged for their help and outstanding technical assistance. We are grateful for the Biocenter Oulu transgenic facility for their excellent ES cell work and the blastocyst injections.

Anthony Heape, Ritva Saastamoinen, Eija Ruottinen, Marja-Leena Karjalainen, Auli Kinnunen, Seppo Lähdesmäki, Teija Luoto, Irmeli Nykyri, Anne Vainionpää and Pertti Vuokila are acknowledged for their valuable help in practical matters. Risto Helminen, Antti Viklund and Kimmo Halt are acknowledged for all their help concerning computers.

Haluan kiittää äitiäni, sisaruksiani Jaria, Pirjoa ja Tapania sekä heidän perheitään siitä tuesta ja kannustuksesta, jota he ovat osoittaneet minulle näiden vuosien aikana. Syvimmät kiitokseni avomiehelleni Jarille ja tyttärelleni Emilialle, että olette jakaneet kaikki nämä vuodet kanssani. Kiitos, että olette jaksaneet ja ymmärtäneet minua sinä aikana, jolloin väitöskirjan kirjoittaminen on vienyt ajatukseni täysin pois luotanne. Ilman teidän tukeanne tätä väitöskirjaa ei olisi olemassa. Lopuksi, tämä väitöskirja on omistettu edesmenneelle isälleni. Kiitos isä, että jaksoit aina uskoa minuun.

This work was supported by the Academy of Finland, Biocenter Oulu, Sigrid Juselius Foundation, Tauno Tyni Foundation and the European Renal Genome Project.

Oulu, May 2013

Tiina Jokela

Abbreviations

APC	adenomatous polyposis coli
ApoE	apolipoprotein E
AQP	aquaporin
BFP	blue fluorescent protein
Bmp	bone morphogenetic protein
bp	base pair
β -Catenin	catenin (cadherin associated protein), beta 1
CamK II	calcium/calmodulin-dependent kinase
cDNA	complementary DNA
CD	collecting ducts
CFP	cyan fluorescent protein
CKI	casein kinase I
CMV	cytomegalovirus
CN	calcineurin
Dkk	dickkopf
Dox	doxycycline
DNA	deoksiribonucleic acid
DT	distal tubule
Dvl	disheveled
E	embryonic day
EGFP	enhanced green fluorescent protein
Emx	empty spiracles homolog
ER	estrogen receptor
Fgf	fibroblast growth factor
Fz	frizzled homolog
γ GT	γ -glutamyl transpeptidase
Gdnf	glial cell line-derived neurotrophic factor
GFP	green fluorescent protein
GOI	gene of interest
Gsk3 β	glycogen synthase kinase 3 beta
GUS	β -glucuronidase
Hox	homeo box
4OH-TM	4-hydroxy-tamoxifen
Jnk	Jun kinase
Ksp	kidney-specific cadherin

Lef	lymphoid enhancer binding factor
LBD	ligand-binding domain
LEF	lymphoid enhancer binding factor
LRP	low density lipoprotein receptor-related protein
LTR	long terminal repeat
MET	Mesenchyme to Epithelial Transition
MM	metanephric mesenchyme
MMTV	mouse mammary tumor virus
Neph	nephrin
Nes	nestin
NFAT	nuclear factor of activated T cells
NLK	nemo-like kinase
NM	nephrogenic mesenchyme
Osr	Odd-skipped related
Pax	paired box-containing gene
PCP	planar cell polarity
PCR	polymerase chain reaction
PKC	protein kinase C
PT	proximal tubule
Ret	ret proto-oncogene
Ren	renin
RFP	red fluorescent protein
Rho	Ras homolog gene family
RNA	ribonucleic acid
ROCK	Rho-associated kinase
Ror2	receptor tyrosine kinase-like orphan receptor 2
Ryk	Ryk receptor-like tyrosine kinase
Sgl	sodium - glucose cotransporter
Six	sine oculis-related homeobox gene
sFRP	secreted Frizzled-related protein
Src	sarcoma, proto-oncogene
TAL	thick ascending loop of Henle
TCF	transcription factor (T-cell specific)
TET	tetracycline
TRE	tetracycline response element
UB	ureteric bud
WD	Wolffian duct

WIF1	Wnt-inhibitory factor-1
Wnt	wingless related MMTV intergration site
WT1	Wilms tumour1
YFP	yellow fluorescent protein

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals (I–III).

- I Jokela T & Seppo Vainio (2007) Conditional tamoxifen Cre induced mutagenesis in the embryonic kidney in organ culture. *Genesis* 45(12): 757–761.
- II Shan J¹, Jokela T¹, Skovorodkin I & Vainio S (2010) Mapping of the fate of cell lineages generated from cells that express the Wnt4 gene by timelapse during kidney development. *Differentiation* 79(1): 57–64.
- III Shan J¹, Jokela T¹ & Vainio S (2009) Generation of an allele to inactivate Wnt4 gene function conditionally in the mouse. *Genesis* 47(11): 782–788.

¹These authors contributed equally to this work.

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1 Introduction

Embryonic lethality is a frequently encountered problem in genetically modified mice because of the essential role of various genes in early embryonic development. Thus, analysis of its functions at subsequent stages is impossible. With Cre/loxP technology this problem is partly solved and conditional gene deletion can be established.

The Cre recombinase of bacteriophage P1 recognizes a specific 34-bp DNA sequence called loxP. Cre mediates intramolecular and intermolecular site-specific recombination between two loxP-sites. The Cre/loxP recombination system has become a useful tool in addressing a wide variety of biological problems. In particular, its use enables the generation of genetically modified animals and plants and the genetic manipulation of embryonic stem cells. Its uses include the design of conditional mutations, precise chromosome rearrangements, removal of unwanted DNA sequences and targeted DNA integration. It has been particularly useful in the design of genetically modified mice, for the complementary purposes of generating sophisticated mouse models of human disease and for more precise understanding of gene function in an animal (Sauer 1998).

This thesis will introduce 4-hydroxytamoxifen (4OH-TM) induced Cre-mediated recombination *in vitro* using kidney culture system as a model. Recombination was studied by LacZ-derived staining, which was the indication of the efficient Cre-mediated recombination. This study was among the first using 4-hydroxytamoxifen for Cre-mediated recombination *in vitro* in kidney culture assays; it can thus open new windows on kidney research *in vitro*.

Wnt4 is a member of a large family of secreted signaling molecules and is an important gene in kidney and gonad development. In addition, *Wnt4* function has been shown to be fundamental for the development of other organs including pituitary, adrenal and mammary glands. The studies in the past have been made using the conventional *Wnt4* knock-out, which is lethal after birth. This prevents studying the roles of *Wnt4* postnatally and in diverse organs where *Wnt4* gene function is expressed. In this thesis we generated two different *Wnt4* lines, *Wnt4*^{EGFPCre} and floxed *Wnt4*, to overcome this problem.

2 Review of the literature

2.1 Standard techniques for the genetic modifications in the mouse genome

The exponential increase in the number of genes identified by the genome sequencing projects has generated a need for functional description of an enormous number of different genes. For this, new genetic tools have been developed allowing precise genetic modifications in germ line of complex organisms, such as the mouse. The mouse is an excellent experimental model to study human biology and disease because of its anatomic, physiologic, and genetic resemblance to humans. Other advantages of the mouse are its relatively short life cycle, small size, good productivity and the fact that its genome can be manipulated by molecular means.

For several decades it has been possible to add genes to the mouse genome and to make gene replacements and modifications. Although diverse techniques and methods have been developed to manipulate the mouse genome, all presently used methods fall in two basic technical approaches: the pronuclear injection of transgenes into fertilized oocytes or embryonic stem cell-mediated gene targeting.

“Classical” transgenic mice are derived from direct pronuclear injection of transgene DNA constructs into one-cell embryos. The method is mainly used for overexpression of endogenous genes or for expressing genes, which are not naturally present in the wild-type mouse genome. Pronuclear injection results in an unpredictable number of transgene copies being incorporated at random locations in the recipient genome. Due to the random nature of transgene integration, position site-dependent effects may alter transgene expression. These effects may produce transgene silencing, alter the cell and tissue specificity of the transgene or affect overall level of expression.

Unlike in the pronuclear injection method, where exogenous transgenes integrate randomly, gene targeting allows introducing specific modifications to endogenous genomic sequences. Gene targeting takes advantage of the embryonic stem (ES) cells, the pluripotent derivatives of the inner cell mass of the early-stage embryo, which have the unique ability to contribute to all the tissues of a host embryo including its germ line. Over the past two decades, various kinds of genetic modifications have been introduced in ES cells, ranging from random insertions (enhancer or gene trap approaches and classical transgenic constructs)

to homologous recombination. For homologous recombination, a targeting construct is introduced to ES cells prior to being injected into the blastocyst. The targeting construct contains a modified version of the endogenous gene, drug resistance gene (neomycin, puromycin, hygromycin) for the selection of recombinant ES cells and polyA addition site. In distinction to the so-called transgenic mice, mouse lines derived from homologous recombination in ES cells are referred to as gene-targeted mice, demonstrating that a specific gene has been manipulated. Usually, this manipulation has been used to inactivate the gene of interest (GOI, creating a knock-out), even though more delicate mutations (even a single nucleotide) have also been made.

Another interesting application of gene targeting is knock-in technology, in which any gene of interest can be placed under the transcriptional control of an endogenous gene. Knock-in mice were originally derived as a means to visualize a gene's expression during development and in the adult mouse with the help of targeted recombination of the reporter gene. The knock-in design later evolved to contain the replacement of a gene for the sequence of a similar isoform of the protein. Therefore, analogous protein isoforms could be tested for redundancy.

Conventional targeting exhibits two particular limitations. Because the modified gene is transmitted through the germ-line, mice derived from these ES cells are affected by gene dysfunction throughout ontogenesis. This might lead to early lethality if the protein is essential for development, thus precluding analysis of the gene function later in life. Inactivation of a widely expressed gene may result in a complex phenotype affecting multiple tissues. Thus, analysis of the modified gene's functional significance in a particular tissue might be difficult.

Some strategies have been developed to assist gene mutations that are restricted to specific organs or tissues, maintaining expression of the wild type gene in the rest of the body. These approaches are based on the activity of site-specific recombinases, like Cre recombinase, to produce conditional gene knock-outs in which gene targeting can be spatially and temporally regulated.

Another application of the Cre/loxP recombination system is its use as a tool for lineage-tracing and fate mapping studies. The aim of this technique is to identify the fate of a particular cell type during differentiation and development. Cre recombinase expression is directed into the cell type under study, either by transgenesis using cell-type-specific regulatory elements or by targeting Cre recombinase encoding *cDNA* into a gene locus specifically expressed in the appropriate cell type. Cre recombinase activity is then detected with a transgenic reporter construct. The reporter construct usually consists of a floxed stopper

element followed by a gene encoding a detectable lineage tracer, such as β -galactosidase or fluorescent proteins. In this setting, expression of Cre recombinase leads to irreversible excision of the floxed stopper element, activation of the tracer gene and thus permanent marking of the Cre-expressing cells and their descendants (This chapter was based on the following reviews: Babinet & Cohen-Tannoudji 2001, van der Wayden *et al.* 2002, Misra & Duncan 2002, Hall *et al.* 2009 Gama Sosa *et al.* 2010).

2.2 Basic properties of Cre recombinase

Cre recombinase of the bacteriophage P1 is a site-specific DNA recombinase of the integrase family (Hamilton & Abremski 1984). It is a 38-kDa protein that recognizes a 34-base-pair (bp) long site called loxP on the P1 genome and efficiently catalyzes DNA recombination between pairs of loxP sites. The loxP site consists of an 8-bp asymmetric core spacer sequence and two 13-bp-long palindromic flanking sequences (Figure 1) (Sauer 1998).

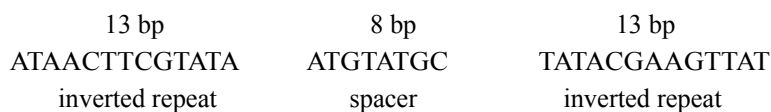


Fig. 1. The recognition site of Cre recombinase. The *loxP* site consists of a single core spacer and two flanking inverted repeats.

The result of Cre-mediated recombination depends upon the location and orientation of the loxP sites, which can be either in *cis* or in *trans*. When loxP sites are on the same DNA strand (*cis* location) in opposite orientation, inversion of DNA segments takes place. Excision happens when loxP sites are in same orientation on the same DNA strand. When two loxP sites are located on different strands of DNA (*trans* situation), an insertion of one DNA into another or translocation between two molecules (chromosomes) will take place (Nagy 2000, Tian *et al.* 2006).

Because Cre recombinase does not require accessory proteins or high-energy cofactors for its activity, it is suitable for use in mammalian cells such as mouse cells. Additionally, Cre is a very stable protein and generation of DNA constructs with a wide variety of promoters driving Cre expression can be accomplished quite easily.

2.3 Spatial and temporal regulation of the Cre recombinase

Cre/loxP technology has been extensively used to engineer the genome of experimental mice. The ability to inactivate an endogenous gene in a spatially and temporally controlled manner is not only beneficial for by-passing early lethal phenotypes, but also permits biological questions to be addressed with exquisite accuracy. To achieve cell/tissue-specific deletions of the chosen genes, the activity of Cre recombinase can be regulated spatially or temporally or combining both for spatio/temporal control. For the spatial control Cre is expressed under a cell- or tissue-specific promoter. Additional temporal control is achieved by regulating the Cre activity by inducible systems such as tamoxifen- or tetracycline-regulated systems, both of which are discussed in more detail in the following chapters.

