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Sanna Junttila

STUDIES OF KIDNEY INDUCTION *IN VITRO* USING GENE EXPRESSION PROFILING AND NOVEL TISSUE MANIPULATION TECHNIQUES

UNIVERSITY OF OULU GRADUATE SCHOOL;
UNIVERSITY OF OULU,
FACULTY OF BIOCHEMISTRY AND MOLECULAR MEDICINE;
BIOCENTER OULU;
CENTER FOR CELL-MATRIX RESEARCH

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Supervised by
Professor Seppo Vainio

Reviewed by
Docent Sanna Lehtonen
Docent Satu Kuurre

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Abstract

For decades, the mammalian kidney has served as a model system for studying developmental processes, such as induced epithelialization, branching morphogenesis, and cell differentiations. The possibility to recapitulate and follow the renal organogenesis ex vivo in organ culture set-ups has provided a large amount of molecular and cellular information about sequential events during development. However, certain limitations remain when combining traditional organ culture set-ups with modern molecular technology. This thesis seeks to address these disadvantages.

In the experimental part of the thesis, the traditional organ culture set-ups were studied, modified, and optimized to meet the needs of functional genetic screening. First, the traditional transfilter- induced nephrogenesis was characterized with a panel of nephron segment specific markers to reveal the differentiation level of *in vitro* developing mouse renal tissue. A comprehensive genome wide time course microarray analysis was also performed to *in vitro*-induced metanephric mesenchyme.

Next, to improve the accessibility of genetic tools into the three- dimensional organ in culture, the classic kidney culture set-ups were modified to tolerate dissociation and re-aggregation before the induction of nephrogenesis. This step was achieved with the aid of preservative growth factors offering a 24- hour window to manipulate the genetic and cellular composition of the explant. The dissociation and re-aggregation per se had not particular effect on the progress of the nephron differentiation. Demonstrations of the addition and removal of cells, as well as a virus vector mediated gene knock in and knock down are presented.

The gene expression data, together with the novel organ manipulation and culture techniques presented in this thesis, provide a useful guide and specific tools to further characterize the details of nephron development and differentiation in functional manner.

Keywords: kidney development, microarray, nephrogenesis, organ culture

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Tiivistelmä

Nisäkkäiden munuainen on toiminut vuosikymmeniä mallielimenä tutkittaessa kehitysbiologisia tapahtumasarjoja, kuten epitelisaatiota, haaroittumismorfologiaa sekä solujen erilaistumista. Munuaisaihioita voidaan viljellä laboratorio-olosuhteissa, jolloin kehityksen aikaisia muutoksia päästään seuraamaan lähes reaaliaikaisesti. Perinteisten kudosviljelytekniikoiden tarjoamat mahdollisuudet solujen molekulaariseen muokkaukseen ovat kuitenkin varsin rajalliset. Tässä väitöskirjassa esitettävät tulokset pyrkivät osaltaan vähentämään näitä rajoitteita.

Väitöskirjan kokeellisessa osassa tarkastellaan lähemmin klassista munuaiskudosviljelyä sekä esitetään siihen tehtyjä optimointeja, joiden avulla kudosviljelyä pyritään hyödyntämään geenien toiminnan tutkimuksessa. Aluksi perinteisellä tavalla reikäisen kalvon läpi indusoitu nefroni karakterisoitiin tarkasti hyödyntäen useita erilaistumista osoittavia merkkimolekyylejä. Lisäksi samalla tekniikalla tuotettujen munuaiskudosviljelmien geeniekspressiota tutkittiin mikrosiruanalyysillä.

Klassisia kudosviljelytekniikoita muokattiin soveltuvammaksi moderneille geneettisille työkaluille. Munuaiskudos hajotettiin ensin solususpensioksi, jonka jälkeen solut muodostivat uudelleen kolmiulotteisen, kudosmaisen rakenteen. Hyödyntämällä suojaavia kasvutekijöitä, hajotus kyettiin tekemään jo ennen nefronien muodostumisen alkua. Näin saavutettin 24 tunnin aikaikkuna indusoimattoman kudoksen geneettiselle muokkaukselle. Väitöskirjassa esitelläänkin demonsrtaatiot solujen lisäämisestä ja poistamisesta sekä virusvälitteisestä geenin aktivoinnista ja hiljennyksestä hyödyntäen uutta kudosmanipulaatio ja –vilejelytekniikkaa.

Nefronin kehityksen aikaisen geeniekspression kartoitus sekä tässä tutkimuksessa kehitetyt uudet kudosmanipulaatio ja -viljelytekniikat tarjoavat yhdessä työkaluja molekyylitason yksityiskohtaiseen tutkimiseen.

Asiasanat: geeniekspressio, kudosviljely, munuaisen kehitys, nefroni

To my family

"Tutkija syntyy, kuten mikä biologinen olio tahansa, perinnöllisten tekijäin ja ympäristön yhteisvaikutusten summana. Perintötekijöistä tärkein on kohtalaisen älyn ohella jokaisessa ihmisessä asuva uteliaisuus, jonka selvimmin ja pelkistettynä tapaa lapsessa.

– –

Jos riittävään älyyn ja kehittyneeseen uteliaisuuteen vielä liittyy ripaus itsetuntoa, kunnianhimoa ja kilpailumieltä sekä luovaa mielikuvitusta, on tutkijan verso hyvin varustettu ympäristön hioville vaikutuksille."

-Lauri Saxén, 2000

A researcher is born, like any biological creature, as a sum of interactions between inheritable factors and the environment. The most important inheritable factor, in addition to moderate intelligence, is the curiosity in human beings, which is most apparent and simplified in a child.

– –

If adequate intelligence and sophisticated curiosity is combined with a pinch of self-esteem, ambition, competitive spirit, and creative imagination, a young researcher is well-equipped to the polishing influence of the environment.

-Free translation retelling Lauri Saxén, 2000

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My deepest, everlasting gratitude goes to my fiancé Markus; without you I wouldn’t have succeded. I needed this, and you knew it, after all. Finally, for my adored kids, Pihla and Joonas: *kiitos ja anteeksi.*

Oulu, Finland, October 2014 *Sanna Junttila*

Abbreviations

Ankrd6	Ankyrin repeat domain 6
AQ1	Aquaporin 1
BMP7	Bone morphogenetic protein 7
Brn1	Brain-specific homeobox/POU domain protein 1
Ca^{2+}	Calsium
Camk2γ	Calcium/calmodulin-dependent protein kinase II gamma
CD	Collecting duct
CD24	Small cell lung carcinoma cluster 4 antigen
CD31	CD31 antigen, see PECAM-1
Celsr2	Cadherin, EGF LAG seven-pass G-type receptor 2
Cited1	Cbp/P300-Interacting Transactivator, With Glu/Asp-Rich Carboxy-Terminal Domain, 1
ClcnKb	Chloride Channel, Voltage-Sensitive Kb
CM	Cap mesenchyme
cMyc	V-Myc Avian Myelocytomatosis Viral Oncogene Homolog
Cre	Causes recombination/Cyclization recombinase
DCT	Distal convoluted tubule
Dis/re	Dissociated and reaggregated
Dll1	Delta-like 1
dMM	Dissociated metanephric mesenchyme
DNA	Deoxyribonucleic acid
dpp	Days post partum
drMM	Dissociated and reaggregated metanephric mesenchyme
DTL	Descending thin limb of Henle's loop
Dvl2	Dishevelled segment polarity protein 2 E11.5 etc.Embryonic day 11.5 etc.
EGFP	Enhanced Green fluorescent protein
Emx2	Empty Spiracles Homeobox 2
ES	Embryonic stem
eSC	Embryonic spinal cord
Eya1	Eyes Absent Homolog 1
FACS	Fluorescence-activated cell sorting
FGF2, -8	Fibroblast growth factor 2 or 8
FGFrl-1	Fibroblast growth factor receptor -like 1
Foxd1	Forhead box D1
Gata3	GATA Binding Protein 3

GBM	Glomerular basement membrane
GDNF	Glial cell-derived neurotrophic factor
GSK3beta	Glycogen synthase kinase 3 beta
GUDMAP	Genitourinary Database Molecular Anatomy Project
hESC	human embryonic stem cells
hiPSC	human induced pluripotent stem cells
Hoxa11	Homeobox A11
Hoxc11	Homeobox C11
Hoxd11	Homeobox D11
Hoxb7	Homeobox B7
IM	Intermediate mesoderm
iMM	Intact metanephric mesenchym
iPS	Induced pluripotent stem
iPSC	Induced pluripotent stem cells
Jag1	Jagged 1
JNK	JUN N-Terminal Kinase
Jun	*Jun* proto-oncogene
Klf4	Kruppel-Like Factor 1
Ldb1	LIM Domain Binding 1
Lef	Lymphoid enhancer-binding factor 1
Lgals3	Lectin, galactoside-binding, soluble, 3; Galectin-3
Lhx1	LIM homeobox 1
LIF	Leukemia inhibitor factor
LoxP	Locus of X-over P1
MAP	Mitogen-Activated Protein
Map3k7	Mitogen-activated protein kinase kinase kinase 7
Mapk8	Mitogen-activated protein kinase 8
Mapk9	Mitogen-activated protein kinase 9
mESC	Mouse embryonic stem cells
MET	Mesenchymal to epithelial transformation
mK3/4	Mouse kidney 3 or 4 cells
MM	Metanephric mesenchyme
mRNA	Messenger ribonucleic acid
NCC	Sodium-chloride cotransporter, see Slc12a3
Nfatc4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4
Nfix	Nuclear factor I/X (CCAAT-binding transcription factor)