2.3.1 Promoter driven spatio/temporal control

Controlling the expression of Cre with cell-type or tissue-specific promoters leads to the spatial regulation of Cre. It can even exhibit a precise temporal pattern if the chosen promoter is active only at a certain stage of development or in a certain physiological condition. In such studies, standard gene-targeting techniques (which were introduced in Chapter 2.1) are used to generate a mouse in which an essential region of GOI is floxed, thus containing two loxP sites flanking the DNA segment to be deleted. Tissue/cell specific expression of Cre results in the inactivation of this allele. Before Cre-mediated recombination, the floxed (also referred to as conditional) allele should have wild-type activity. In general, loxP sites are placed in introns, but have been inserted in 5'- (Dragatsis *et al.* 2000, Sakai *et al.* 2001) or 3'-flanking regions (Guy *et al.* 2001, Brakebush *et al.* 2000) without interfering gene expression. By crossing a conditional mouse line with a mouse line that expresses Cre in a cell/tissue-specific manner, offspring are produced in which the conditional allele is inactivated only in those tissues or cells that express Cre recombinase (Figure 2). The same strategy can be used for cell/tissue-specific expression of a transgene, when cell/tissue-specific promoter is separated from the coding region of a gene of interest by loxP flanked 'STOP' sequences.

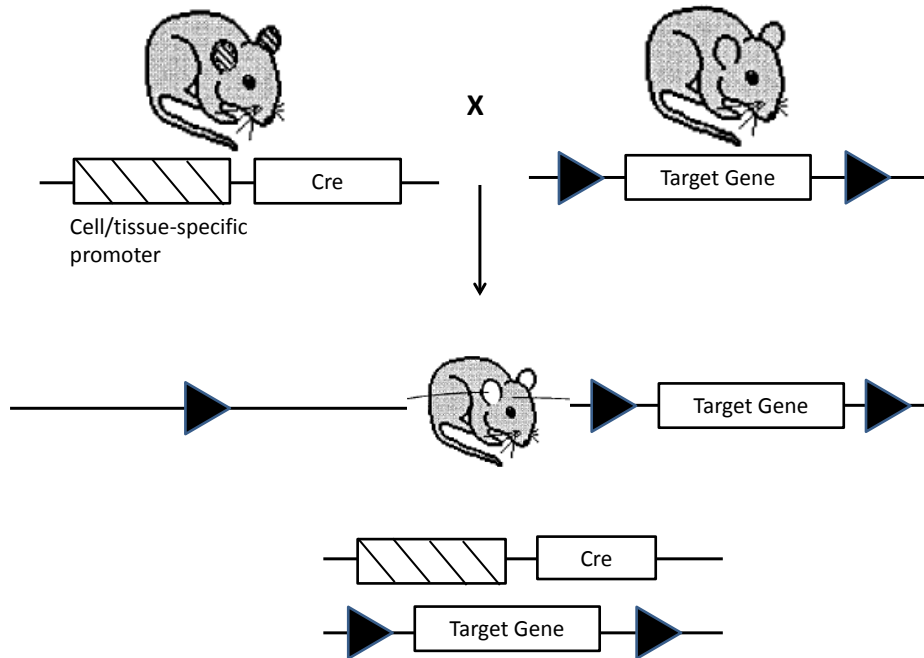


Fig. 2. Conditional gene deletion by Cre recombinase. The target endogenous gene is modified so that it is flanked by two directly repeated loxP sites. Mating of the loxP mouse with a Cre mouse will generate a double-transgenic mouse in which the loxP-modified gene has been deleted in those tissues in which the Cre has been expressed. In the example shown, Cre is expressed in the ears (black striped) of the Cre mouse. Thus, the deletion of the loxP-modified target gene is confined solely to the ears (white) of the double-transgenic mouse (modified from Sauer 1998).

2.3.2 Tamoxifen-inducible system

In the tamoxifen induction system, a fusion gene ($Cre-ER^T$) is formed between Cre and a mutated ligand-binding domain (LBD) from the estrogen receptor, ER. This mutated ER^T is insensitive to its natural ligand, estradiol, but instead binds the synthetic ligands tamoxifen or 4OH-tamoxifen (4OH-TM) (Metzger *et al.* 1995, Feil *et al.* 1996). In the absence of tamoxifen, the Cre- ER^T fusion protein is bound to the heat-shock protein, Hsp90, and is located in the cytoplasm, making Cre inactive. When tamoxifen is present, it binds to the estrogen receptor, ER, displacing Hsp90. This causes translocation of the Cre- ER^T fusion protein to the nucleus and initiation of recombination (Birling *et al.* 2009).

A wide range of *Cre-ER^T* mouse lines have been created and used for tamoxifen-inducible Cre-mediated recombination in cell culture, embryos, in utero and in adult mice (Danielian *et al.* 1998, Vooijs *et al.* 2001, Hayashi & McMahon 2002, Bosenberg *et al.* 2006, Nakamura *et al.* 2006).

New estrogen receptor-based Cre-LBD fusion proteins with enhanced sensitivities to their inducers and reduced background activity have been developed: Cre-ER^{T2}, ERiCreER and MerCreMer (Indra *et al.* 1999, Sohal *et al.* 2001, Casanova *et al.* 2002). The Cre-ER^{T2} recombinase is a fusion protein that contains three mutations in the human ER and is efficiently activated by tamoxifen, but not by endogenous estrogens (Feil *et al.* 1997, Indra *et al.* 1999). In the MerCreMer recombinase, Cre is fused to two mutated murine estrogen-receptor (Mer) binding domains and is insensitive to 17 β -estradiol but sensitive to the estrogen antagonist tamoxifen (Sohal *et al.* 2001). Casanova *et al.* (2002) generated ER-iCre-ER by fusing two ER^{T2} domains onto both ends of the iCre recombinase (a codon-improved Cre recombinase). The ERiCreER had a twofold increased activity in cell culture assays compared to the previously described MerCreMer Cre double fusion protein.

2.3.3 Tetracycline-inducible system

Another method of inducing Cre expression is the tetracycline-inducible system. There are two modifications of this system: the reverse tetracycline-controlled transcription activator (rtTA) system (Gossen *et al.* 1995) and the tetracycline-controlled transcription activator (tTA) system (Gossen & Bujard 1992). The basic elements for both of these systems are a regulatory unit, a tetracycline-responsive element and an induction agent, tetracycline (tet) or doxycycline (Dox). A regulatory unit consist of the tTA or rtTA, which are fusion proteins of the Tet repressor (TetR or rTetR) binding domain and the transcription activation domain of virion protein 16 of the herpes simplex virus, VP16 (Gossen & Bujard 1992). The second part, the response element TRE, contains tet operator sequences (tetO) of *Escherichia coli*, which are the binding sites of TetR or rTetR. In the rtTA system, the Tet repressor has four amino acid substitutions that result in altered binding characteristics and create the reverse TetR (rTetR) (Gossen *et al.* 1995).

In the tTA (Tet-OFF) system, addition of tetracycline or Dox prevents binding of tTA to tetO, and transcription of the target gene will not take place. In the absence of the inducing agent, tTA binds to tetO and activates the transcription of

the chosen target gene. In the rtTA (Tet-ON) system, mutated rtTA binds to tetO in the presence of tetracycline or Dox and the transcription of the target gene will start. In the absence of tetracycline or Dox, rtTA is not able bind to tetO and there is no transcription activation of the target gene (Figure 3) (Zhu *et al.* 2002, Aiba & Nakao 2007).

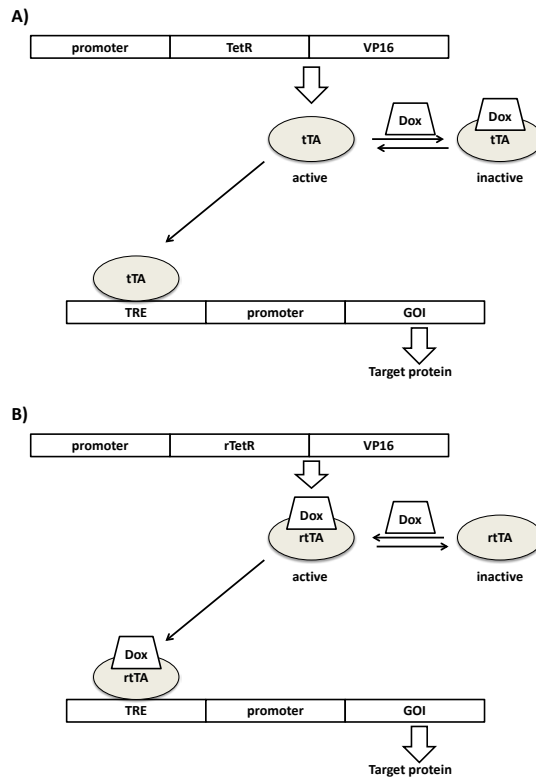


Fig. 3. Tetracycline inducible systems. A) Tet-OFF-system: In the absence of induction agent doxycycline (Dox), the tetracycline-controlled transcription activator (tTA) binds to Tet repressor (TRE) binding domain and activates transcription of Cre or GOI. Dox administration results in incapability of the tTA protein to bind to the TRE sequence and expression of Cre/GOI is eliminated. **B) Tet-ON-system:** the reverse tetracycline-controlled transcription activator (rtTA) requires Dox for binding to TRE to activate transcription of Cre/GOI. Depletion of Dox results in incapability of the rtTA protein to bind to the TRE sequence and expression of Cre or GOI is eliminated. GOI=gene of interest.

Regulating gene expression with a tetracycline-inducible system combined with Cre/loxP is a promising but at the moment insufficiently explored alternative compared to the tamoxifen-inducible system. Based on the characteristics of two available methods (rtTA or tTA) that work through opposite mechanisms, the rtTA (Tet-ON) system with Cre/loxP appears more desirable. Utomo *et al.* (1999) and Guo *et al.* (2005) utilized the Tet-ON system, demonstrating that Cre transcription can be induced in the presence of Dox and blocked by its withdrawal. An earlier report by St-Onge *et al.* (1996) showed only limited control of Cre-mediated recombination when applying (tTA) Tet-OFF system. As tTA system needs permanent attendance of tetracycline or Dox to prevent Cre expression, long-term maintenance of the necessary inducers could be in principle demanding and increase the possibility of unwanted side effects.

In the past, the Tet-systems to drive Cre expression have involved the use of three genetic constructs: the floxed allele, the tTA or rtTA under the control of a specific promoter, and the Tet operator/promoter fused to Cre. To achieve tetracycline-mediated control, a transgenic line with tetO DNA sequences (Cre tetO allele) is crossed with two other lines: one that encodes the tTA or rtTA and one that contains the floxed allele of the gene of interest. The main disadvantage of this tertiary system is its complexity that requires the generation, careful characterization, and mating of three different transgenic lines.

The development of single constructs, such as tetOCre: ROSArtTA (Heinonen *et al.* 2001) and R26rtTA-TRECre (Backman *et al.* 2009), which contains all of the desired Tet-system elements, has made the system more flexible. Thus, to achieve tetracycline-mediated induction followed by Cre-mediated recombination of the floxed gene, only two mouse lines are needed for the breeding set-up. One mouse line contains Cre and all the tetracycline system basic elements, while the other contains the floxed gene of interest.

2.4 Flp/FRT system

Even though the Cre/loxP was the first well-studied recombination system and has been the most widely used one for genetic manipulation in mice, new systems such as the Flp/FRT have been developed. The Flp recombinase is a product of *Saccharomyces cerevisiae* *FLP* gene. Similar to Cre, Flp recognizes short specific DNA sequences, which contain two 13-bp inverted repeats surrounding an 8-bp asymmetric core region. Detection of the FRT (Flp recombinase Recognition

Target) sites results in similar genetic alterations including excision, inversion, and translocation of targeted DNA sequences as the Cre recombinase.

The utilization of FLP in mammalian cells has shown less efficient recombinase activity than Cre because of the thermal instability of the FLP protein at 37°C (Buchholz *et al.* 1996). However, a thermostable mutant, Flpe, has been identified and shown to be highly effective in mice, exhibiting a fourfold increase in recombination efficiency (Buchholz *et al.* 1998). Recently, a mouse codon-optimized Flp gene (FLPo) has been constructed with similar recombination efficiency to Cre when tested in ES cells (Raymond *et al.* 2007).

Although the use of the Flp/FRT system may not be as widespread as Cre/loxP system, it is useful as a supplementary genetic tool. Regardless of comparable DNA recognition sites and action mechanisms, the Flp/FRT and Cre/lox systems do not cross-react with each other. Flpe deleter lines where Flpe is under a ubiquitous promoter or targeted at the ROSA26 locus to give rise to expression in all cells have been published (Farley *et al.* 2000, Rodriguez *et al.* 2000, <http://cre.jax.org>). Such lines have been employed in the conditional targeting approach to efficiently eliminate the selection markers like neo for producing F1-alleles, since the removal does not disturb the loxP-flanked region (Bouchard *et al.* 2004). Inducible Flp-LBD lines in which Flp has coupled to ligand-binding domains of estrogen receptors and Flp/FRT reporter lines have been generated (Hunter *et al.* 2005, <http://cre.jax.org>).

2.5 Reporter proteins

Several Cre mouse lines have been reported and all of them need careful characterization of the Cre function. It is important to detect the Cre-mediated recombination in a standard and most simple way at the individual cell level. Functioning of the Cre/loxP-mediated recombination system can be tested by the use of reporter mouse strains that provide a means to monitor specificity of Cre recombination *in vivo*. It is important to take into consideration that there does not appear to be a linear relationship between the intensity of the reporter protein produced and the level of Cre-mediated recombination.

Although for most reported Cre lines, recombination properties have been validated by reporter gene studies, the efficiency of Cre recombination can be locus-dependent, and therefore the recombination pattern obtained with a particular reporter gene does not necessarily predict that of other floxed genes. Indeed, the chromatin structure at the locus of interest, the state of DNA

methylation and the transcriptional activity seem to affect the recombination efficiency. It has also been demonstrated that the ability of a floxed target gene to be recombined can vary from one cell type to another. This can be potentially explained by differential availability of Cre to its recognition sites because of cell type and development-specific chromatin conformations. Before starting a phenotypic analysis with Cre-lines, it is thus mandatory to monitor and quantify recombination at the target locus by methods such as Southern blot or PCR/qPCR.

There are many reporter proteins in use: β -galactosidase, luciferase, β -glucuronidase (GUS: commonly used in plants) (Jefferson *et al.* 1987, Kim *et al.* 2012), and a wide variety of fluorescent proteins with different colors. Of these, β -galactosidase, luciferase as well as green and red fluorescent proteins are extensively used in cell cultures or in the mouse.

Several single and double reporter mouse lines have been generated for Cre-mediated recombination. Numerous single reporters are targeted at the ROSA26 locus with *LacZ* (Soriano 1999, Mao *et al.* 1999) or with different fluorescent proteins (Mao *et al.* 2001, Shrinivas *et al.* 2001, Luche *et al.* 2007).

Most dual Cre-reporters have combined an enzymatic reporter like *lacZ* or alkaline phosphatase with a fluorescent protein, most commonly with green or red (Lobe *et al.* 1999, Novak *et al.* 2000). Also dual Cre-reporters with two-color fluorescent proteins exist (Muzumdar *et al.* 2007, de Casperi *et al.* 2008, Hartwich *et al.* 2012). Different Cre-reporter lines will be discussed next in more detail.

2.5.1 β -galactosidase

One of the first reporter proteins, and still very popular, is β -galactosidase (β -gal). It is encoded by the bacterial gene *LacZ*. In bacteria, β -gal cleaves the disaccharide lactose into glucose and galactose. β -gal can be detected using a variety of substrates, which yield colored or fluorescent soluble products. These are helpful when quantifying β -gal activity or visualizing transduced cells live *in vivo*. However, when transduced *LacZ* containing cells are localized, chromogenic substrates that yield a precipitated product are desirable. The most common such substrate is 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal). When β -gal cleaves the glycosidic linkage in X-gal, a soluble blue monomer is produced. Therefore, X-gal staining provides a visual assay of *LacZ* activity.

At present, several different *LacZ* expressing mouse lines have been established and are widely used. A popular reporter line is floxed *ROSA26 LacZ* line, which contains a loxP-flanked stop cassette followed by *LacZ* gene in the *ROSA26* locus. As a result, *LacZ* will be activated after Cre-mediated recombination (Soriano 1999). On another floxed line, Mao *et al.* (1999) modified the gene-trapped proviral *ROSA26* locus (Zambrowicz *et al.* 1997) so that β -galactosidase/neomycin phosphotransferase fusion gene (β -geo) is expressed only after Cre-mediated excision of loxP-flanked DNA sequences.

In the double reporter lines, such as *LacZ*/human placental alkaline phosphatase (hPLAP), commonly referred to as (Z/AP), *LacZ* switches to alkaline phosphatase expression after exposure to Cre recombinase activity (Lobe *et al.* 1999). In another application *LacZ* was coupled with enhanced green fluorescent protein, EGFP, in double reporter mice, Z/EG (*LacZ*/EGFP). This line expresses *LacZ* and after Cre-mediated recombination, EGFP (Novak *et al.* 2000). *LacZ* and *hPLAP* genes in the Z/AP line and *LacZ* and *EGFP* genes in the Z/EG line are under ubiquitously expressed cytomegalovirus (CMV) enhancer/chicken beta-actin hybrid gene promoter, CAGG (Lobe *et al.* 1999, Novak *et al.* 2000).

2.5.2 Fluorescent proteins

Fluorescent proteins (Day & Schaulefe 2008, Daya & Davidson 2009) have become important tools for cell-lineage tracing and gene expression profiling in biological research. Although histochemical or immunocytochemical detection of enzymes such as β -galactosidase (which was already introduced in the previous chapter) is simple, the sensitivity may be problematic and vary depending on the amount of protein synthesized or by other factors that can influence its subcellular distribution. This may lead to an underestimation of the extent of staining. Furthermore, when using β -galactosidase as a reporter, there is a need for sample fixation and substrate permeation to the tissues, which restricts its use. On the contrary, fluorescent proteins offer the advantage that they can be visualized in tissues without fixation and cells can be isolated using fluorescence-activated cell sorting (FACS) without exogenous substrates. In animal models, such as mice, the green and red fluorescent proteins and their color variants have been widely used as reporters. The properties of fluorescent proteins and generated animal models which utilize fluorescent proteins will be discussed next in more detail.

Green fluorescent protein (GFP) is a protein originally isolated from the jellyfish, *Aequorea victoria* (Chalfie *et al.* 1994). It is a 27-kDa protein that emits

green when exposed to blue light. Mature GFP is able to produce intense fluorescence without any cofactors in many different organisms. GFP can be visualized in living cells without any treatment of the observed cells or tissues, and is therefore particularly useful for monitoring gene expression in cultured cells, organs, whole embryos and animals. Due to the potential for widespread usage, many different mutants of GFP have been created including color mutants: blue fluorescent protein (BFP), cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) derivatives (Srinivas *et al.* 2001, McRae *et al.* 2005).

An improved version of the wild-type GFP gene has been developed, the enhanced green fluorescent protein (EGFP). EGFP is a red-shifted GFP variant with humanized codon usage, which shows a four- to 35-fold brighter green fluorescence over GFP (Kawamoto *et al.* 2000).

Several EGFP or its color variant reporter mouse lines that utilize the Cre mediated recombination have been developed (Shrinivas *et al.* 2001, Mao *et al.* 2001, Kawamoto *et al.* 2000, Muzumdar *et al.* 2007, de Casperi *et al.* 2008). These include *EYFP* and *ECFP* reporter lines, where *EYFP* or *ECFP* *cDNAs* were targeted into the ROSA26 locus, preceded by a loxP-flanked stop sequence (Shrinivas *et al.* 2001). A similar strategy was used by Mao *et al.* (2001) when generating the *EGFP* reporter line. Besides employing the ROSA26 locus, fluorescent protein can be expressed under ubiquitous promoters. Kawamoto *et al.* (2000) created the *CAG-EGFP* reporter line where EGFP is under the modified chicken beta-actin promoter/CMV enhancer.

Since the discovery of GFP, there has been a need to develop mouse reporters in other colors like green and with longer wavelength excitation and emission spectra. For these, red fluorescent proteins (RFP) have turned out to be favorable options. dsRed from *Discosoma striata* was the first RFP which was characterized. Since dsRed is tetramer *in vivo*, new monomeric variants like mRFP1 (Campbell *et al.* 2002) and its modified version, mCherry (Shaner *et al.* 2004) were produced. The advantages of mCherry over mRFP1 are its fast maturation, high photostability and brightness. Furthermore, mCherry is codon-optimized (Shaner *et al.* 2004). Several reports have already shown that mCherry is an appropriate RFP in mouse embryonic stem cells as well as in transgenic mouse strains (Fink *et al.* 2010, Hartwich *et al.* 2012, Maye *et al.* 2011, Nowotschin *et al.* 2009, Viotti *et al.* 2011). In addition to mCherry, a dimeric RFP called tdTomato was generated from mRFP1. tdTomato is currently the brightest of the available fluorescent proteins including the RFPs and GFP variants (Day & Shaulefe 2008). Besides the above-mentioned proteins, many other novel FPs

have been cloned from corals and engineered to improve their utility for live-cell imaging (reviews Day & Shaulefe 2008, Daya & Davidson 2009). Red fluorescent proteins have been used to generate mouse lines for the Cre-mediated recombination (Luche *et al.* 2007, Vintersten *et al.* 2004).

Green fluorescent and red fluorescent proteins have been used together for generating double fluorescent reporter mice. In mT/mG double reporter mouse line, Muzumdar *et al.* (2007) coupled tdTomato (mT) with EGFP (mG) and targeted the constructs at the ROSA26 locus. In IRG line, de Casperi *et al.* (2008) took advantage of another RFP, namely DsRed-Express, and combined it with EGFP. DsRed-Express and EGFP are expressed ubiquitously under CMV enhancer/chicken beta-actin promoter. More recently Hartwich *et al.* (2012) reported the pink mouse line which switches expression from the red fluorescent protein mCherry to EGFP after Cre-mediated recombination. Both fluorescent proteins are expressed ubiquitously from the CMV enhancer/chicken beta-actin (CAGGS) promoter. The benefit of the above-mentioned dual reporters is that the presence of two fluorescent proteins allows *in vivo* monitoring of recombined and non-recombined cells upon Cre-mediated recombination.

2.5.3 Luciferase

Luciferase is a generic name for enzymes which are commonly used in nature for bioluminescence. Bioluminescence is a chemical reaction where energy is released in the form of light emission. The substrate molecule, luciferin, reacts with oxygen and a catalyzing enzyme, luciferase, to emit light in such a reaction.

Luciferases have been found for example in bacteria, fungi, in marine invertebrates and vertebrates as well as in insects (Greer & Szalay 2002, Haddock *et al.* 2010). In the last decades, many luciferase genes such as *Luc* from the firefly have been isolated, sequenced and cloned. Thus, luciferase genes can be synthesized and inserted into organisms or transfected into cells. This allows observation of biological processes, testing the activity of transcription from specific promoters, imaging of gene expression, tracing bacterial and viral infection *in vivo* and visualizing the proliferation of tumor cells in animal *in vivo* imaging (Greer & Szalay 2002).

Genetically modified transgenic mice for the bioluminescence and Cre/loxP system approaches have been reported. In the *ROSA26 L-S-L-Luc/+* reporter mouse, luciferase (*Luc*) cDNA and a floxed stop-cassette was introduced into the ROSA26 locus (Safran *et al.* 2003). In the *LucRep* reporter mouse, β -actin

promoter drives the luciferase gene expression after Cre-mediated recombination (Lyons *et al.* 2003).

2.6 The Wnt protein family

The name “Wnt” is an amalgam of *Wingless* and *Int1* (Nusse & Varmus 1992, van Amerongen & Nusse 2009). The *Int1* gene was originally identified as a proto-oncogene, when it was found that upon activation by the mouse mammary tumor virus (MMTV), *Int1* contributed to the tumor formation (Nusse & Varmus 1982, van Amerongen & Nusse 2009). Later when the *Drosophila melanogaster* segment polarity gene *Wingless* was cloned, it was shown to be a homologue to *Int1* (Rijsewijk *et al.* 1987).

The Wnt protein family (Willert & Nusse 2012) consists of secreted cysteine-rich glycoproteins that have roles in many cell biological and developmental processes. Wnt signaling regulates stem cell maintenance, cell proliferation and differentiation, cell-fate specification, cell mobility and cell polarity. Wnt proteins have also a role in gastrulation and in the development of numerous tissues/organs including kidney, liver, heart and neuronal circuitry (Clevers 2006, Freese *et al.* 2010, Behari 2010, Park & Shen 2012, Monroe *et al.* 2012). Wnt signaling has also been linked to human cancers and some degenerative diseases, such as Parkinson’s and Alzheimer’s disease (Sancho *et al.* 2009, de Ferrari *et al.* 2007, Pulkkinen *et al.* 2008, Kawakami *et al.* 2009).

In mice, 19 different *Wnt* genes have been identified: *Wnt1*, *Wnt2*, *Wnt2b*, *Wnt3*, *Wnt3a*, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt6*, *Wnt7a*, *Wnt7b*, *Wnt8a*, *Wnt8b*, *Wnt9a*, *Wnt9b*, *Wnt10a*, *Wnt10b*, *Wnt11* and *Wnt16*. Wnt proteins are around 40 kDa in size and contain a N-terminal signal sequence that targets them for secretion (Port & Basler 2010). Wnts are lipid-modified and posttranslationally glycosylated (Hartrink 2011).

Wnt proteins bind to seven-pass transmembrane Frizzled (Fz) receptors (Bhanot *et al.* 1996, Wang *et al.* 1996, Yang-Snyder *et al.* 1996), which interact closely with lipoprotein receptor-related (LRP) 5/6 co-receptors (Brown *et al.* 1996, Tamai *et al.* 2000, Wehrli *et al.* 2000, He *et al.* 2004). Besides these receptors, Wnts may also interact with single-pass transmembrane receptor tyrosine kinases (RTKs) such as receptor tyrosine kinase-like orphan receptor 2 (Ror2) (Nishita *et al.* 2006, Liu *et al.* 2008) and muscle-specific receptor tyrosine kinase (MuSK) (Jing *et al.* 2009). Also Ryk receptor-like tyrosine kinase (Ryk) (Lu *et al.* 2004, Fradkin *et al.* 2010) and protein tyrosine kinase 7 (PTK7) have

been linked to Wnt signaling (Peradziryi *et al.* 2012). Wnt signaling has shown to be regulated by heparan sulfate proteoglycans (HSPGs). HSPGs are cell surface and extracellular macromolecules (Perrimon & Bernfield 2000, Dhoot *et al.* 2001). The sulfation state of HSPGs regulates activation of Wnt signaling (Dhoot *et al.* 2001, Ai *et al.* 2006). Sulfated heparins, inhibits Wnt activation to the Fz receptor.

2.7 Wnt signaling pathways

Historically, Wnt signaling pathways have been divided into to the canonical (β -catenin-dependent) and non-canonical (β -catenin-independent) pathways (reviews: Veeman *et al.* 2010, Verkaar & Zaman 2006, van Amerongen and Nusse 2009, Rao & Kuhl 2010, Monroe *et al.* 2012). In reality this division is not so apparent because some Wnts can stimulate both pathways depending on the cellular context.