Nlk	Nemo-like kinase
NPHS1	Nephrosis 1, Congenital, Finnish Type (Nephrin)
Oct3/4	Octamer-Binding Protein 3/4, POU Class 5 Homeobox 1
Osr1	Odd-skipped related transciption factor 1
Pax2	Paired box 2
PCP	Planar cell polarity
PCT	Proximal convoluted tubule
Pecam1	Platelet/endothelial cell adhesion molecule 1
Plcb1	Phospholipase C, beta 1 (phosphoinositide-specific)
Podxl	Podocalyxin
Ppp3ca	Protein phosphatase 3, catalytic subunit, alpha isozyme, calcineurin
PTA	Pretubular aggregate
RC	Renal corpuscle
Ret	Ret proto-oncogene or receptor tyrosine kinase Ret
RhoA	Ras homolog family member A
RIMM-18	Rat immortalized metanephric mesenchyma cell line, 18
RNA	Ribonucleic acid
RNAi	RNA interference
Rock1	Rho-associated, coiled-coil containing protein kinase 1
RV	Renal vesicle
Sall1	Spalt-like transcription factor 1
SD	Slit diaphragma
Sdc3	Syndecan 3
shRNA	Short hairpin-RNA
siRNA	Short interfering RNA
Six1/2	SIX homeobox 1 or 2
Slc12a1	Solute carrier family 12 (sodium/potassium/chloride transporter), member 1
Slc12a3	Solute carrier family 12 (sodium/chloride transporter), member 3
Slc34a1	Solute carrier family 34 (type II sodium/phosphate contransporter), member 1
Sox2	SRY (sex determining region Y)-box 2
Tak1	TGF-beta activated kinase 1/MAP3K7 binding protein 1
TAL	Thick ascending limb of Henle's loop
Tcf7	Transcription factor 7 (T-cell specific, HMG-box)
UB	Ureteric bud
Vegfa	Vascular endothelial growth factor A

Wnt4	Wingless-type MMTV integration site family, member 4
Wnt9b	Wingless-type MMTV integration site family, member 9B
WT1	Wilm's tumor 1 homolog

Original articles

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Junttila S, Saarela U, Halt K, Manninen A, Pärssinen H, Lecca RM, Brändli AW, Sims-Lucas S, Skovorodkin I, and Vainio SJ (2014) Functional Genetic Targeting of the Embryonic kidney Progenitor Cells *Ex vivo.* JASN. In press.

II Junttila S*, Halt K*, Myllärinen T, Yan W, and Vainio SJ (2014) Identification of the expression patterns of dynamic genes in kidney mesenchyme induced with embryonic spinal cord. Manuscript.

*Equal contribution

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1 Introduction

An organ is a selection of different tissue types that serve defined functions. Its development employs a sequence of cell differentiation and morphogenesis processes. Many of these complicated processes are shared generally with the development of different organs. According to C.H: Waddington,

> *"The word 'morphogenesis' – – when used strictly should mean the moulding of cells and tissues into definite shapes." (Waddington 1956).*

This 'moulding' occurs through e.g., cell polarization, guided cell migration, inductive epithelial-mesenchymal interactions and branching morphogenesis.

The research in developmental biology utilizes model-systems, and the value of certain model-system arises from the balance of simplicity and complexity – a good model simplifies circumstances, but still sufficiently reflects the phenomenon *in vivo*. The vertebrate kidney has served as a model-system in organogenesis for almost a century. A technical turning point was experienced in the 1950's when Clifford Grobstein established the Trowell-type (Trowell 1954) transfilter culture system for mammalian embryonic kidneys. Now 60 years later, this culture system is utilized in laboratories to reveal the secrets behind kidney development.

The mammalian kidney develops in a spatial and temporal sequence through three different forms: The pronephros, the mesonephros, and the metanephros. In the course of the embryogenesis, the first two forms degenerate or become a part of the reproductive system. The metanephros then forms the permanent secretory organ – the adult kidney. The cellular and molecular mechanisms in charge of moulding the kidney have been extensively investigated. Utilizing both, transgenic mice, the sequencing of the genome and the advances in molecular tools, understanding of these processes has increased enormously. However, there still remains much to reveal.

The development of metanephros is based on a mutual interaction between two progenitor tissues – the epithelial ureteric bud and the metanephric mesenchyme. Both are derivatives of the intermediate mesoderm, but at the onset of metanephric development they are separate until the epithelial counterpart invades the mesenchyme upon inviting signals. The mesenchymal cells trigger the epithelial bud to branch dichotomously in an iterative fashion and eventually this branching tubule forms the collecting duct system of the mature kidney. At the same time, the invading epithelial bud causes condensation of the mesenchymal

cells around its tips. Subsequently, signals from the ureteric bud induce a transformation in a portion of the condensing cells. After a sequence of morphogenetic events, the mesenchymal-to-epithelial transformed cells form all parts of the functioning unit of the adult kidney – the nephron.

Nephron formation – or nephrogenesis – can be followed *in vitro* in organ culture. Since Grobstein's pioneering experiments, it is possible to separate the two counterparts. Both can be cultured alone under certain conditions. For an isolated metanephric mesenchyme, the inductive signals for nephrogenesis can be provided by other inducers instead of the natural inducer, the ureteric bud. One of the most prominent nephrogenic inducers is the embryonic spinal cord. When these two tissues; the metanephric mesenchyme and the embryonic spinal cord, are placed in close contact in a culture, a robust induction of nephron formation takes place. (Grobstein 1953a, Saxén 1987).

This described experimental nephrogenesis set-up is an easy- to- access model-system for developmental studies. However, certain limitations restrict its applications. The metanephric mesenchyme, when uninduced and isolated, rapidly enters apoptosis (Koseki *et al*. 1992, Saxén 1987). Despite numerous trials to establish a stable cell line featuring the metanephric mesenchyme, the experimental nephrogenesis still relies on freshly isolated embryonic tissues. Another limitation in the organ culture system is the limited penetration of manipulative agents (Lee *et al.* 2008).

This thesis focuses on nephrogenesis and particularly its experimental modelling. Through a review of the literature, past and present experimental applications are bound together. The outlines of the nephrogenesis, i.e., the commitment and morphogenesis, are dealt with both at the cellular and molecular level. The existing knowledge is appreciated, and research questions are raised to fill some of the remaining gaps. The experimental part of this thesis particularly answers these questions: (1) Which genes are expressed dynamically during experimental nephrogenesis; and (2) can we study their functions in a cost-effective, but reliable, manner?

2 Review of the literature

2.1 Development of the Metanephros

The mammalian permanent excretory organ – the metanephric kidney – appears at midgestation in mice. It is actually the third and last excretory organ derived from the intermediate mesoderm (IM); pronephros, mesonephros, and metanephros that appear in the cranial to caudal sequence. (Saxén 1987).This thesis focuses on metanephric development and particularly on nephron formation or nephrogenesis.

2.1.1 Patterning and Development of the Kidney Precursors

The IM is a narrow cell population located between the axial and lateral plate mesoderm in the developing embryo. The IM becomes specified, as the cells migrate through the primitive streak during gastrulation (Barak *et al*. 2005). These cells generate the majority of the urogenital system, including the metanephros.

The transcriptional regulator *Osr1* is expressed in the IM cells and identifies the founders of the principal tissue primordia of the metanephric kidney, namely, the epithelial nephric duct and the mesenchymal nephric cord (Mugford *et al*. 2008). The epithelial nephric duct is characterized with a $Pax2^{+}Lhx1^{+}Gata3^{+}$ expression. The duct extends in a cranial to caudal direction and later is referred to as the Wolffian duct. From its most caudal portion, a bud emerges and grows dorsally to form the ureteric bud (UB), which eventually gives rise to the collecting duct (CD) tree of the metanephric kidney. (Bouchard *et al*. 2002, Grote *et al*. 2006, Pedersen *et al*. 2005, Torres *et al*. 1995, Tsang *et al*. 2000). The mesenchymal nephric cord, identified by key transcription factors, including *Osr1, WT1, Hoxa11, Hoxc11, Hoxd11, Sall1, Six1* and *Eya1,* extends parallel to the nephric duct. The most caudal end will form a morphologically distinct region, the metanephric mesenchyme (MM) (Little & McMahon 2012). The MM cells will generate the most of parts of the mature metanephric nephron.

The development of the metanephros is considered to begin when the UB extends into the adjacent MM. The key regulators of the primary UB outgrowth are *GDNF*, as secreted by the MM and the Ret receptor, located at the UB cells. The position and restriction of the budding into a single discrete bud is managed

mainly through a tightly regulated GDNF expression and the sorting of nephric duct cells according to the level of Ret signalling. Cells with high Ret signalling will undergo active rearrangements to form the first ureteric tip at the area where GDNF signalling is allowed in the MM. GDNF/Ret signalling predominantly controls also later the bificuration of the UB, which continues throughout kidney development to form eventually the tree-like collecting system of the kidney. (Reviewed in Costantini & Kopan 2010).

The primary MM is a complex tissue of progenitor cell populations specified already before the UB ingrowth. The identified progenitor populations include at least *Six2*$^{+}$*Cited1*$^{+}$ cap mesenchyme (CM), *Foxd1*$^{+}$ stromal progenitors and vascular progenitors (Kobayashi *et al*. 2008). The first mentioned are the nephron-committed mesenchymal cells that will generate all the segments of the nephron. The *Foxd1*$^{+}$ population will contribute to the nonepithelial renal interstitium and to the glomerular vasculature associated mesangial cells and the non-glomerular associated pericytes (Das *et al*. 2013, Humphreys *et al*. 2010). The vascular progenitors may contribute to the vasculogenesis in glomerulus (Sequeira Lopez & Gomez 2011 and unpublished data of Vainio lab).

2.1.2 Morphogenesis of the Nephron

An Overview of the Nephron Induction

Ureter derived signals induces mesenchymal-to-epithelial transition (MET) in the CM cell population. These cells begin to aggregate to form the so-called pretubular aggregate (PTA) that appears just beneath the tips of UB at the T-branch (Figure 1). The cells in PTA, while undergoing MET will become polarized and form an epithelial renal vesicle (RV) with lumen (Figure 2). Right after the MET, the newly formed RV will fuse with the ureteric tip epithelium to form a continuum of the ureteric tree and the nephron (Georgas *et al*. 2009). The RV is already polarized in the proximal-distal axis with respect to the gene expressions with, the latter being closer to the UB tip and the former more distant. This polarization is then followed with an unwinding of the RV to form so-called comma-shaped and S-shaped bodies (Figure 2). At the stage of the S-shaped body, the lumens of the ureteric tree and the newly formed nephron will connect (Georgas *et al*. 2009). The distal and mid-regions of the S-shaped body will contribute to the connecting tubule and the loop of Henle, while the proximal

segment of the S-shaped body will form the renal corpuscle (RC) and proximal convoluted tubules (PCT) (Figure 2).

In RC, the podocytes and the Bowman's capsule are CM derivatives. A third glomerular component, the capillary loop as well as the supportive mesangial cells are of stromal origin. Podocyte differentiation begins with a columnar epithelial cell and goes on through transformation, wherein the cells acquire some mesenchymal-like characteristics, but remain as an atypical epithelial cell. Podocytes then loose their lateral cell attachments except at their base, and extend themselves as foot processes nearly completely around the capillary loops. Finally, the podocyte cell bodies become independent of each other, but still remain attached through interdigitated foot processes. (Quaggin & Kreidberg 2008). (Figure 2, note the grey cells inside the RC).

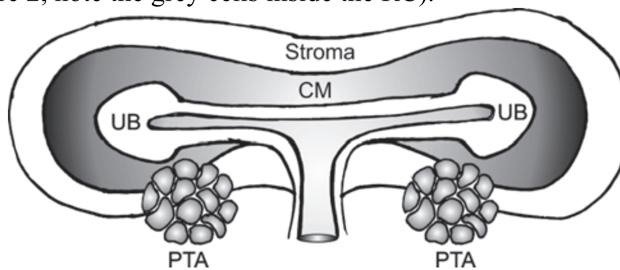

Fig. 1. The ureteric bud (UB) has invaded the metanephric mesenchyme (MM) consisting of the cap mesenchyme (CM) and the stroma. At the armpit of the T-branch, the CM is induced to form the pretubular aggregates (PTA).

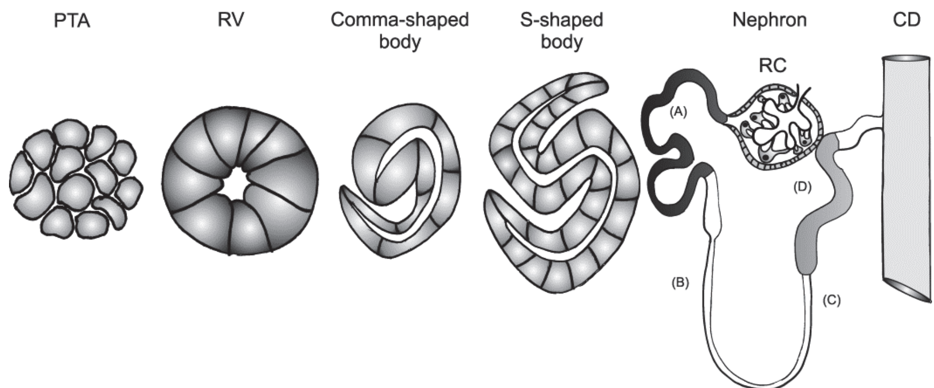

Fig. 2. Stepwise morphogenesis of the nephron. PTA - pretubular aggregate; RV - renal vesicle; RC - renal corpuscle; CD - collecting duct. (A) - proximal convoluted tubule; (B) - descending limb of Henle's loop; (C) - ascending limb of Henle's loop; (D) - distal convoluted tubule.

Glomerular Vascular Development

Two main theories for the renal vasculature development exist,, namely, the angiogenetic and vasculogenetic mechanisms – known as sprouting from existing capillaries and in situ formation of capillaries from progenitor cells in the MM population, respectively. The former is supported by the xenograft experiments where the chick and quail capillaries invade the mouse kidney explant grown on chorio-allantoic membranes (Sariola 1985). The second mechanism is based on experiments showing that the vasculature is genetically related to the early MM and vascular progenitors are found in the MM population at the beginning of the kidney development (Hyink *et al*. 1996, Robert *et al*. 1998). Currently, both mechanisms are thought to contribute to the vascular development of the kidney (Sequeira Lopez & Gomez 2011).

In the proximal cleft of the S-shaped body, the differentiating podocytes begin to express high levels of Vegfa, attracting an angioblast population from the surrounding interstitium to form glomerular capillaries (Eremina *et al*. 2007). The basal laminas of the endothelial cells and podocytes fuse to form a thick basement membrane called the glomerular basement membrane (GBM). This membrane barrier divides the glomerular space into two compartments, an inner containing the capillaries and mesangial cells, and an outer containing the podocytes and the space into which the filtrate passes. (Quaggin & Kreidberg 2008).

Mesangial cells are thought to be of stromal origin and are found adjacent to the endothelial cells as a nexus at the base of the capillary network. Their role is possibly to help maintain the structure of the capillary loops and perhaps help the vasculature respond to various physical stimuli. (Yamanaka 1988).

Patterning of the Loop of Henle

The loop of Henle differs from other portions of the nephron; it is a long, straight tubular segment that rests parallel to the orientation of the CD. While other portions, the glomerulus and distal and proximal tubules are situated in the cortex, the loop of Henle reaches from the cortex or medulla into the papilla of the kidney (Figure 3). (Little *et al*. 2010). It has been suggested that CD derived Wnt9b play a role in the patterning of the loop of Henle. This promotion of convergent extension would ensure thinning and elongation of the tubule alongside the CD. (Karner *et al*. 2009, Lienkamp *et al*. 2012).

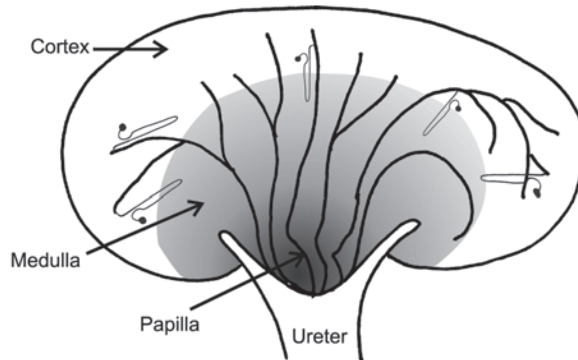

Fig. 3. A schematics of the kidney 3D architecture. The induction of new nephrons takes place in the cortex, and collecting duct branching proceed from center to out. Thus, patterning the organ. All the glomeruli and proximal- and distal tubules are located in the cortex whereas some of the loops of Henle and collecting ducts extend from the cortex to the medulla and papilla where the collecting ducts drain into the ureter.

2.1.3 Cessation of nephrogenesis

After primary budding and invation of the MM, the UB undergoes around ten generations of repeated branching. This process is followed by CD elongation and fewer branching to ultimately form the collecting system of the kidney. The branched ureteric tree remains connected to the nephric duct, which is left outside the kidney and later is connected to the bladder. The branching number, pattern, and elongation extent together determine the kidney size and shape. (Cebrian *et al*. 2004, Costantini & Kopan 2010, Little *et al*. 2010, Srinivas *et al*. 1999).