2.7.1 Canonical/ β -catenin-dependent pathway

The classical and best characterized Wnt signaling pathway is the Wnt/ β -catenin pathway, which is highly conserved between different species. In the absence of Wnt activity, β -catenin is recruited to a protein assembly, the destruction complex, which consists of Axin and adenomatous polyposis coli (APC) and glycogen synthase kinase-3 β (GSK-3 β) and casein kinase I (CKI). These kinases successively phosphorylate β -catenin at several N-terminal sites. β -catenin is first phosphorylated by CKI at serine45, followed by GSK-3 β -mediated phosphorylation at serine33, serine37 and threonine41 residues. Axin and APC act as scaffolding proteins, which assist the interaction of these kinases. The phosphorylated β -catenin is recognized and ubiquitinated by the beta-transducin repeat-containing protein (β TrCP) and subsequently degraded by the proteasome (Rao & Kuhl 2010, Monroe *et al.* 2012).

If the Wnt ligand is present, it binds to cell membrane Fz receptor and a co-receptor LRP 5/6 and activates Disheveled (Dvl). This leads to the inhibition of the β -catenin destruction complex through an incompletely understood mechanism followed by stabilization of cytosolic β -catenin. As a consequence, stabilized β -catenin translocates to the cell nucleus and associates with T cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors (Hoppler & Kavanagh 2006) to activate the transcription of the Wnt target genes.

The mechanism underlying Wnt-mediated inhibition of the β -catenin destruction complex has been intensively investigated. Recently, it has been suggested that Wnt signaling occurs on LRP signalosomes, which contain receptor complexes bound to Dvl polymers. In response to signalosome formation, LRP5/6 is phosphorylated by GSK-3 β and CK1. The phosphorylated LRP5/6 recruits Dvl and Axin and the destruction complex disintegrate. As a consequence, β -catenin is released from the destruction complex enabling Wnt signal transduction (Bilic *et al.* 2007, MacDonald *et al.* 2009, Niehrs & Shen 2010, Zeng *et al.* 2005). The schematic presentation of canonical Wnt signaling pathway is shown in Figure 4.

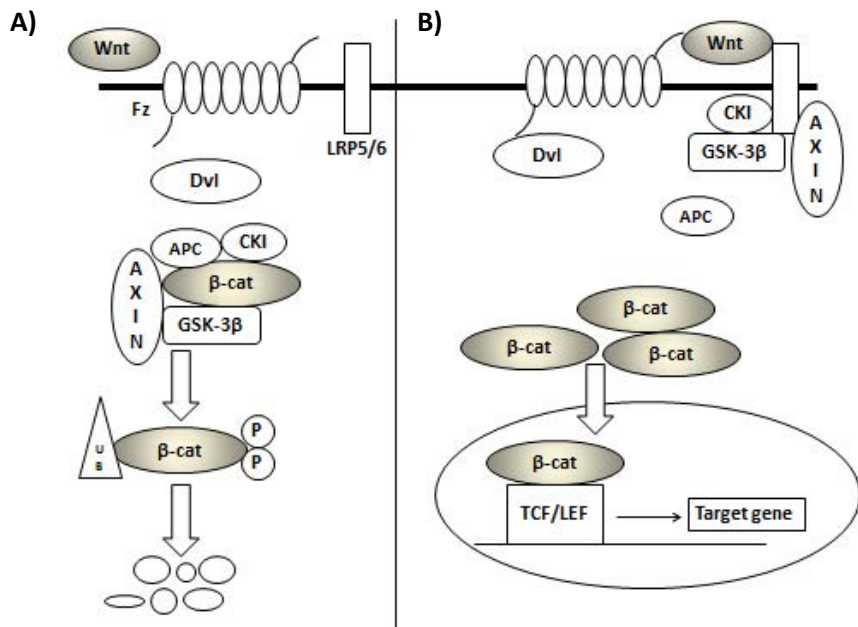


Fig. 4. Canonical Wnt signaling pathway. A) When Wnt is not present, β -catenin (β -cat) is phosphorylated in the destruction complex containing Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3 β (GSK-3 β) and casein kinase I (CKI), ubiquitylated and eventually degraded by the proteasome. **B)** The binding of Wnt to its receptors Frizzled and lipoprotein receptor-related (LRP) 5/6 at the cell surface leads to phosphorylation of LRP5/6 by CKI and GSK-3 β and recruitment of Dvl and Axin. This results in disintegration of the β -cat destruction complex, β -cat stabilization, nuclear translocation and transcription of its target genes through interacting with T cell factor/Lymphoid enhancer factor (TCF/LEF) transcription factors.

2.7.2 Non-canonical Wnt signaling pathways

In some circumstances, Wnts do not co-operate with LRP receptors nor stabilize β -catenin. Wnts can activate several alternative intracellular cascades through Fz receptors and Dvl (reviews: Veeman *et al.* 2010, Verkaar & Zaman 2006, van Amerongen and Nusse 2009, Rao & Kuhl 2010, Monroe *et al.* 2012).

The existence of a calcium-mediated pathway that is directly activated by Wnt ligands binding to Fz receptors was originally described in *Danio rerio* and *Xenopus laevis*. It was shown that Wnt5a and rat Frizzled-2 (RFz-2) induced cytoplasmic Ca^{2+} fluxes in a G protein-dependent manner in *Danio rerio* embryos (Slusarski *et al.* 1997a, Slusarski *et al.* 1997b). Later it was found that Wnt5a and RFz-2 induced the G protein-dependent activation of Ca^{2+} sensitive protein kinase C (PKC) in *Xenopus laevis* embryos (Sheldahl *et al.* 1999). Other Ca^{2+} sensitive proteins such as calcium/calmodulin-dependent kinase (CaMK) II and calcineurin (CaN) have been proposed as downstream effectors of the Wnt/ Ca^{2+} pathway. Through calcineurin the Wnt/ Ca^{2+} pathway has been reported to connect to nuclear factor of activated T cells (NFAT) (Burn *et al.* 2010). On the other hand, CaMKII has been found to activate a nemo-like kinase (NLK), which antagonizes Wnt/ β -catenin signaling (Ishitani *et al.* 2003). A possible role for Dvl in Wnt/ Ca^{2+} signaling has recently been tested. Dvl was found to have a mild ability to activate calcium signaling in the calcium flux, PKC, and CamKII assays (Sheldahl *et al.* 2003). The Wnt/ Ca^{2+} signaling have been shown to be active in convergent extension (CE) movements in gastrulation (Wallingford *et al.* 2001).

The planar cell polarity (PCP) pathway was originally identified in *Drosophila melanogaster* (Strutt *et al.* 1997, Theisen *et al.* 1994, Vinson & Adler 1987). In *Drosophila*, PCP signaling controls the polarized behavior of cells in several distinct structures, such as the wing hair, bristles on the body and the ommatidia of the eye (Strutt 2003). A group of PCP factors including Fz and Dvl are involved in all of these processes. However, participation of any Wnt ligand in *Drosophila melanogaster* PCP signaling is uncertain and it may be that this pathway is Wnt ligand-independent. In vertebrates some Wnts appear to control processes that bear a resemblance to the *Drosophila* PCP pathway, such as gastrulation movements (Kikuchi *et al.* 2009). It has also been proposed that a similar signaling mechanism may be involved in cochlear hair cell polarity, heart development, neuronal migration and cancer (Veeman *et al.* 2003). The PCP signaling mediated by Fz and Dvl leads to activation of small GTP binding Rho

family kinases, RhoA and Rok, and further downstream the Jun-N-terminal kinase (JNK) or Rho-associated kinase, ROCK.

It is also known that besides Fz receptors, other receptors can mediate non-canonical Wnt signaling. Members of the Ror and Ryk families can interact with Wnt proteins through their extracellular cysteine-rich domain (CDR) in the case of Ror proteins or their Wif-domain in the case of Ryk proteins. Ror2, member of the receptor tyrosine kinase-like orphan receptor (Ror) family, activates non-canonical Wnt signaling through JNK. Ror2 has been linked to cardiac development (Takeuchi *et al.* 2000). Wnt3a and Wnt5a bind to the Fz-CRD of Ror2 to mediate cell polarization and migration (Nishita *et al.* 2006, Liu *et al.* 2008). Ryk, receptor-like tyrosine kinase, operates through the proto-oncogene Src (Wouda *et al.* 2008). The Wnt/Ryk pathway has a role in neuronal development. Protein tyrosine kinase 7 (PTK7) is an evolutionarily conserved transmembrane receptor. Only recently, it has been shown that PTK7 acts as a Wnt co-receptor, which activates the PCP pathway, but inhibits canonical Wnt signaling. PTK7 is necessary for CE cell movements in *Danio rerio*, *Xenopus laevis* and in mice (Golubkov *et al.* 2010, Wehner *et al.* 2009, Yen *et al.* 2009). Additionally, PTK7 is required for neural crest migration, another PCP signaling regulated process. Inhibition of PCP factors like Fz7 and Dvl in *Xenopus laevis* embryos blocks neural crest migration (De Calisto *et al.* 2005), the same phenotype has been shown in PTK7 knock-down experiments (Shnitsar *et al.* 2008). The schematic presentation of non-canonical Wnt signaling pathways is shown in Figure 5.

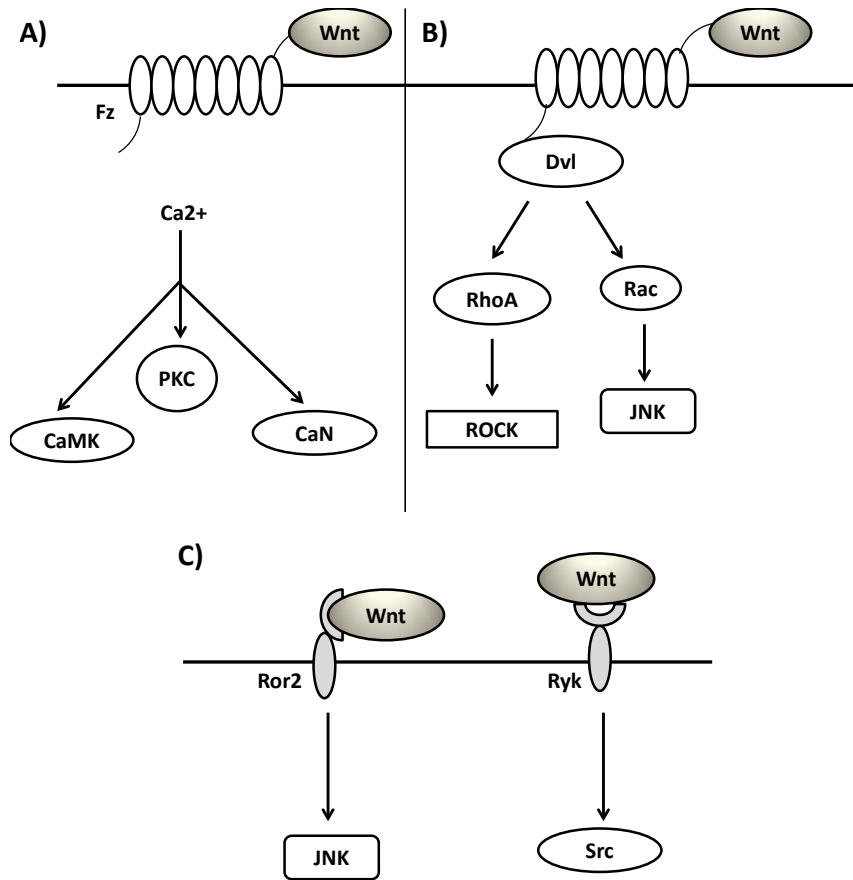


Fig. 5. Non-canonical Wnt signaling pathways. A) Wnt signaling through Frizzled (Fz) receptors can lead to elevation of intracellular Ca^{2+} levels and activation of the calcium-sensitive proteins calcium/calmodulin-dependent kinase (CaMK), calcineurin (CaN) and protein kinase C (PKC). B) The Wnt/planar cell polarity (PCP) pathway is portrayed by activation of small GTP binding Rho family kinases RhoA and Rac and their downstream targets ROCK and Jun-N-terminal kinase (JNK). C) Alternative Wnt receptors Ror2 and Ryk can initiate non-canonical Wnt signaling responses by activating JNK and Src.

2.8 Antagonists and agonists of the Wnt signaling

There are several secreted antagonists of Wnt signaling, including secreted Frizzled-related proteins (SFRP), Wnt inhibitory factors (WIF) and Dickkopfs

(Dkk) (reviewed in Kawano & Kypta 2003). Norrin and R-Spondins can activate Wnt signaling, thereby acting as agonists. The protein Wise/Sclerostin can act both as an activator (agonist) or inhibitor (antagonist) depending on cellular context (Itasaki *et al.* 2003).

The SFRPs are the largest family of Wnt antagonists (reviewed in Bovolenta *et al.* 2008), comprising five members in humans, SFRP1-SFRP5. The founding member Frzb was initially identified through its sequence similarity with the Fz receptors (Hoang *et al.* 1996, Leyns *et al.* 1997). Furthermore, Frzb was shown to bind to Wnt8 and block its signaling in *Xenopus laevis*, supporting the role of Frzb as a Wnt antagonist (Leyns *et al.* 1997, Wang *et al.* 1997). Thus, because of their homology with the Wnt-binding domain on the Fz receptors, SFRPs directly bind Wnts in the extracellular space and prevent their interactions with receptors.

The dickkopf (Dkk) family encodes secreted glycoproteins and consists of four main members in vertebrates, Dkk1-4. Dkk1 was originally identified as head inducer and Wnt antagonist in *Xenopus laevis* embryos (Glinka *et al.* 1998). Dkk proteins (Nierhs 2006, Pietila *et al.* 2011) specifically block the canonical Wnt signaling by interacting directly with the Fz co-receptors LRP5/6 (Semenov *et al.* 2001). Besides this Dkk1 forms a complex with the transmembrane molecules, Kremens. This leads to endocytosis of LRP6 and additional inhibition of Wnt signaling in a target cell (Mao *et al.* 2002).