The CM serves as a progenitor population wherefrom the UB induces nephrons. However, CM exists only transiently. In humans, at around 36 weeks gestation and in mice in the first few days after birth, the last round of MET is initiated (Hartman *et al*. 2007, Rumballe *et al*. 2011). Afterwards, all *Six2*$^{+}$ cells are lost, and no new nephrons form. The mechanisms behind this phenomenon remain unknown. However, two models have been speculated. The first involves an active trigger ending the CM self-renewal and commitment of the rest of the CM cells for differentiation. In the other model, a gradual depletion of CM self-renewing population leads to loss of the true progenitors. Both models may involve changes in gene expression profiles of either the self-renewing cells them-selves or the adjacent niche surrounding the CM. (Little *et al*. 2010).

2.1.4 Nephron Maturation to Functioning Unit

When the last wave of nephrogenesis is over, subsequent tubular elongation and maturation occurs in the proximal tubules, the loop of Henle and CDs. Although the permanent kidney begins to produce urine during its development (in mice around E15 and in humans at 10 weeks gestation) it might take several weeks following birth until the kidney functions properly to maintain homeostasis (Rabinowitz *et al*. 1989). The fetal or embryonic urine, however, plays an important role in the formation of amniotic fluid, which is necessary for symmetrical embryonic growth and lung development (Moritz & Wintour 1999).

The processes of induction, morphogenesis, and differentiation of the metanephros take place in the cortex, while the ureter is branching outwards. Thus, the kidney is growing from the outermost cortex, but maturing inwards. During the developmental process, the kidney gains a three-dimensional architectural structure, which also creates different functional zones in the kidney (Figure 3). The outermost zone – the cortex – consists of proximal parts of the nephron, namely, the RC with glomerulus and the PCT. This part takes care of glomerular filtration and the subsequent reabsorption of filtrated sodium, chloride, water, bicarbonate, glucose, amino acids, potassium, phosphate and calcium. In addition to these, PCT takes care of the secretion of organic ions, such as uric acid, and acts as a site for ammonia production. A distal convoluted tubule (DCT) is also located in the cortical zone and acts as a site for reabsorption of sodium chloride (NaCl) and regulation of calcium excretory. Below the cortex is the medullary zone with loops of Henle. Thin descending limbs are full of water channels making them water permeable, while the thin ascending limbs are water impermeable. Thus, loops of Henle maintain the hypertonic plasma in the medullary interstitium. The CD system reaches from cortex through the medulla until the papilla and, of course, outside the kidney. In the CD, urine is effectively concentrated prior to entering the renal pelvis and eventually the bladder via the ureter (Figure 3). (Satlin *et al*. in Vize *et al*. 2002)).

Specific nephron segments can be recognized according to their gene expression, in particular, specific ion-channel coding genes. In the mature nephron, at least four distinct segments can be recognized by marker genes. The proximal tubule is characterized by *Slc34a1*$^{+}$ (Magagnin *et al*. 1993), a thick ascending limb the of Henle's loop by *Slc12a1*$^{+}$ (Olives *et al*. 1996) a distal tubule by *Clcn-Kb*$^{+}$ (Kobayashi *et al*. 2001), and a distal convoluted tubule by *Slc12a3*$^{+}$ (Campean *et al*. 2001, Loffing *et al*. 2001).

2.2 Historical steps for *in vitro* study of kidney development

The pioneering kidney *in vitro* cultures were done via chick metanephros almost hundred years ago by Reinhoff (Saxén 1987). The most remarkable milestones in the history of experimental nephrogenesis are reviewed in this chapter.

The *in vitro* study of nephrogenesis got a burst when Clifford Grobstein in 1953 introduced the "transfilter technique" for investigation of cell interactions and cell migration (Grobstein 1953b). In the same year, Grobstein also established the technique for separation of the two principal components of the metanephros – the UB and the MM (Grobstein 1953a). In the transfilter technique, these components were able to interact even when separated from each other by thin porous membrane during the culture (Figure 4). These two methods proved to be particularly advantageous in studies on mechanisms of epithelial-mesenchymal interactions and spread along with Grobstein's students, to several labs around the world, including the Finnish Department of Zoology lead by Professor Sulo Toivonen at the University of Helsinki. The techniques were also adopted, and further modified by Grobstein's former *post doc*, Lauri Saxén, and his colleagues. (Leikola 1989). Later, it was learned that the MM could be transfilter induced with several different heterologous tissues, of which the embryonic dorsal spinal cord (eSC) proved to be the most prominent (Figure 4) (Saxén 1987).

The dynamics and kinetics of the inductive signals were then intensively investigated by applying the transfilter techniques. These studies from the late fifties to the end of the seventies led to the conclusions that the nephrogenic inductive signal, while then molecularly still unknown, would be a cell-cell contact –dependent, permissive and needing about 24 hours in a transfilter set-up to fully trigger the tubulogenesis in an isolated MM (reviewed in (Saxén 1987).

Later, Ekblom and colleagues showed that *in vitro* induction of separated MM with eSC led to the development and partition of the three main segments of the nephron: the glomerular epithelium, proximal tubules with brushed border and distal tubules (Ekblom *et al*. 1981).

The dynamics of induced nephrogenesis has been studied also via dissociation and reaggregation of the rudiment. Here, the separated MM was first induced in a transfilter system with eSC and then dissociated into single cells. After reaggregation and a subsequent culture in hanging drops (Auerbach & Grobstein 1958), the roles of different molecules were followed during the rearrangement and morphogenesis of the nephron (Figure 4) (Vainio *et al*. 1992).

Noticeable was finding that the separated MM had to be induced prior to dissociation to avoid cell death (Saxen *et al*. 1988).

From Grobstein, until today, and into the future – the Trowell-type transfilter organ culture system with whole or separated kidney rudiments, has been applied in conjunction with modern molecular experiments to reveal the mechanisms behind the organogenesis of the kidney.

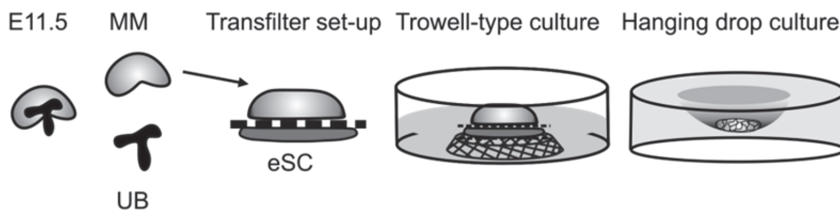

Fig. 4. Shcematic presentation of the classical kidney mesenchyme culture techniques. The sizes of explants are exaggerated for clarity.

2.3 Experimental Nephrogenesis – Present Tools and Possibilities

Since Grobstein, a substantial amount of progress has been made in further deconstructing renal morphogenesis *in vitro* and *in vivo* to understand endogenous development. This knowledge is tremendously valuable when learning about the mechanisms behind the congenital abnormalities that lead to kidney disease. In addition, regenerative medicine is relying on these known mechanisms of normal renal development. Whereas some experimental set-ups have as their aims creating tools for basic research to answer the questions arising from normal development, others again are trials to create methods that can be eventually relevant for clinical use. This chapter discusses the recent advances in the experimental tools used to investigate nephrogenesis, and the limitations to consider when utilizing these same tools.

2.3.1 In vivo Model Systems to Study Nephrogenesis

Genetically Modified Mouse Models

The molecular basis of organogenesis has been investigated, using genetically modified model organisms such as traditional knockout and transgenic mice.

These methods have provided a tremendous amount of information about kidney development (Dressler 1999, Little *et al*. 2010, Little & McMahon 2012, Vainio & Lin 2002). However, creation and breeding of these animals can be expensive and time consuming and in some cases, even impossible. Regarding diverse developmental genes, deleting any gene having a role in embryogenesis, may cause lethality prior to the development of the organ of interest. For example in the case of the *LIM domain-binding protein 1 (Ldb1)* knockout, the mouse embryos die before the onset of kidney development (Mukhopadhyay *et al*. 2003). Thus, conventional *in vivo* knockouts can be inefficient tools for investigating the molecular aspect of organogenesis.

The cre-lox -system is a commonly used conditional knockout method (Sauer 1998). Here the genes are deleted due to the interaction of time and/or site specific promoter driven cre-recombinase with the lox-P sites flanking the gene of interest in different mouse colonies. The promoter for cre-recombinase can also be chemically inducible. However, there still remain some limitations for accuracy in time and space specification. The activation of the cre and the recombination itself takes time. Nevertheless, the cre-lox -based conditional knockout -method can be more informative than traditional gene modified mouse models. For example, the conventional *Lhx1*-knockout mice have renal agenesis due to the failure in primary UB budding. However, *Lhx1* also plays a crucial role at later stages of renal development. This aspect would have been impossible to investigate without the *Hoxb7*cre-mediated conditional knockout of *Lhx1* in MM. (Kobayashi *et al*. 2005). Igarashi (2004) (Igarashi 2004) and Rubera *et al.* (2009) (Rubera *et al*. 2009) wrote comprehensive reviews of the available Cre transgenic lines targeting specific types and regions of the developing kidney. These lists were later completed with the distinct alleles of *Six2*-cre (Kobayashi *et al*. 2008) transgenic mice, which allow for gene inactivation in the cap mesenchyme, and with *Wnt4EGFP*Cre (Shan *et al*. 2010), which inactivates genes from the RV stage of nephron development.