WIF1 was first identified as an expressed sequence tag from human retina with highly conserved orthologues in *Xenopus laevis* and *Danio rerio* (Hsieh *et al.* 1999). It was shown that injection of WIF1 RNA into early *Xenopus laevis* embryos resulted in an induction of a partial secondary axis and abnormal somitogenesis through the Wnt8 inhibition (Hsieh *et al.* 1999). Structurally, WIF1 contains a unique WIF domain (WD), which mediates the Wnt binding of WIF1. Thus, similarly to SFRPs, WIF1 directly binds Wnts in the extracellular space and inhibits their interactions with receptors.

Norrin and R-spondins can activate the Fz/LRP receptors, acting as agonists of Wnt signaling (Kim *et al.* 2008, Ye *et al.* 2009). Norrin is a small, cysteine-rich, secreted protein that shows weak homology to the ligands of the transforming growth factor (TGF)- β family (Meitinger *et al.* 1992). In humans, mutations in the corresponding gene (NDP) cause Norrie disease, which leads to retinal dysfunction (Smallwood *et al.* 2007, Ye *et al.* 2009). Norrin has been identified as a ligand that binds the Fz4 CRD-domain with high affinity (Smallwood *et al.* 2007) and activates the canonical signaling pathway through Fz4 and LRP5 (Xu *et al.* 2004). R-spondins belong to a small protein family

consisting of four members, R-spondin 1-4. R-Spondin proteins have been shown to interact directly with Fz8 and LRP5/6, thereby positively regulating canonical Wnt signaling (Nam *et al.* 2006). Furthermore, it has been suggested that R-spondins regulate canonical Wnt signaling by competing with Dkk1 for binding to Kremen and LRP5/6 (Kim *et al.* 2008).

2.9 Introduction to kidney function and development

The kidney is an essential organ for the homeostasis of the body. It regulates the balance of electrolytes and blood pressure. The kidney maintains acid-base balance and reabsorbs water, glucose and amino acids. It also produces hormones including erythropoietin, the enzyme renin and is involved in the synthesis of vitamin D.

Embryologically, the kidney is derived from the intermediate mesoderm and develops through three spatially and temporally distinct forms: the pronephros, the mesonephros and the metanephros.

The most primitive kidney form, the pronephros, is present in all vertebrates during embryonic development. It is often not functional and degenerates later on, but is necessary for osmoregulation in larval stages in amphibians and fish in osmoregulation. The pronephros is a functional and permanent kidney in some primitive fishes, like hagfish, in adult stage (Saxen 1987).

The mesonephros is the permanent kidney of adult fish and amphibians. In higher vertebrates the mesonephros contributes to the hematopoietic stem cell development. In male mammals some of the mesonephric tubules differentiate into the efferent ducts and proximal epididymis (Robaire *et al.* 2000). The metanephros differentiates into the permanent kidney in higher vertebrates such as mammals, avians and reptiles (Gilbert 2006) and is discussed in detail in the following chapter.

2.10 Metanephros

Structurally, the mammalian metanephros contains an inner medullary region, an outer medullary region and a cortical region. This gross structure is necessary for the functionality of the metanephric kidney, since it establishes an osmotic gradient between the cortex and medulla. This drives the extraction of water from the urine. The functional unit of the kidney, the nephron, consists of a renal

corpuscle, proximal and distal tubules, loop of Henle and the collecting duct system.

Renal corpuscle is the initial blood-filtering component of a nephron and consists of a glomerulus and Bowman's capsule. The renal tubule receives plasma filtrate from the glomerulus and processes it into urine. The proximal tubule reabsorbs most minerals and other nutrients from the tubular fluid and passes them to blood. The loop of Henle helps establish the hypertonic environment of medullary interstitial fluid. The distal tubule continues the return useful materials from the filtrate to the blood by actively pumping small molecules out of the tubule lumen. The collecting duct system is the final component of the kidney to influence electrolyte and fluid balance. It collects the urine from the nephron and passes it to the ureter for secretion.

The metanephros begins to develop in mouse at E10.5-11. The differentiation of metanephric kidney depends on a series of reciprocal inductive interactions between the metanephric mesenchyme and the Wolffian duct-derived ureteric bud (Constantini & Kopan 2010).

The development of the metanephric kidney starts after the Wolffian duct has extended caudally along the body axis and has formed an outgrowth, the ureteric bud. The ureteric bud is an epithelial tissue that invades the metanephric blastema and induces mesenchymal cells to condense to form a cap around the ureteric tip. The cap condensate begins to form pretubular aggregates that undergo a mesenchyme-to-epithelial (MET) transition to form the renal vesicle. From this point, the renal vesicle goes through a wide-ranging morphogenesis, elongating to form first the comma-shaped, then the S-shaped bodies before maturing into the nephron, the excretory unit of the kidney (Vainio & Lin 2002, Valerius & McMahon 2008, Little & McMahon 2012). The S-shape body gives rise to three different segments in the mature nephron: the proximal segment differentiates into glomerular podocyte cells, the mid-section becomes the proximal tubule and the loop of Henle, and the distal end forms the distal tubule, which fuses with the ureteric bud branches (Figure 6) (Reidy 2009, Dressler 2009).

At the same time with the induction of the first nephrons, the ureteric bud continues to branch. This branching process is essential for the growth and additional differentiation of the kidney (Constantini & Kopan 2010). During the branching morphogenesis, each tip acts as an inductive place to start nephron formation after the tip has first elongated and branched to generate new epithelial tips. Finally, the branches of the ureteric bud form the collecting-duct system of the kidney.

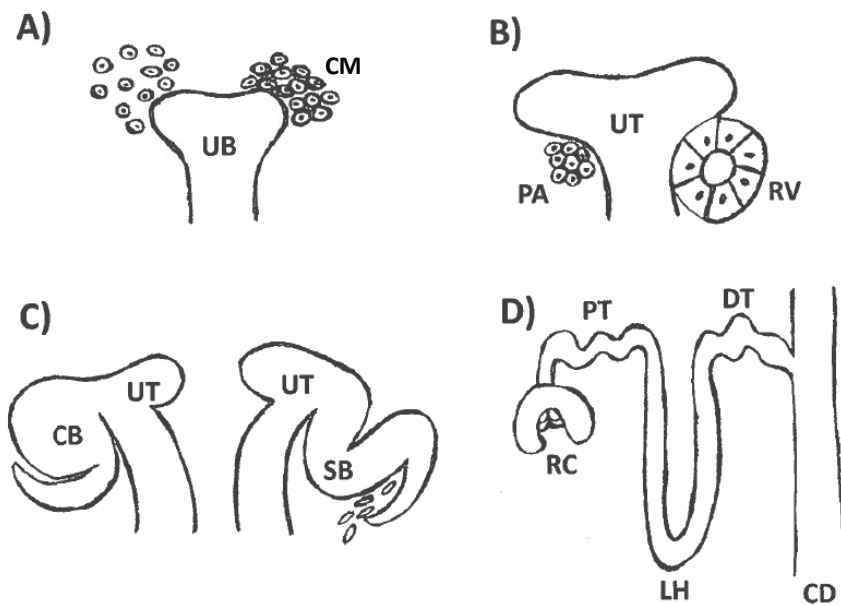


Fig. 6. Overview of nephrogenesis during kidney development: formation of individual nephrons through MET and elongation. A) Around the tip of the branching ureteric bud (UB), the metanephric mesenchyme (MM) condenses to form cap mesenchyme (CM). B) CM proximal to the ureteric trunk (UT) and immediately adjacent to its basement membrane condenses to form pretubular aggregate (PA). Cells of the PA then undergo changes to produce a renal vesicle (RV) with a polarized epithelium (MET). C) Cells of the RV proliferate and segment, forming intermediate stages referred to as comma-shaped (CB) and s-shaped (SB) bodies. Endothelial cells are attracted into the cleft at the proximal end of the SB to begin to form the glomerular vasculature. D) Elongation, including patterning and segmentation, proceeds to form the mature nephron: renal corpuscle (RC), proximal tubule (PT), loop of Henle (LH) and distal tubule (DT). The branching ureter forms the collecting-duct (CD) system (modified from Little *et al.* 2010).

2.11 Wnt signaling in kidney induction

Several Wnts are expressed in developing kidney having either roles in ureteric branching or nephron induction. In mouse, *Wnt6*, *Wnt7b*, *Wnt9b* and *Wnt11* expression is detected in the ureteric bud epithelium, while *Wnt2b* and *Wnt4* are found in the kidney mesenchyme (Figure 7).

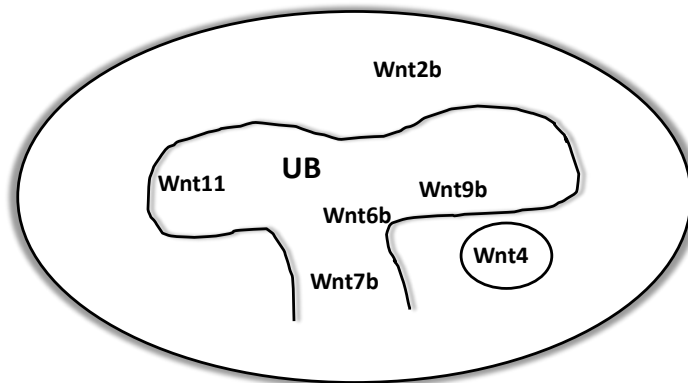


Fig. 7. Wnt signalling molecules in the developing kidney. *Wnt2b* and *Wnt4* expression is detected in the kidney mesenchyme. *Wnt6*, *Wnt7b*, *Wnt9b* and *Wnt11* are present in the ureteric bud (UB) epithelium.

Wnt6 is present in the bud at early stages of kidney development, starting from invasion of the ureteric epithelium into the metanephric mesenchyme and expanding in the branching ureter tips during later development. *Wnt6*-expressing cells are able to induce kidney tubule formation and *Wnt4* expression *in vitro*. Additionally, *Wnt6*-expressing cells can rescue tubulogenesis in mesenchymes from *Wnt4* mutant embryos. Thus, *Wnt6* might function as an epithelial signal that triggers kidney tubulogenesis by activating *Wnt4* signaling (Itaranta *et al.* 2002).

Wnt7b expression is restricted to the ureteric trunk epithelium that gives rise to the collecting duct network and ureter of the kidney, but is not present at the ureteric tips. When *Wnt7b* activity is specifically removed from the mouse epiblast lineage with *Sox2Cre* line (Hayashi *et al.* 2002), cortical epithelial development remains normal but the medullary zone fails to differentiate. The *Wnt7b^{c3/-}:Sox2Cre* mutants do not develop proper collecting ducts due to the orientation problems in cell division. Furthermore, *Wnt7b* signaling is essential for elongation of the loop of Henle, which remains truncated in the *Wnt7b^{c3/-}:Sox2Cre* mutants (Yu *et al.* 2009). *Wnt7b* is produced by macrophages to stimulate repair and regeneration in the kidney injury model. By genetically deleting *Wnt7b* in macrophages with leukocyte-specific *Csf1R-icre* transgene (Deng *et al.* 2010), repair of kidney injury is greatly diminished. This study emphasizes the role of the macrophage *Wnt7b* as a critical factor in the regeneration of the kidney epithelium (Lin *et al.* 2010).

Wnt9b expression is found in the metanephric duct and in the stalk of the ureter, but is absent from the ureteric tip. In the *Wnt9b* mutant, kidney mesenchyme fails to aggregate and undergo tubulogenesis and UB branching does not proceed beyond the T-stage. The *Wnt9b*-deficient kidney mesenchyme does not express *Wnt4*, suggesting that *Wnt9b* acts upstream of *Wnt4*, perhaps through the canonical Wnt signaling pathway (Carroll *et al.* 2005). More recently it was suggested that *Wnt9b* regulates planar cell polarity and kidney tubule morphogenesis through the non-canonical Rho/Jnk signaling. Mice carrying a hypomorphic mutation of *Wnt9b* or mice that had a floxed allele of *Wnt9b* deleted with either *KspCre* or the tamoxifen inducible *CaggCreErTm* developed cystic kidneys. Cyst formation on these mouse lines were the result of defects in planar cell polarity (Karner *et al.* 2009). *Wnt9b* has been shown to have a role in the maintenance and differentiation of progenitor cell population within kidney by over-expressing *Wnt9b* in the *Six2*-positive cap mesenchyme (Kiefer *et al.* 2012). Karner *et al.* (2011) demonstrated that Wnt9b/ β -catenin signaling is active in the nephron progenitors and is required for their renewal/proliferation using a combination of approaches in the *Wnt9^{-/-}* mice, in the conditional *Wnt9b* knock-outs with *KspCre* and β -catenin gain-of-function and β -catenin loss-of-function experiments.

Wnt11 is expressed in the ureteric bud and its expression remains in the tips of the growing ureter (Kispert *et al.* 1996). *Wnt11* controls the ureteric branching morphogenesis through glial cell-derived neurotrophic factor (GDNF) and Ret tyrosine kinase receptor. In the *Wnt11^{-/-}* newborn mouse kidneys are smaller due to ureteric branching defects. *Wnt11* functions by maintaining normal expression levels of GDNF, which in turn activates GDFN/Ret signaling to upregulate *Wnt11*, indicating that these factors co-operate in a positive feedback loop to control the ureteric branching (Majumdar *et al.* 2003).