Non-mammalian Model Systems for Nephrogenesis

The regulatory pathways in mammalian kidney development can be dissected using much more simple organisms as models because organisms from invertebrates to humans all possess some kind of excretory organs designed to perform the same task, namely, to remove metabolic wastes from the body. Also, genetic pathways involved in other aspects of invertebrate development may

serve as hints for studying mammalian kidney development due to the numerous orthological genes that exist between species.

Within invertebrates, the soil worm *Caenorhabditis elegans* and the fruit fly *Drosophila Melanogaster* have served as informative models despite the simplicity of their excretory organs. In *C. elegans*, the excretory system consists of a single large H-shaped excretory cell, a pore cell, a duct cell, and a gland cell (Barr 2005). In *Drosophila*, the "kidney" consists of malpighian tubules that develop from the hindgut and perform a secretion, re-absorption and filtering function (Jung *et al*. 2005). *C. elegans* and *Drosophila* both share many genetic pathways and orthologs with mammals. Also, the availability of genetic tools and sequenced geneomes and short reproductive cycle make them attractive as model systems.

Although each of the three mammalian kidney forms – the pronephros, the mesonephros and the metanephros – differ in their overall organization and complexity, they all have the nephron as their basic structural and functional unit. Similar developmental pathways seem to be responsible for the induction and their responses to induction in all kidney forms (Vize *et al*. 1997). Given the similarities between the development of simple and more complex kidneys (Raciti *et al*. 2008), the pronephros has recently emerged as an attractive model to use to study kidney development and disease. In teleost, like the zebrafish, the mesonephros serves as the permanent functioning kidney. However, usually the pronephros is used as a model system in the zebrafish. The zebrafish pronephric glomerulus expresses many of the same genes found in mammalian glomeruli and it contains podocytes and fenestrated endothelial cells. (Drummond 2005). The transparent larva of the zebrafish enables the visualization of pronephric defects during development without the need to sacrifice the organism. Also, availability of the genome sequence and the ability to knock down gene function rapidly by using morpholino antisense oligonucleotides advances the use of the zebrafish as a model organism.

The larval kidney of amphibian *Xenopus leavis* serves as an easy- to- access model for pronephric development. The frog eggs can be fertilized *in vitro*, and then all subsequent development occurs in either water or a simple salt solution. Its large size and external development make the frog embryos easy to manipulate. Thus, grafting, explanting small pieces of tissue, and microinjection of DNA, mRNA, or protein are all possible. The knowledge gained by such systems can then be transferred to higher vertebrate systems where it may shed additional light on metanephric development. (Jones 2005).

2.3.2 In vitro Model Systems to Study Nephrogenesis

Mesenchyme Derived Cell Lines

The ultimate goal of renal regenerative medicine is to be able to create an unlimited supply of patient specific cells that resemble the renal progenitors for kidney regeneration and repair. A stable, MM-derived cell line could serve as an endless source of material. *In vitro* differentiation and the maintenance of clinically relevant renal progenitor cells are not, however, routine as yet. None of the currently available cultured rodent MM cells have lost the potential to undergo MET These include the BSN cell line (Sakurai *et al*. 1997), RIMM-18 (Levashova *et al*. 2003), and primary rat E13 MM cells (Rosines *et al*. 2010). Actually, no long-term culture systems exist that can propagate *Six2*$^{+}$ nephron progenitors. The MET potential is thus lost in favor of immortalization (Little & McMahon 2012). However, such a cell line would be very useful; as for all *in vitro* experiments dealing with epithelializing MM, the cells must at the moment be manually isolated from animal embryos.

Two immortalized cell lines representing either early metanephric mesenchyme (mK3) or MM undergoing MET (mK4) have been well characterized via microarray analyses (Valerius *et al*. 2002). Although the competence to differentiate into nephric tubules has been lost also in these cell lines, they still serve as useful, repeatable tools for initial screening and testing of candidate genes.

The human and mouse podocytes have been cultured *in vitro* for decades. The human and rat primary podocytes used originally in cultures later proved to be not useful after nephrin and podocin were found to mark differentiated podocytes as these cell cultures do not express these genes. However, currently several conditionally immortalized podocyte cell lines have been established from humans, mice and rats. These cell lines are used to study podocyte cell functions *in vitro*. (Ni *et al*. 2012). In addition to podocytes, glomerular endothelial, mesangial and proximal tubular cells have been conditionally immortalized and cultured *in vitro* (Sarrab *et al*. 2011, Satchell *et al*. 2006, Wilmer *et al*. 2010).

Stem Cell Based Strategies

Embryonic stem (ES) cells are pluripotent stem cells derived from inner cell mass of fertilized eggs in mammals. Mouse and human ES cells are self-

renewable and able to give rise to any cell types in the body. These ES cells have been traditionally used to create transgenic mice, and later their potential for regenerative medicine has also been studied. *In vitro* differentiation of ES cells into specific cell types of various organs has gained great attention. Researchers have put effort into establishing methods to efficiently induce mouse and human ES cells into renal progenitors (Reviewed in Takasato *et al*. 2014b). ES cell capacity to differentiate toward renal lineage has been tested by introducing naïve ES cells into developing kidneys (Rak-Raszewska *et al*. 2012, Steenhard *et al*. 2005). However, this integration has proven to be less successful.

A breakthrough in the usage of pluripotent stem cells in regenerative research occured when Takashi and Yamanaka identified four factors, namely, *Oct3/4, Sox2, cMyc,* and *Klf4,* thet were sufficient to reprogram the human or mouse somatic cells to closely resemble ES cells. (Takahashi & Yamanaka 2006, Takahashi *et al*. 2007). Indeed, these cells, called induced pluripotent stem cells (iPSCs), are phenotypically indistinguishable from ES cells. Thus, the knowledge accumulated using ES cells to generate renal lineage cells is potentially applicable to iPSC as well. The iPSC technology enables the creation of patient-specific pluripotent cells lines. Thus, these cells will erase two of the major problems using stem cell approaches in regenerative medicine; the immune rejection when using patient's own cells, and the ethical issue derived from the use of human fertilized eggs. (Osafune 2010).

In recent years, there has been a great interest to reprogram or direct the mouse and human pluripotent stem cells to either mature kidney cells or renal progenitors (Araoka *et al*. 2014, Bruce *et al*. 2007, Kim & Dressler 2005, Kobayashi *et al*. 2005, Lin *et al*. 2010, Mae *et al*. 2010, Mae *et al*. 2013, Morizane *et al*. 2009, Narayanan *et al*. 2013, Nishikawa *et al*. 2012, Song *et al*. 2012, Taguchi *et al*. 2014, Takasato *et al*. 2014a, Vigneau *et al*. 2007). These studies rely on an understanding of embryology and natural niche which the renal progenitor cells meet when progressing from inner cell mass through mesoderm and IM. These studies applied different combinations of growth factors and chemical compounds including LIF, Activin-A, BMPs, GDNF, FGFs, GSK3 inhibitors, and Retinoic Acid. The outcomes from directive differentiation have varied between evidence for cell expressing individual nephric markers, to nephric progenitor cells and even to self-organizing developing kidney. (Reviewed in Takasato *et al*. 2014b, Takasato *et al*. 2014).

In addition to directive differentiation of pluripotent stem cells, a few other approaches have been employed to reach self-renewing renal progenitor cell

source: isolation of long term self-renewing embryonic stem cells (Fuente Mora *et al*. 2011, Lusis *et al*. 2010, Murray & Woolf 2014), directing the kidney derived stem cells (Murray & Woolf 2014) and direct transcriptional reprogramming of adult cells to nephron progenitors (Hendry *et al*. 2013).

The lack of one definitive gene unique to the nephron progenitor cell lineage is now challenging the decision to define the success using directed differentiation (Takasato *et al*. 2014b). The ultimate test for such cells is the capability to form functioning nephron. The question then rises as to which target is more preferable; the generation of cell types of a mature kidney, or the differentiation of the stem cells into kidney progenitor cells?

Three-dimensional Tissue Cultures

The classical organ culture experiments using isolated MM have proven to be especially useful to test the hypotheses for whether a particular gene is required in the MM or UB. Gene function characterization has become possible in organ culture set-up due to the emergence of the chemical inhibitors specific to various signal transduction pathways (e.g. mitogen-activated protein (MAP) kinase inhibitors (Fisher *et al*. 2001) or Notch pathway inhibitors (Cheng *et al*. 2003)). However, there is of course a limited selection available. Several studies have described the use of antisense oligonucleotides and small interfering RNA (siRNA) molecules to inhibit gene expression in kidney organ culture (Davies *et al*. 2004, Durbeej *et al*. 1993, Rothenpieler & Dressler 1993, Sainio *et al*. 1994, Sariola *et al*. 1991). However, it seems that the culture conditions do affect the response to the oligonucleotides and their incorporation is variable with respect to cell type and stage of differentiation. Moreover, the oligonucleotides appear to be toxic for rodent embryonic kidneys in experimental conditions and do not penetrate well into the epithelial sheets in these organ cultures. Thus, the classical organ culture set-up provides only a limited opportunity to study the molecular foundations of nephrogenesis. (Lee *et al*. 2008).