In the mesenchyme, *Wnt4* is detected in the condensing mesenchyme and pretubular aggregates (Stark *et al.* 1994). *Wnt4* has been suggested to act as an autoinducer of the mesenchyme-to-epithelial (MET) transformation during nephron differentiation. In the absence of *Wnt4*, metanephric mesenchyme fails to form pretubular aggregates and nephron differentiation does not take place or is greatly reduced (Stark *et al.* 1994, Kobayashi *et al.* 2005), leading to severe kidney defects and early postnatal death in the *Wnt4^{-/-}* mouse (Stark *et al.* 1994, Vainio 2003). *Wnt4*-expressing cells are capable of inducing tubule formation in the kidney mesenchyme *in vitro* (Kispert *et al.* 1998) and can also rescue the

tubulogenesis deficiency in the *Wnt4* mutant kidney mesenchyme (Kispert *et al.* 1998, Itaranta *et al.* 2006).

Wnt2b is expressed in the perinephric cells of the early metanephros. The role of *Wnt2b* in kidney development has not been investigated in detail, but apparently *Wnt2b* is not a critical factor in the induction since *Wnt2* mutant mice do not show kidney phenotype (Goss 2009, Tsukiyma & Yamaguchi 2012). However, Lin *et al.* (2001) were able to reconstitute kidney organogenesis *in vitro* when separated ureteric bud was cultured with *Wnt2b*-expressing cells. Thus, *Wnt2b* may have a function in the ureteric bud morphogenesis.

2.12 Some transcription factors and signaling molecules in kidney development

Besides the Wnt protein and Wnt signaling pathways, many other transcription factors and signaling molecules have been shown to have a role in kidney organogenesis. In this chapter those transcription factors and signaling molecules that have been associated to Wnt proteins or Wnt signaling are discussed in more detail.

Empty spiracles homolog 2 (*Emx2*), a homeobox transcription factor, is expressed initially in the ureteric bud. In *Emx2*^{-/-} mice, the ureteric bud grows and invades the metanephric mesenchyme normally, but the tubules are not induced and the kidney development fails (Miyamoto *et al.* 1997). Some early kidney development markers, such as *Wt1*, *Gdnf*, *Pax2*, *Ret* and *Lim1*, are expressed in the *Emx2* mutants, suggesting that *Emx2* acts downstream of these genes and is not regulating the ureteric budding or branching. The expression of *Wnt4* is lost in the mesenchymal cells in *Emx2* mutant mice (Miyamoto *et al.* 1997). As *Wnt4* is an important initiating signal for tubulogenesis, it is likely that *Emx2* regulates the ureteric bud-derived signals to the kidney mesenchyme and is involved in tubule induction *in vivo* (Vainio & Lin 2002).

Fibroblast growth factor 8 (*Fgf8*) is a secreted signaling molecule that is expressed at E11.5 in the uninduced metanephric mesenchyme (Perantoni *et al.* 2005) and later in the developing nephros (Grieshammer *et al.* 2005). *Fgf8* null mice die at the gastrulation stage (Meyers *et al.* 1998). Conditional inactivation of *Fgf8* function from the metanephric mesenchyme with *Pax3Cre* (Grieshammer *et al.* 2005) and *T (brachyury) Cre* (Perantoni *et al.* 2005) leads to impaired kidney development. The mutant kidneys remain small with deficient nephron formation

and lack the expression of *Lim1* and *Wnt4*, factors which are essential for nephrogenesis (Grieshammer *et al.* 2005, Perantoni *et al.* 2005).

Sine oculis homeobox 2 (*Six2*) is a highly conserved transcription factor that is expressed in the induced metanephric mesenchyme. *Six2* gene expression is maintained throughout kidney development but no expression is detected in adult mouse kidneys (Humphreys *et al.* 2008). *Six2* is required for suppression of nephrogenesis and progenitor renewal in the developing kidney. In this, *Six2* may act, at least in part, to block *Wnt9b* action (Kobayashi *et al.* 2008). *Six2* deficiency results in kidney malformations and also leads to a reduced number of nephrons in animal models (Self *et al.* 2006, Kobayashi *et al.* 2008).

Wilms tumor homolog (*Wt1*) is a zinc-finger transcription factor that has several isoforms with different roles in the developing urogenital system. In humans, *WT1* mutations are associated with childhood kidney tumours, and cause the Wilms tumour/aniridia/genitourinary anomalies/mental retardation (WAGR), Denys–Dash and Frasier syndromes, which are characterized by gonadal and kidney abnormalities. In mice, *Wt1* is expressed in the intermediate mesenchyme and later in the pronephric duct (Kreidberg *et al.* 1993). *Wt1* is highly expressed also in glomerular podocytes (Pelletier *et al.* 1999, Miller-Hodges & Hohenstein 2012). In *Wt1*^{-/-} mice, the ureteric bud fails to grow out of the Wolffian duct and the metanephric mesenchyme subsequently becomes apoptotic leading to a complete failure of kidney development (Kreidberg *et al.* 1993, Donovan *et al.* 1999). Knockdown of *Wt1* function with small interfering RNA molecules (siRNA) that are used to inhibit a gene of interest at the mRNA level, results in an almost complete loss of nephrons in vitro. Additionally, *Wt1* is controlled by *Pax2* and is needed for expression of *Wnt4* (Davies *et al.* 2004). *Wnt4* has been shown to be a down-stream target of *Wt1* in cell culture assays (Sim *et al.* 2004, Essafi *et al.* 2011, Murugan *et al.* 2012), in kidney mesenchyme in vivo (Essafi *et al.* 2011) and in the *Xenopus laevis* pronephros (Murugan *et al.* 2012) Thus, *Wt1* is essential for the nephron formation at the mesenchymal-to-epithelial (MET) stage.

2.13 Mouse lines for conditional mutagenesis in the kidney

The basics and mechanism of the Cre/loxP system as well as inducible systems coupled to it was already described in the previous chapters. In this chapter different Cre-lines to kidney research field will be introduced.

At the moment, there are a number of genetically modified kidney-specific Cre mice which potentially permit gene modification to the specific cells or different segments within kidney, such as mesonephric duct, podocytes, epithelial renal tubules, collecting duct network, mesenchyme progenitor cells and stromal cells. Table 1 summarizes some of the kidney-specific Cre mouse strains that are presently accessible (reviews in Lantinga-van Leeuwen *et al.* 2006, Wu 2007, Kohan 2008).

Table 1. Cre mouse lines with Cre expression in the kidneys.

Mouse	Promoter	Renal expression	Reference
Ah-Cre	Cyp1A1	CD, glom	Sansom <i>et al.</i> (2004,2005)
ApoE-Cre	Human apoE	PT, DT	Lehste <i>et al.</i> 2003
AQP2-CreTag	AQP2 (aquaporin-2)	CD (principal cells)	Nelson <i>et al.</i> 1998
Bmp7-Cre	Bmp7	CD, UB, NM	Oxburgh <i>et al.</i> 2004
γGT-Cre	γGT	PT (cortical)	Iwano <i>et al.</i> 2002
Hoxb7-Cre	Hox-b7	UB, CD	Oxburgh <i>et al.</i> 2004
iL1-sglt2-Cre	Sglt2	PT	Rubera <i>et al.</i> 2004
Ksp1.3/Cre	Ksp-cadherin	Tub.epith.(mainly CD, TAL)	Shao <i>et al.</i> 2002
MMTV-Cre	MMTV- LTR	Not specified	Wagner <i>et al.</i> 2001
Neph-Cre	Nphs1(nephrin)	Glom (podocytes)	Eremina <i>et al.</i> 2002
Nes-Cre1	nestin (rat)	Not specified (kidney)	Dubois <i>et al.</i> 2006
Osr1-Cre	Osr1	intermediate mesoderm	Mugford <i>et al.</i> 2008
Pax2-Cre	Pax2	Glom, tubules, CD	Ohyama & Groves 2004
Pax3-Cre	Pax3	metanephric mesenchyme	Engleka <i>et al.</i> 2005
Pax8-Cre	Pax8	Glom, tubules	Bouchard <i>et al.</i> 2004
Rarb2-Cre	Rarb2	mesenchyme progenitor cells	Kobayashi <i>et al.</i> 2005
Ren1d-Cre	Ren1d -locus (renin)	Juxtaglomerular cells, afferent arterioles	Sequeira Lopez <i>et al.</i> 2004
Six2-Cre	Six2	MM cap mesenchyme	Kuure <i>et al.</i> 2007 Kobayashi <i>et al.</i> 2008
Tcf21/Pod1-Cre	Tcf21/Pod1	metanephric mesenchyme	Maezawa <i>et al.</i> 2012

Table 2 summarizes a few of the tamoxifen inducible Cre-mouse lines that can be used in kidney research. Examples for Cre-ER regulated gene expression in kidney includes the first adult mouse model of nephrogenic diabetes insipidus by tamoxifen-induced Cre-mediated *AQP2* gene deletion (Yang *et al.* 2006). In addition to ablating gene function, the Cre-ER system has been employed in a mouse model which permits inducible activation of *Pax2* within the podocytes. Following activation of *Pax2*, healthy adult mice developed renal disease and proteinuria connected to inhibition of the podocyte key regulator molecule Wilms

tumor1 (*Wt1*) and reduction of its target gene *nephrin* expression in the glomerulus (Wagner *et al.* 2006).

Table 2. Inducible Cre mouse lines with expression in the kidneys.

Mouse	Promoter	Renal expression	Reference
Cited –CreER(T2)	Cited1	cap mesenchyme	Boyle <i>et al.</i> 2008
Cre-ER(TM)	pCAGG	universal	Hayashi & McMahon 2002
K18iresCreER(T2)	K18-keratin	epithelial cells.	Wen <i>et al.</i> 2003
bMCM86	pCAGGS	glom (podocytes)	Bugeon <i>et al.</i> 2003
KspCad-CreER(T2)	Ksp-cad	epithelial cells	Lantinga-van Leuwen <i>et al.</i> 2006
Osr1eGFPcCreER(T2)	Osr1	intermediate mesoderm	Mugford <i>et al.</i> 2008
Sall1CreER(T2)	Sall1	MM	Inoue <i>et al.</i> 2010
R26cre-ER(T)	Rosa26	universal	Vooijs <i>et al.</i> 2001
Tie2ER(T2)Cre	Tie2	glom (endothelium)	Forde <i>et al.</i> 2002

In the kidney, the Tet-ON system with the podocin promoter to drive rtTA has been employed to temporally regulate podocyte-specific Cre expression (Shigehara *et al.* 2003). Another example of the Tet-ON system is the usage of the paired box-containing gene 8 (*Pax8*) promoter coupled to rtTA to control Cre expression in the nephron and to efficiently knock-out glucosylceramide synthase (Traykova *et al.* 2007).

The Flp/FRT system has not been utilized to achieve kidney-specific gene expression or knock-outs.

2.14 Kidney as an *in vitro* model organ

For many decades the embryonic kidney has been a classical developmental model organ when studying the molecular and cellular interactions of organogenesis. In the traditional Trowell-Type Tissue culture system the whole embryonic kidney is cultured on small-diameter culture dishes supported by metal grids. Pieces of Millipore or Nuclepore filters are placed on top of the grids. In such culture conditions ureteric bud branches and nephrons form within a few days of culture. *In vitro* kidney development is not complete as the neurogenic and endothelial compartments of the kidney do not develop in such cultures.

In classical tissue recombination experiments by Grobstein and Saxen (Grobstein 1956a, Grobstein 1956b, Saxen 1987, Saxen & Lehtonen 1987) the metanephric mesenchyme (MM) is separated from the ureteric bud (UB) by enzymatic treatment and cultured on the filter with heterologous inducer tissues

such as spinal cord (spc). Without the UB, the MM fails to form epithelial tubules and degenerates. Spc can mimic the ureter as an inducer of mesenchymal tubulogenesis. The early research suggested that tubule induction in the kidney depends on inductive signaling and that direct cell-cell contacts are needed between inducer tissue and MM. The signals produced *in vitro* by spc may be the same as the signals involved in tubule induction *in vivo*.

Murine MM can be also induced with some chemicals such as lithium, (2'Z,3'E)-6-Bromoindirubin-3'-oxime (BIO) and Leukemia inhibitory factor (LIF). Lithium ions and BIO can induce nephrogenesis in isolated mouse and rat mesenchymes by inhibiting the GSK-3 β activity, which leads to the activation of the canonical Wnt pathway (Kuure *et al.* 2007). LIF alone or in combination with FGF2, TGF α or TGF β 2/FGF2 is able to induce tubule formation in isolated rat mesenchymes (Karanova *et al.* 1996, Plisov *et al.* 2001).

The kidney organ-culture settings have become a useful tool because many of the processes in kidney organogenesis that happen *in vivo* also occur *in vitro* (Constanti *et al.* 2011). These processes include UB branching, the formation and segmentation of nephrons and the fusion of nephrons to collecting ducts. The vascular component of the glomerulus does not form *in vitro* (Vaughan & Quaggin 2008). Additionally to what is, isolated UB or MM can be grown independently (Grobstein 1953). The isolated UB will grow and branch without any MM if cultured in proper supporting elements (Matrigel or collagen) and supplied with suitable growth factors. This is beneficial to separate the processes of UB branching and nephrogenesis and to examine the secreted factors generally provided by the other tissues to stimulate these processes. The isolated MM undergoes nephrogenesis if cultured with either heterologous tissues or with factors that are normally provided by the UB (Sariola 2002).

As already mentioned in the beginning of this chapter, the standard conditions for kidney culture is to grow it on a filter at the air-medium interface. This causes the kidney to flatten and grow in two rather than three dimensions. Even though in this way the three-dimensional (3D) organization of the kidney is largely lost, kidney imaging can be more easily performed (Constanti *et al.* 2011).

The time-lapse image settings for cell-lineage tracings and fate mapping experiments take advantage of either the Cre/loxP technology coupled to fluorescent proteins or transgenic mice directly expressing fluorescent proteins (FP). Several mouse lines that express GFP or other FPs in specific kidney lineages are available (Takasato *et al.* 2004, Levinson *et al.* 2005, Jain *et al.* 2006, Kobayashi *et al.* 2008, Chi *et al.* 2009).

The first transgenic direct FP-line, *Hoxb7/EGFP* (Srinivas *et al.* 1999), labels the Wolffian duct (WD) and UB lineages and has been beneficial for studying the rate and the pattern of WD, ureter and UB morphogenesis (Watanabe & Costantini 2004). *Hoxb7/EGFP* as well as other FP transgenic lines can be visualized in combination with bright-field or phase imaging to observe UB branching or other processes like nephron formation in the milieu of overall kidney growth. Transgenes such as *Hoxb7/EGFP* or other similar applications can also be combined with mutant backgrounds to study how different mutations affect developmental processes.

Another approach to image/track specific cell lineages in the developing kidney is to use mouse lines expressing Cre recombinase together with Cre-dependent conditional reporter lines. Such an approach allowed the identification and characterization of a stem-like population within the developing metanephros. The cap mesenchymal cells expressing *Six2* have the potential to give rise to all epithelial cell lineages in the nephron and to self-renewal (Kobayashi *et al.* 2008). Similar observations were made with *Cited1* (Boyle *et al.* 2008). In one study, *Osr1*-positive cells were capable of making either epithelial or stromal precursors prior to E10.5 (Mugford *et al.* 2008), consistent with the wide expression pattern of *Osr1* in the intermediate mesoderm and in the lateral plate mesoderm. However, when the metanephric mesenchyme was induced, the *Osr1*-positive population made only epithelial cells, suggesting that the stromal progenitor cells had now switched off *Osr1*, thus is a discrete lineage (Mugford *et al.* 2008). *Pax3-Cre* (Grieshammer *et al.* 2005) and *Rar2b-Cre* (Kobayashi *et al.* 2005) lines were used to address the fate of MM cells in the developing kidney. These results indicated that the MM gives rise to nephronic epithelium *in vivo*.

3 Outline and aims of the present study

The Cre/loxP-system has been studied *in vivo* and the results point out that dose-dependent recombination induced by tamoxifen was possible in various tissues (for example kidney, heart, lung and liver) in the adult mouse, in the embryo and in cultured cells. However, using inducible Cre/loxP system in tissue culture conditions is still poorly characterized.

Wnt4 is an important gene in kidney and gonad development. Defects in the *Wnt4* gene expression lead to impaired kidney development (Stark *et al.* 1994) and partial sex reversal (Stark *et al.* 1994, Bernard & Harley 2007). The studies in the past were made using the conventional *Wnt4* knock-out, which is lethal after birth. Therefore, new mouse lines are needed in order to reveal *Wnt4* function at any stage during the life cycle or in other organs.

Therefore the aims of this study were:

1. To set up a method and find the conditions for the tamoxifen-induced Cre/loxP mediated activation of the reporter gene LacZ in the kidney cultures.
2. To generate a mouse model where EGFP-Cre was targeted at the locus of a *Wnt4*. This allele allows real-time profiling of *Wnt4* gene expression by monitoring the expression of EGFP and as well as mapping the fate of those cells that expresses or has expressed the *Wnt4* gene. It also provides a tool for conditional mutagenesis in the kidney starting from the renal vesicle stage of nephron development. Additionally, the function of the *Wnt4* gene can be investigated in diverse organs where *Wnt4* is active.
3. To generate an allele and subsequent mouse model for conditional inactivation of the *Wnt4* gene.

4 Materials and methods

The methods used in this thesis are summarized in Table 3. Table 4 summarizes the mouse strains used in experiments and table 5 shows the collection of primers utilized in different genotyping PCR reactions. More detailed descriptions can be found in the original articles I-III.

Table 3. Methods used in this study.

Method	Original publication
Cloning of the construts	II,III
Cryosectioning	III
Hematoxylin Eosin staining	I-III
Immunohistochemistry	I
LacZ staining	I,II
Light and immunofluorescence microscopy and analysis	
Mouse breeding and PCR genotyping	I-III
Organ culture	I-III
RNA isolation and Quantitative real-time PCR	I,II
Section in situ hybridization	III
Time-lapse video movies and analysis	II
Whole mount in situ hybridization	II

Table 4. Mouse strains used in thesis experiments.

Mouse strain	generated	used in
Cre-ER Tm	Vooijs <i>et al.</i> 2001	I
ROSA26LacZ	Soriano 1999	I
Wnt4 ^{+/EGFP^{Cre}}	EGFP Cre fusion protein and neo cassette was targeted in to the first exon of the Wnt4 locus	I, II
ROSA26YFP	Shrinivas <i>et al.</i> 2001	II
floxed Wnt4	loxP-sites were placed to flank exons three to five	III
MeuCre40	Leneuve <i>et al.</i> 2003	III
CAGCre	Sakai & Miyazaki 1997	III

Table 5. PCR-primers.

Primer	Sequence
Cre1	5'GCACGTTACCCGCATCAAC3'
Cre2	5'CGATGCAACGAGTGATGAGGTTTC3'
ROSA26LacZ-1	5'AAAGTCGCTCTGAGTTGTTAT3'
ROSA26LacZ-2	5'GCGAAGAGTTTGCCTCAACC3'
ROSA26LacZ-3	5'GGAGCGGGAGAAATGGATATG3'
ROSAYFP-1	5' AAAGTCGCTCTGAGTTGTTAT3'
ROSAYFP-2	5' GCGAAGAGTTTGCCTCAACC3'
ROSAYFP-3	5' GGAGCGGGAGAAATGGATATG3'
Wnt4-flox1	5'TAGGTCTCAGAATCCAAGGT3'
Wnt4-flox2	5'GGTTCTTGACAGTCTGGCTG3'
Wnt4-flox(null)	5'AGCTCTTGCCACCAAGGAT3'
Wnt4-L (qRT-PCR)	5'CTGGAGAAGTGTGGCTGTGA3'
Wnt4-R (qRT-PCR)	5'GGACGTCCACAAAGGACTGT3'

5 Results

5.1 4OH-TM at the concentration of 0.5 μ M effectively induces the Cre-mediated recombination and activates LacZ reporter in a dose- and stage-dependent manner (I)

The tissue interactions in the developing metanephros have been studied in assays that take advantage of the classic experimental methods of embryology, the organ culture (Grobstein 1956a, Grobstein 1956b, Saxen 1987, Saxen & Lehtonen 1987). Earlier research has indicated that tamoxifen-inducible Cre-mediated recombination can be used successfully *in vivo* and in the cell culture models (Danielian *et al.* 1998, Vooijs *et al.* 2001, Hayashi & McMahon 2002, Bosenberg *et al.* 2006, Nakamura *et al.* 2006). Therefore, we wanted to examine whether the TM-inducible Cre recombination approach could be used effectively *in vitro* in kidney culture settings.

The dose-dependent induction of 4OH-TM was first studied in kidney culture assays. β -galactosidase enzyme expression which could be visualized by X-gal staining was the read-out for the successful 4OH-TM-induced Cre-mediated recombination. Low concentrations of 4OH-TM were not effective; instead, 0.5 μ M 4OH-TM combined to 24 hours of exposure showed the most effective recombination in the E11.5 kidney explants (Fig. 1 in I). Before proceeding to further experiments, we wanted to make sure that 0.5 μ M 4OH-TM was not toxic to the kidney explants *in vitro*. With Pax2 antibody immunostaining, we demonstrated that the degree of ureteric bud branching and nephron precursor development was initiated and morphogenesis of the kidney explants was not changed (Fig 2. A-D in I). Based on the above-mentioned results, 24-h exposure and 0.5- μ M concentration were chosen for the coming experiments.

Since 4OH-TM induced recombination was accomplished *in vitro* with E11.5 kidney explants, the stage-dependent induction was examined next. We found that when applying the 4OH-TM to the kidney explants, which were dissected from later phases of kidney development, the induction capacity was reduced. We hypothesized that this was due to the incomplete capacity of the inducer, 4OH-TM, to migrate through the thicker cortex of the more developed kidney explants. To address in more detail the potential incapacity of the 4OH-TM to induce recombination hanging drop assays were used as cultivating methods. However, this did not improve the induction efficiency (Fig. 3 in I).

After specifying the effectiveness of 4OH-TM Cre-mediated recombination in the whole kidney *in vitro*, we also analyzed whether recombination could be applied in the classical tissue culture system. For this, E11.5 mesenchymes were cultured with 0.5 μ M 4OH-TM for 48 h and with spinal cord, the heterologous inducer of kidney tubulogenesis (Saxen and Lehtonen 1987). Under such settings activation of the LacZ gene was accomplished, since the mesenchymes were stained for the LacZ. But when the mesenchymes were sectioned, it became evident that not all the cells were LacZ positive (Fig. 4A–D in I).

We next sought out if more effective recombination could be achieved if the competence of the mesenchyme for tubulogenesis were preserved for several hours by culturing the kidneys in the presence of Bmp7 and Fgf2 (Dudley *et al.* 1999). By preventing apoptosis of the kidney mesenchyme, these factors extend the time available for 4OH-TM induced recombination prior to nephron induction. In this setting, the mesenchymes showed significant recombination of the LacZ gene in the developing nephrons, which expressed the Pax2 protein (Fig. 4 E, F in I).

In conclusion, we identified the settings for the effective recombination of the reporter gene *in vitro* in kidney culture settings.

5.2 $Wnt4^{+/EGFPCre}$ and $Wnt4$ floxed mouse lines are useful new tools for conditional mutagenesis (II, III)

Wnt4 gene function has been previously studied in the conventional *Wnt4* mutant mice. In these mice, defective *Wnt4* signaling leads to impaired kidney development and these mice die soon after birth. We generated two *Wnt4* mouse lines, the $Wnt4^{+/EGFPCre}$ allele and $Wnt4^{lox/lox}$ for conditional inactivation of the *Wnt4* gene and for addressing the roles of *Wnt4* function. In $Wnt4^{EGFPCre}$ allele, the *Wnt4*-promoter activates the expression of the Cre-recombinase and the EGFP protein in the organs or cells where *Wnt4* gene is expressed. By using the *Rosa26LacZ* line, we verified that LacZ staining was observed in the organs where *Wnt4* function has been reported to be active, namely kidney, lung, adrenal gland, female and male gonads and spinal cord (Fig. 2 in II). Further examination during kidney development revealed that the $Wnt4^{+/EGFPCre}$ allele recapitulated the expression pattern of the exogenous *Wnt4* gene (Fig. 3 and 4 in II).

In the floxed *Wnt* allele, two loxP sites were targeted to flank exons three to five. Upon Cre-mediated recombination floxed *Wnt4* mouse should have the same phenotype as the conventional *Wnt4* mutant mice, which possess the same

modification of the *Wnt4* gene. *Wnt4* gene activity was inactivated conditionally by using the *Wnt4*^{+/*EGFP*Cre} mouse line. We observed the same phenotype that has been previously reported in the conventional *Wnt* mutant mice (Stark *et al.* 1994). (Fig 3 A and B in III, Fig. 3 E and F in III). Histological analysis of the conditional mutant kidneys demonstrated that the mutant kidneys remained for the most part morphologically undifferentiated, although some nephron structures were rarely detected (Fig. 3 C, D in III). Similar observations have been made with the conventional *Wnt4* mutant mice.

We confirmed that the *Wnt4*^{*EGFP*Cre/*c*} mutants do indeed lead to a loss of *Wnt4* expression. QRT-PCR analysis demonstrated that at E14.5, there were no major changes in mRNA levels between wild-type, *Wnt4*^{*c*/*+*} and *Wnt4*^{*c*/*c*} kidneys, while *Wnt4* expression was severely diminished in *Wnt4*^{*EGFP*Cre/*c*} mutants (Fig. 4C in III).

Taking together all mentioned above, we demonstrated that *Wnt4*^{+/*EGFP*Cre} and *Wnt4* floxed mouse lines are useful new tools for conditional mutagenesis and can be used to address the role of Wnt4 function and signaling in research.

5.3 *Wnt4* gene contributes to the nephrons, some of the cells around the stalk of the developing ureter and also certain presumptive medullary stromal cells (II)

We used the embryos from the crosses of *Wnt4*^{+/*EGFP*Cre} males with *ROSA26YFP* reporter females (Shrinivas *et al.* 2001) to set up time-lapse movies of the embryonic kidneys in organ culture in order to analyze the behaviour of the pretubular cells that express or have expressed *Wnt4* gene. Such an approach allowed detailed visualization of the fate of pretubular cells from cultured kidneys prepared at E11.5, E12.5 or E13.5 (Suppl. movies 2–4 in II). Analysis of the movies made with the *Wnt4*^{+/*EGFP*Cre}: *Rosa26YFP* kidneys revealed how the *Wnt4*^{*EGFP*Cre}-marked YFP cells generated the nephron structures through kidney organogenesis (Suppl. fig. 1 in II: compare D and F to C).

Besides forming the nephron structures, the first few YFP-positive pretubular mesenchymal cell aggregated in some movies developed for a while towards nephron-like structure but these structures then appeared to disintegrate (Supplementary movies 2 and 3 in II, white arrows). New YFP-positive cells came out in the central part of the kidney in connection with the disintegration process and moreover became localized between the developing tubules and the branches of the ureteric tree (Suppl. Fig. 2 C and D, arrows). *Wnt4*^{*EGFP*Cre}-marked

YFP-positive cells were detected in the areas that fused to the stalk of the developing ureter (Suppl. movies 2–4 in II, see Suppl. fig. 2A–D in II, arrows).

In conclusion, we demonstrated that most of the nephron-associated cell lineages were derived from cells that had expressed the *Wnt4* gene.