Recently, there has been interest to developing a different kind of dissociation and reaggregation method as applied to the kidney rudiment, to improve the access of manipulative agents into the tissue and cells to e.g. test the renal progenitor potential of differentiated stem cells, and to expand the potential renal precursor populations. Joraku *et al.* established a system where mouse renal structures were reconstructed from primary renal cells extracted from adult mice (Joraku *et al*. 2009). Also Ranghini *et al.* used post-natally extracted cells, mouse

kidney derived stem cells which differentiated into podocyte-, mesangial-, and proximal tubule-like cells (Ranghini *et al*. 2013). Unbekandt and Davies were able to dissociate and reaggregate the whole mouse embryonic metanephric kidney rudiment (Unbekandt & Davies 2010). In a later study, Ganeva *et al.* reported an improvement onto this method; they were able to separately dissociate and reaggregate the MM and the UB (Ganeva *et al*. 2011). Rosines *et al.* established a system where cultured rat renal progenitor cells reconstituted renal structures in three-dimensional culture approaches (Rosines *et al*. 2010). However, the methods of Joraku *et al.* and Unbekandt and Davies did not perform the manipulations of UB and MM separately. Ranghini *et al.* note that the stem cell population established in their studies lacked the potential to differentiate into distal tubule or collecting duct cells (Ranghini *et al*. 2013). In the improved dissociation and reaggregation method of Ganeva *et al.* they separate the treatments allocated to UB and MM, but MM is allowed to be induced prior to or during the treatments.

Most recently, aggregations of directi vely differentiated pluripotent stem cells have been studied. In these studies either differentiated cells alone or in mixture with primary embryonic kidney cells have been shown to organize into three-dimensional renal structures expressing several specific marker genes. (Hendry *et al*. 2013, Mae *et al*. 2013, Taguchi *et al*. 2014, Takasato *et al*. 2014a).

2.3.3 Kinetics of Experimental Nephrogenesis

In vivo, the kidney develops according to a well-known spatial-temporal fashion. The new nephrons are induced at the cortex and are maturing inwards. Thus, throughout kidney development *in vivo*, several different nephrogenic stages will overlap. In contrast, induction with eSC is robust and the difference in morphogenetic stages is restricted mainly to the time needed for the first induced cells to be replaced with new, as yet uninduced cells via random migration movements. This process takes approximately 12 hours. (Saxén 1987).

In the transfilter induction set-up, it takes one to two hours for the in-growth of cytoplasmic processes from eSC into the pores of the Nuclepore filter to make a contact with the mesenchyme. The early morphogenesis of the experimentally induced MM can be subdivided into five different, but partially overlapping periods (Figure 5). The first 12 hours is a period of adaptation and shows low metabolism; probably due to the establishment of contact between MM and eSC. The induction period itself takes altogether 24 hours, which are characterized by

high DNA/RNA synthesis, peaking at 24 hours after initial contact with the eSC. The induction is completed during the third period, up until 36 hours after initial contact. This stage is characterized by the appearance of pre-tubular aggregates and a gradually decreasing DNA synthesis. The fourth period is characterized by the polarization of epithelializing cells and lumen formation and thus the appearance of the RV. The switch to the fifth period takes place on day three when the newly formed epithelial tubules begin to elongate and segment. (Saxen & Lehtonen 1978, Saxén 1987, Vainio *et al*. 1965).

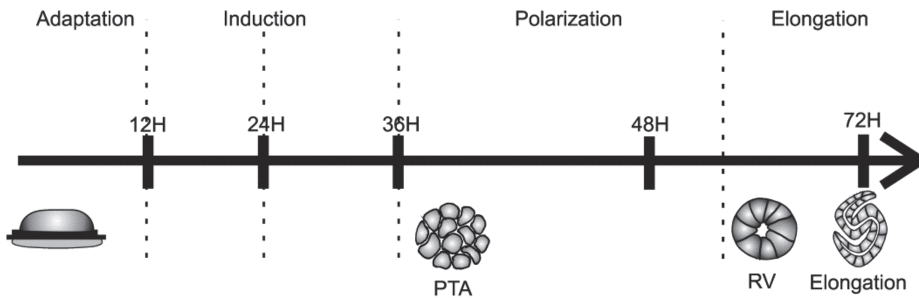

Fig. 5. The sequence of differentiation periods during transfilter induction according to Saxén (1987).

2.3.4 Induction Strategies In vitro

In addition to inducing tissues, namely, the UB and the heterologous inducers, several different kinds of nephrogenesis- inducing strategies have been established. The downside in using eSC as an inductor is the uncharacterized nature of its inducing signal. Although it probably is a Wnt-related signal, other undefined molecular players still exist in this system, and they may have a biasing effect on *ex vivo* induction. The purest and simplest way to induce the nephrogenesis in isolated MM is to use purified Wnt proteins. Unfortunately, due the complex, lipid and sugar-groups containing structure of the members of Wnt-family, thus far, to produce and extract for example Wnt4 has not been successful. However, cells expressing different Wnt proteins have been used for induction (Itaranta *et al*. 2002, Kispert *et al*. 1998).

Nephron induction in isolated MM with soluble factors has gathered research interest for years. Thus far, the only successes have been in rat MM with leukemia

inhibitor factor (LIF) (Barasch *et al*. 1999) and in mouse MM with lithium ions (Davies & Garrod 1995) and chemical GSK3β inhibitors such as Bromoindirubin-3-oxime`(BIO) or CHIR99021 (Araoka *et al*. 2014, Kuure *et al*. 2007, Mae *et al*. 2013, Taguchi *et al*. 2014, Takasato *et al*. 2014a). LIF, although working with rat MM, had a poor effect on mouse MM as an inducer (Barasch *et al*. 1999) and lithium ions only induced the mesenchymal condensation, but no further stages of tubulogenesis (Davies & Garrod 1995). Instead, the GSK3β inhibitors induced spontaneous nephron formation in both rats and mice extending to the development of renal corpuscle, and proximal and distal tubules (Kuure *et al*. 2007). The GSK3β inhibitors have been utilized, for example, in studies of CM progenitor potential of extracted MM cells or directed hiPSC (Araoka *et al*. 2014, Mae *et al*. 2010, Mae *et al*. 2013, Park *et al*. 2012, Taguchi *et al*. 2014, Takasato *et al*. 2014a). The functional segmentation of these nephrons remains to be investigated in detail. Notable is that both lithium ions and chemical GSK3β inhibitors bypass the cell surface receptor mediated signalling (Wnt9b) but when using transient application, the signalling events downstream of β-catenin stabilizations occur endogenously (Kuure *et al*. 2007).

2.4 Molecular Basis of Nephrogenesis

The formation of a fully differentiated, functional adult kidney requires a precise regulation and integration of a number of distinct developmental processes. Over the last two decades, developmental biologists have tried to characterize the molecular basis for the regulation of those processes. The role and involvement of some molecules have already been characterized, mainly based on gene ablation and both cell and organ culture work. In this section, some of the important molecules involved in that regulation of the nephrogenesis are reviewed.

2.4.1 Maintenance of the Self-renewing Progenitor Population

As important as the initiation of polarization in MM cells is the maintenance of the self-renewing progenitor population. The development of the nephric tree is reiterative, from relatively small populations of progenitor cells developing up to 13,000 nephrons in the mice and up to 1.5 million in humans. The maintenance of CM progenitors ensures continued ureteric branching via the production of branching factors. At the same time, CM serves as a source for new nephrons until the whole kidney is constructed. Thus, a paradigm exists in the population of

CM; at the same time, the subset of it should differentiate while the rest must be maintained and remain self-renewable.

Expression of *Wt1* transcription factor is essential for MM survival. *Wt1* is expressed within the early MM and the expression persists within renal progenitors throughout development. *Wt1* expression is also found within the proximal part of forming nephrons (Armstrong *et al*. 1993). Loss of *Wt1* leads to bilateral renal agenesis (Kreidberg *et al*. 1993). Similar phenotype is seen in deletion of *Fgfr1* and *Fgfr2*; and in simultaneous removal of the MM-specific *Fgf20* and the *Fgf9* (Barak *et al*. 2012, Poladia *et al*. 2006, Sims-Lucas *et al*. 2011). In addition to WT1 and FGFs, the BMP signalling is essential for MM survival and maintenance. A severe renal dysplasia is seen in Bmp7-null mouse (Dudley *et al*. 1995). Recent study indicates WT1 in regulating early MM survival through FGF and BMP signaling in antagonistic manner (Figure 6) (Motamedi *et al*. 2014). Some FGF, such as Fgf16 and Fgf20, which are expressed in the developing kidney, are direct targets of WT1 regulation. Loss of WT1 in early MM reduces FGF and also FGF target gene expressions. At the same time, loss of WT1 expression induces ectopic pSMAD signalling, a downstream effector of BMP action leading to apoptosis (Figure 6). (Motamedi *et al*. 2014).

Motamedi *et al.* hypothesize that in early MM, the presence of *Fgfs* and other factors might be sufficient to direct BMP action towards non-canonical, growth-promoting pathways (Motamedi *et al*. 2014). Indeed, BMP7 functions to activate the JNK-Jun signaling axis directly in $Six2^{+}$ cap mesenchyme (and $PAX2^{+}$ progenitors) leading to a proliferative signal (Figure 6) (Blank *et al*. 2009). At later stages, BMP7 induced pSMAD signalling is priming the $Six2^{+}$ progenitor cells for subsequent MET, instead of apoptosis as in early MM (Figure 6)(Brown *et al*. 2013, Motamedi *et al*. 2014). These changes in cellular responses of MM cells to BMP/SMAD signalling is highlighting the complexity of BMP signalling, which clearly depends on the cellular context, developmental timing and the presence of pathway modulators (Motamedi *et al*. 2014).

The self-renewal CM is defined by the expression of essential transcription factors Six2 and Cited1. The $Six2^{+}Cited1^{+}$ population is refractory to inducing signals of WNT9b/β-catenin as they are $Wnt4^{-}$ and $LEF1^{-}$ (Figure 7) (Kobayashi *et al*. 2008). Thus, these cells compose the self-renewing CM. A subset from this cell population is primed for induction of MET via BMP7/pSMAD signaling which causes loss of Cited1 expression and promotes transition of nephron progenitor cells from the $Six2^{+}Cited1^{+}Wnt4^{-}$ compartment to the $Six2^{+}$ only

(Figure 6 and 7) (Brown *et al*. 2013). Now, the Cited1$^-$Six2$^+$Wnt4$^-$ become inducible by WNT9b/β-catenin signalling and can enter the MET.

Interestingly, recent studies suggest that Wnt9b collaborates with Six2 in maintaining the expression of a subset of CM-specific genes. Cells receiving the same Wnt9b/β-catenin signal either proliferate or differentiate, depending on the cellular environment in which the signal is received. Interpretation of that signal is dependent, at least in part, on the activity of Six2. Six2$^+$ cells that receive the Wnt9b signal are maintained as progenitors, whereas cells with reduced levels of Six2 are induced to be differentiated by Wnt9b (Karner *et al*. 2011). Another study emphasises the amount of Wnt9b signalling when determining the fate of Six2$^+$ cells. According to Park *et al.,* there is a distinction between early CM progenitor compartments: complexes of Six2 and β-catenin are found from Six2$^+$only population but not in Six2$^+$ Cited1$^+$ population. . In self-renewing CM cells (Six2$^+$Cited1$^+$) Six2 and Lef/Tcf factors form a regulatory complex that promotes progenitor maintenance via preventing β-catenin from activating genes such, as *Fgf8* and *Wnt4*. Entry of β-catenin into the Six2/Lef/Tcf complex promotes nephrogenesis instead. Thus, in differentiating nephron progenitor cells (Six2$^+$Cited1$^-$), a high dosage of Wnt9b elevates the β-catenin level and lowers the Six2 level. (Park *et al*. 2012).

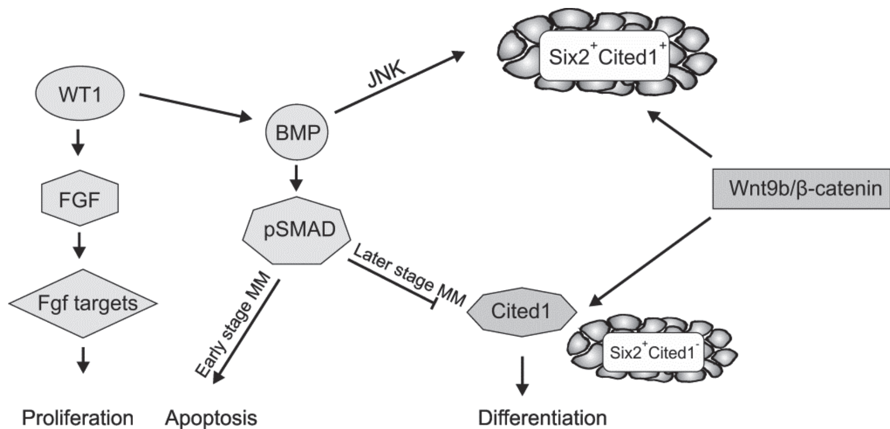

Fig. 6. Model of molecular regulation of maintenance, self-renewal and differentiation of the nephron progenitor cells.

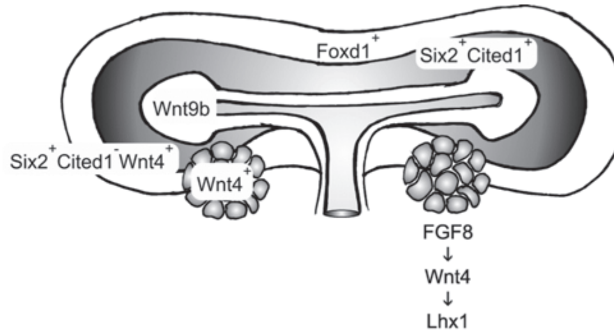

Fig. 7. Nephrogenic niche. The self-renewing cap mesenchyme is Six2+Cited1+ whereas the cells induced by the ureteric derived Wnt9b are Six2+Cited1-Wnt4+. The Wnt9b triggers Fgf8 expression in the pretubular aggregate. Fgf8 in turn triggers Wnt4 expression, which then triggers Lhx1 expression. The stromal precursors are Foxd1+.

2.4.2 Induction of Mesenchymal-to-Epithelial Trasformation

When the UB has grown into the MM, the cells adjacent to the UB organize to form corona around the tip of the UB. As mentioned, the cells in corona are called cap mesenchyme (CM) and consist of a self-renewing Six2$^+$Cited1$^+$ population. A subset of CM respond to the signals from the UB branch stalk by losing the *Cited1* expression and then begin to condensate further. The signal derived from the UB branch stalk is likely Wnt9b, which is essential to trigger, in a paracrine manner, the PTA formation via β-catenin stabilization or canonical Wnt-signalling (Carroll *et al*. 2005, Park *et al*. 2012). However, whereas the Wnt9b is expressed ubiquitously all over the ureteric tree, the PTA emerges always at the armpit of the terminal branches of the ureteric tree. This patterning reflects the compartmentalization of CM according to Cited1 expression (Brown *et al*. 2013) and as above was speculated, the via Six2 blocking Wnt9b signalling in some, but not all, nephron progenitors (Park *et al*. 2012). The transcription factor *Emx2*, also expressed in UB, is one critical player for inducing another Wnt family member essential to MET, the Wnt4 (Miyamoto *et al*. 1997).

Wnt4 is cell-autonomously expressed in the PTAs and critical for proper progression of the MET (Stark *et al*. 1994) (Figure 7). Wnt4 signalling is specifically required for the activation of *Lhx1*, which in turn is essential for further development of the PTA as is described in Chapter 2.4.3 (Kobayashi *et al*. 2005). The same activation can be achieved by genetically elevating the β-catenin

levels, suggesting a continuing role for canonical Wnt signalling also during the MET. However, constitutive canonical pathway activation blocks the MET (Kuure *et al*. 2007), suggesting a possible non-canonical component to Wnt4 signalling. This component might involve Ca^{2+}-dependent Wnt-signalling, because the inhibition of calcium signalling disrupted renal morphogenesis in experimental the set-up (Burn *et al*. 2011, Tanigawa *et al*. 2011).

Upstream of *Lhx1* and *Wnt4* is the signalling molecule *Fgf8* (Grieshammer *et al*. 2005, Perantoni *et al*. 2005), and still upstream of that the *FGF receptor like 1 (FGFrl-1)*. It might be just FGFrl-1 which is the modulator between Wnt9b and Fgf8. (Gerber *et al*. 2009). Interestingly, both Wnt9b and Wnt4 can be replaced with Notch-signalling in experimental set-up. Notch activation was cabable of inducing MET in the absence of both Wnt9b and Wnt4. This raises the question about the relationship between Notch ans Wnt in the early nephron development. (Boyle *et al*. 2011). In zebrafish pronephros, Notch function is required upstream of Wnt4 to promote formation of the proximal domain (Naylor & Jones 2009). Boyle *et al*. speculate that the primary function for Wnt4 in the PTA might be to regulate expression of Notch pathway components (Boyle *et al*. 2011), which in turn have a role in the polarization of RV as described in Chapter 2.4.3.