6 Discussion

6.1 Induction of 0.5 μ M 4OH-TM for 24 hours is efficient for wide-spread recombination of LacZ reporter in kidney and mesenchyme cultures from E11.5 embryos (I)

Ubiquitously expressed *Cre-ER*TM mouse lines have been used effectively in cell culture, embryos and in adult mice. On the other hand, applying TM-inducible Cre/loxP-system in tissue cultures needs further investigation.

For the tissue culture applications metanephric kidneys between E11.5 and E12.5 were chosen for the experiments, because at that time the formation of the kidney has reached the point where the metanephric kidneys can be easily detected and dissected from the existing embryos. In addition, at this point most of the important genes involving kidney development have already started or are starting to be expressed. This is an essential fact to consider in situations where Cre-mediated recombination is used to study the inactivation of the functional genes in kidney development.

It has been published by several investigators that when using the *Cre-ER*TM mouse line both the synthetic ligands TM and 4OH-TM can be utilized for the activation of Cre-mediated recombination. TM has usually been the form for the *in vivo* conditions and it is known to be pricier and more toxic than 4OH-TM. 4OH-TM has mostly been used for the *in vitro* experiments, although Starnes *et al.* (2007) and Indra *et al.* (1999) have shown that 4OH-TM can also be used *in vivo*. Borgna and Rochefort (1981) have demonstrated that *in vivo* tamoxifen is hydroxylated to the primary active metabolites 3.4-dihydroxytamoxifen and 4OH-TM, the latter being about 100-fold more powerful as an antiestrogen than tamoxifen. Taking these observations into consideration, 4OH-TM was chosen for the experiments.

The timing for the 4OH-TM-induced recombination was 24 or 48 hours. This was selected according to the earlier studies (Hayashi & McMahon 2002, Voojjs *et al.* 2001) and most significantly due to the fact that TM- or 4OH-TM-inducible Cre-mediated excision takes time. Thus, the total delay between the induced Cre-mediated recombination and the actual effect examined in the whole organism or tissues can vary between several hours or days (Nagy 2000, Hayashi & McMahon 2002, Nakamura *et al.* 2006).

The 4OH-TM induction illustrated dose-dependent recombination between 1 nM to 10 μ M of 4OH-TM, with higher doses giving higher degrees of recombination. The recombination was not efficient at lower concentrations (1 nM and 10 nM), which can implicate the situation where filter settings used in this study may diminish the penetration of the 4OH-TM to the tissues and 4OH-TM is under the threshold for activation of Cre-ERTM. 0.5 μ M and 2 μ M were able to completely activate the recombination, which was observed as 100% recombination efficiency by the LacZ-derived staining in the kidney explants. Over 10- μ M concentrations of 4OH-TM led to complete apoptosis of the cultured kidneys. Thus, the recombination efficiency has to be weighed against the toxicity of high concentrations of TM or 4OH-TM. Other investigators (Danielian *et al.* 1998, Hayashi & McMahon 2002) have reported that TM at higher concentrations is toxic *in vivo* and when injected to pregnant mothers lead to death of the unborn embryos. Starnes *et al.* (2007) verified that 4OH-TM administration to wild-type mouse strain C57BL/6J at a dose typical of those used to activate Cre-ER fusion protein activity in transgenic lines (Indra *et al.* 1999) leads to outstanding microarchitectural alterations in femoral bones of intact male and female mice. It has been shown that tamoxifen stimulates osteoblast proliferation and differentiation *in vitro* (Qu *et al.* 1999) and that estrogen and androgen receptors are also present in chondrocytes, bone marrow stromal cells, as well as osteoclasts and their progenitors (Bellido *et al.* 1995, Benz *et al.* 1991, Couse & Korach 1999, Eriksen *et al.* 1999, Komm *et al.* 1988). Thus, in this way tamoxifen might act as an agonist of bone formation *in vivo* (Starnes *et al.* 2007).

Several experiments have also verified the toxicity of the Cre recombinase itself. Studies on cultured cells lacking exogenous loxP sites have shown that Cre recombinase can cause decreased growth and DNA damage (Loonstra *et al.* 2001). However, not much proof has been provided for a Cre-induced phenotype *in vivo*, besides reports showing a Cre-related phenotype in mice where high levels of Cre expression in neuronal progenitors caused deficiency in brain development (Forni *et al.* 2005) and a transgenic mouse line in which male sterility was shown to occur (Schmidt *et al.* 2000). Originally, the expression of Cre recombinase in mice was expected to be innocuous because it was thought that the mouse genome would not have endogenous loxP sites. However, the above-mentioned studies (Loonstra *et al.* 2001, Forni *et al.* 2005, Schmidt *et al.* 2000) demonstrate that so-called pseudo or cryptic loxP sites exist also in the mouse genome and that Cre-mediated recombination is possible through these sites. However, the widespread expression of Cre in many other transgenic mouse

lines, including both male and female germ lines, demonstrates the rare occurrence of such interactions *in vivo*. Thus, Cre-mediated recombination can be securely used to address the functions of genes in developmental processes.

In this study, the LacZ activation was not effective on the kidneys dissected from E13.5 or older embryos and cultured on the filter settings. The penetration of the 4OH-TM through the kidney cortex might be lowered on these settings. Using hanging drop technique this problem was partly solved. It cannot be excluded that the chosen 24-hour culturing time might not be sufficient to the recombination to take place. Indeed, earlier studies have demonstrated that prolonging the time to 48 instead of 24 hours leads to more efficient recombination when the tamoxifen concentration was not changed (Hayashi & McMahon 2002).

On the mesenchyme cultures supplemented with Bmp7 and Fgf2 (Dudley *et al.* 1999), three different observations were made. Firstly, after 24 hours of culturing with these growth factors, mesenchymes still appeared as a soft tissue mass. There were no signs of induction in the mesenchymes, demonstrating that the growth factors did not trigger nephrogenesis. Also, the LacZ activation was not initiated by these factors. Secondly, 4OH-TM-inducible Cre recombination was achieved only if the growth factors were used to pretreat the mesenchymes prior to the 4OH-TM treatment. If 4OH-TM was added jointly with growth factors to the mesenchyme cultures in the beginning, apoptosis of the cultures was observed (data not shown). This may imply that the 0.5 μM concentration of the 4OH-TM was too high for the delicate structure of the mesenchyme and that a short 6- to 9-hour pretreatment with growth factors is needed to prevent apoptosis (Dudley *et al.* 1999). Thirdly, the use of 1.0 Nucleopore filter did not diminish the effect of induction or tubulogenesis. This is in line with the observations made by Kispert *et al.* (1998) when using different pore size filters on trans-filter cultures to trigger tubulogenesis. Also in our experiments, the cultivation time after 4OH-TM induction was 96 hours, prolonging the time for effective induction to take place as well as tubulogenesis to happen.

In general and taking all the results observed together, one can propose that 4OH-TM-induced Cre-mediated recombination can be used to study kidney development *in vitro* and can now be applied to study the genes important for kidney development. Good candidates would be the created floxed *Wnt4* allele as well as floxed *Fgf8*, both being important players in nephrogenesis (Stark *et al.* 1994, Kispert *et al.* 1998, Perantoni *et al.* 2005).

6.2 $Wnt4^{+/EGFP\text{Cre}}$ and $Wnt4$ floxed mouse lines are useful for studying $Wnt4$ expression in diverse tissues and at different time points during development (II, III)

To have a better understanding of the $Wnt4$ function in various organs and at different developmental stages, we generated $Wnt4^{+/EGFP\text{Cre}}$ and floxed $Wnt4$ mouse lines. In the $Wnt4^{+/EGFP\text{Cre}}$ knock-in line the EGFP Cre fusion *cDNA* and Neo selection cassette were targeted into the $Wnt4$ locus. This knock-in allele preserves only one fully functional $Wnt4$ allele operating, because the targeting construct replaces the ATG-codon and the first exon of the endogenous $Wnt4$ gene with the EGFP Cre fusion *cDNA* and Neo selection cassette. Despite this, $Wnt4^{+/EGFP\text{Cre}}$ heterozygous mice were normal, fertile and did not show any kidney defects. Similar observations have been made with the conventional $Wnt4$ heterozygous mice (Stark *et al.* 1994). Therefore, the $Wnt4^{+/EGFP\text{Cre}}$ line can be used effectively in experiments, for example when addressing the roles of particular genes in kidney development.

Characterization of the $Wnt4^{+/EGFP\text{Cre}}$ line showed EGFP-derived fluorescence and $Wnt4^{EGFP\text{Cre}}$ -marked Rosa26LacZ recombination in the pretubular aggregates of the E14.5 kidney. In addition to this, LacZ staining was seen in the mesonephros, lung, adrenal gland, some compartments of testis and ovary and spinal cord. All above-mentioned are organs where the $Wnt4$ gene is either expressed or is necessary for organogenesis (Gavin *et al.* 1990, Parr *et al.* 1993, Vainio *et al.* 1999). Time-lapse analysis with the Cre reporter YFP revealed that the $Wnt4$ gene acts to form the nephron structures. This result is similar to the idea that $Wnt4$ acts as an auto-inducer of nephrogenesis once activated (Stark *et al.* 1994, Kispert *et al.* 1998). Besides contributing to the nephrons, the first few presumptive nephrons may have also some other fates. In some of the time-lapse movies the early pretubular mesenchymal cell aggregates developed for a while to form early structures resembling nephrons that then disintegrated. It might be that the first few developing nephrons are in fact vestigial, so that they do not develop beyond a certain stage and represent temporary components in the developing kidney (Kampmeiner 1926).

In a floxed $Wnt4$ allele, two *loxP* sites were targeted to flank exons 3 to 5. The $Wnt4$ gene was inactivated with *CAGCre* and $Wnt4^{+/EGFP\text{Cre}}$, both of which led to severe kidney defects, similar to the conventional $Wnt4$ knockout (Stark *et al.* 1994). In addition and similar to $Wnt4^{-/-}$, the conditional inactivation with

Wnt4^{+/*EGFP**Cre*} caused the female embryos to undergo partial sex reversal (Vainio *et al.* 1999, Heikkila *et al.* 2005, Naillat *et al.* 2010).

Wnt4^{+/*EGFP**Cre*} and *Wnt4*^{lox/lox} mouse lines have also been successfully used by other investigators. Weyer *et al.* (2011) created megalin- and/or cubilin-deficient mice using conditional Cre-loxP approach and *Wnt4*^{+/*EGFP**Cre*} line. Murugan *et al.* (2012) activated conditionally Lodavin gene expression (an avidin-tagged LDL receptor) with the *Wnt4*^{+/*EGFP**Cre*}. Heliot *et al.* (2013) addressed the function of the *Hnf1b* during nephrogenesis by conditionally inactivating this gene with *Wnt4*^{+/*EGFP**Cre*} in mouse nephron precursors, which led to defects in nephron segmentation and patterning. Floxed *Wnt4* line has been used to address the role of *Wnt4* in postnatal and adult thymopoiesis (Heinonen *et al.* 2011).

After we reported the *Wnt4*^{lox/lox} line, two additional floxed *Wnt4* mouse lines were created by other investigators (Boyer *et al.* 2010, Kobayashi *et al.* 2011). Boyer *et al.* (2010) studied the roles of *Wnt4* in the ovarian development. They also showed that deletion of *Wnt4* with *CMV-Cre* line led to the same kidney defects as observed with our floxed *Wnt4* line. This result is also in line with the phenotype observed in the conventional *Wnt4*^{-/-} line (Kispert *et al.* 1998). Kobayashi *et al.* 2011 used their line to study sexual differentiation aspects. They crossed their line with ubiquitously expressing Cre-line (Tallquist & Soriano 2000), and reported that the outcome was the same as in the *Wnt4*^{-/-} mice even though the data were not shown (Stark *et al.* 1994, Vainio *et al.* 1999). Furthermore, the development of *Wnt4-GFP-Cre* (*Wnt4*^{GC/+}) mouse line corresponding to the *Wnt4*^{+/*EGFP**Cre*} presented in this thesis has been mentioned (Mugford *et al.* 2009), even though it has not yet been fully characterized or used in research.

In conclusion, we have shown that the created *Wnt4*^{+/*EGFP**Cre*} and *Wnt4*^{lox/lox} mouse lines are functional and effective. In addition, these two mouse lines have already been distributed to the research community and used successively for conditional gene targeting by other investigators.

7 Conclusions and future perspectives

This thesis presented results from studies in which 4OH-TM-induced Cre-mediated recombination was examined in tissue culturing settings. Furthermore, this thesis presented generation and characterization of two novel *Wnt4* mouse lines for the conditional gene targeting.

The first part of the project revealed that 4OH-TM -induced conditional gene activation of the reporter gene can be effectively accomplished *in vitro* in organ culture settings. Recombination efficiency near to 100% can be achieved in kidney explants and in kidney mesenchyme cultures, when proper concentration of 4OH-TM and induction time are used. In the future, culture conditions developed in this study will be valuable tools for studying the roles of various genes important for kidney organogenesis.

The second and third part of the study revealed that *Wnt4* gene function can be effectively recombined by means of conditional mutagenesis. The loss of *Wnt4* function leads to impaired kidney and gonad development, thus showing that the created *Wnt4* mouse lines are functional and effective. In the future, these *Wnt4* mouse lines will serve as useful new tools for addressing the role of *Wnt4* gene function in different developmental processes.

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Original articles

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- II Shan J, Jokela T, Skovorodkin I & Vainio S (2010) Mapping of the fate of cell lineages generated from cells that express the Wnt4 gene by timelapse during kidney development. *Differentiation* 79(1): 57–64.
- III Shan J, Jokela T & Vainio S (2009) Generation of an allele to inactivate Wnt4 gene function conditionally in the mouse. *Genesis* 47(11): 782–788.

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ISBN 978-952-62-0154-2 (Paperback)

ISBN 978-952-62-0155-9 (PDF)

ISSN 0355-3221 (Print)

ISSN 1796-2234 (Online)