There is some doubt whether the cellular compaction takes place during the PTA formation or earlier at the formation of the CM. The latter would mean that already prior to the Wnt4 expression, the cells are polarized (Schmidt-Ott *et al*. 2006). However, what is clear is that the MM derived cells are well polarized at the RV stage. This is obvious because of morphological changes; the formation of the basement membrane and the apical lumen and the apical positioning of the nuclei. The basement membrane has already begun to form around the PTA, but is complete at the RV stage. (Saxén 1987).

2.4.3 Proximal-Distal Segmentation of Nephron

The distal domain of the RV is characterized by the expression of *Lhx1* and its transcriptional target *Dll1*, (a Notch ligand) (Kobayashi *et al*. 2005). Downstream from *Lhx1*, *Brn1* (Nakai *et al*. 2003) specifies the distal domain within the RV and is required by the loop of Henle, the macula densa, and the distal convoluted tubule (Figure 8) (Kobayashi *et al*. 2005, Nakai *et al*. 2003).

Wnt9b can induce the *Lhx1* expression, and thus an early Wnt9b gradient from the ureteric tip assumedly provides the polarizing signal for the RV *Lhx1* expression. The RV cells closest to the UB tip and thus in strong influence from

Wnt9b will express distal Lhx1 and the cells further from the tip will acquire proximal fates. (Kobayashi *et al*. 2005).

Conditional inactivation of the *Lhx1* in the MM results in the failure of the RV to polarize in the proximal-distal axis and thus arrests nephron development before the S-shaped body stage. In the chimeric mice, the *Lhx1*$^{-/-}$ ES cell- derived MM cells can contribute to the entire RV, but they are later found only in the Bowman's capsule and the podocytes, the most proximal cells in the nephron. Because the *Lhx1*$^{-/-}$ cells are absent also from the proximal convoluted tubules, this absence suggests that Lhx1 might also have a role in establishing the mid-proximal region.

How then might Lhx1 play its role in determining the proximal fate of the RV cells? Lhx1 activates the expression of *Dll1*, which in turn acts as a first Notch ligand in the RV (Figure 8). This activation might be the trigger for a Notch-dependent process that determines the proximal tubule and the podocyte fate. Additionally, *Dll1* hypomorphic animals lose proximal segments and have severely reduced nephron numbers (Cheng *et al*. 2007). Taken all together, Lhx1 acts downstream of Wnt signalling during the MET and in nephron segmentation, and upstream of Brn1 and Notch inducing the distal fates, (Nakai *et al*. 2003) and proximal fates, respectively.

For proximal cell identity, the Notch activity is required. These cells are divided further into the podocyte precursors and the proximal tubule precursors. The former will lose Notch activity whereas the latter is dependent on the Notch activation (Costantini & Kopan 2010). Notch signalling, including ligands Dll1 and Jag1 is activated in the RV (Cheng *et al*. 2007, Kopan *et al*. 2007). In the *Notch2*$^{-/-}$ mutant, the RV fails to segment probably because of the failure to proliferate and down regulate *Pax2*. Normally, high level expression of Pax2 extends the entire RV, but it is later suppressed in the midsection of the S-shaped body. Thus, Notch2 is involved in separating the distal (dominated by Pax2 and Lhx1) and the proximal (dominated by WT1) regions. In the absence of Notch2 activity, the initial separation into WT1 and Pax2-expressing domains occurs but is halted and reversed. (Kopan *et al*. 2007). Together Notch1 and 2 regulate the PT diameter by keeping the division plane perpendicular to the basement membrane (Surendran *et al*. 2010).

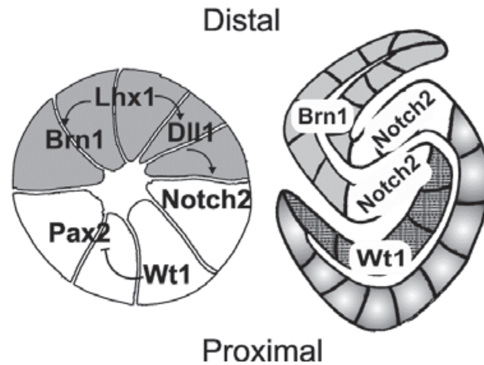

Fig. 8. Molecular regulation of proximal-distal polarization of the renal vesicle and subsequent S-shaped body.

In conclusion, Wnt9b and Six2/Cited1 likely together, with still unknown additional players, position the RV to the armpit of the UB. FGF8/Wnt4 may activate the *Lhx1* expression which secures the distal identity of RV. Lhx1 again activates *Brn1* and *Dll1*. Dll1 is an initiating ligand for *Notch2* activation, which is required for maintaining the proximal identities of RV cells. The Notch2 activation further amplifies its own signal by activating *Jag1*. This produces proliferative burst in the midsection of the comma-shaped body and contributes to the S-shaped body formation. (Kopan *et al*. 2007).

2.4.4 Glomerulogenesis

As this thesis focuses on experimental nephrogenesis which is known to involve only avascular renal corpuscle development, the molecular aspects of glomerulogenesis dealt with here, are restricted to only the few, most well-known players.

The first marker of glomerular development in vertebrates is the restriction of the transcription factor *Wt1* expression to the proximal region of the RV. When the comma-shaped body unwinds to a S-shaped body, the WT1 expression persists in the visceral and parietal epithelium of its proximal segment (Figure 8). Later, in the mature renal corpuscles, WT1 expression is maintained high in the visceral epithelium (podocytes) but it is weak in the parietal epithelium (Georgas *et al*. 2008). The roles of WT1 in nephrogenesis still remain an enigma. Nevertheless, their roles are undoubtedly significant and multifold, taken the renal

and gonadal agenesis in the *Wt1* loss mutation and the four different splice forms of *Wt1* mRNA. The targets of the WT1 regulation also remain mostly unclear; its ability to bind both DNA and RNA is complicating the interpretation of screenings (Quaggin & Kreidberg 2008). WT1 regulates, e.g., *nephrin* (Guo *et al*. 2004), a major component of the podocyte slit diaphragm complex and *podocalyxin (Podxl)* (Palmer *et al*. 2001), a transmembrane protein that may help keep the podocyte cell bodies separated.

At the S-shaped body stage, a primary capillary loop grows into the proximal cleft between the primitive podocytes and the future proximal tubule. The molecular regulation of the capillary loop development is still poorly understood; the small size of the capillary loop and the fact that all available *in vitro* models thus far only develop avascular renal corpuscles, limit the investigation opportunities. More information is available from the composition of the glomerular basement membrane (GBM), the specialized basal lamina between the podocytes, and the endothelial cells of the capillary loops. (Quaggin & Kreidberg 2008). The major components of the GBM are type IV collagen, laminin and heparan sulfate proteoglycan agrin (Miner 1999). During the maturation of the RC, shifts occur both in the laminin and the collagen composition of the GBM. The earliest epithelial cells of the RV mainly express laminin 1 ($\alpha1\beta1\gamma1$). In the nascent form of the GBM, laminin 8 ($\alpha4\beta1\gamma1$) is the isoform present. A second shift occurs upon further maturation of the RC when laminin 10 ($\alpha5\beta1\gamma1$) becomes the major isoform. At the capillary loop stage, laminins 9 ($\alpha4\beta2\gamma1$) and 11 ($\alpha5\beta2\gamma1$) are found, but in adult glomerulus, only laminin 11 is present. (Reviewed in Quaggin & Kreidberg 2008). Upon nephron maturation, type IV collagen goes through a shift from $\alpha1$ and $\alpha2$ collagen subunits to $\alpha3$, $\alpha4$ and $\alpha5$ subunits (Miner & Sanes 1994).

As in the case of capillary loop development, the details of the assembly of the podycytes and especially their interdigitated foot processes, remain poorly understood. There are a set of important players assigned to become involved in maintaining the foot process assembly. Among these is nephrin, a product of the *NPHS1* gene (Kestila *et al*. 1998). It is an essential component of the slit diaphragm (SD), the protein barrier between the foot processes. In addition to its structural role, nephrin also has an important function in signal transduction. Phosphorylation of nephrin leads to cytoskeletal reorganization in the podocytes. (Quaggin & Kreidberg 2008).

2.4.5 Repair Capacity within the Adult Kidney

By definition, a stem cell must be capable of long-term self-renewal, clonogenity and a capacity to differentiate into one or more distinct differentiated end states. The CM serves as the progenitor cell population in embryonic kidney. However, it is lost at birth or shortly afterwards (Hartman *et al*. 2007). Currently, there is a huge debate about the existence of any remaining progenitors in the postnatal kidney. It has long been accepted that the kidney, while not being a regenerative organ, does have a capacity to respond to particularly acute damage. In these repair responses, the proliferation and dedifferentiation of existing terminally differentiated tubular epithelial cells are the recovery mechanisms (Vogetseder *et al*. 2008, Witzgall *et al*. 1994). More recently, other possible repair mechanisms have been proposed including (1) a recruitment of progenitor cells from distant sites in the body (e.g., bone marrow); (2) the presence of a persistent stem/progenitor within the postnatal renal epithelium; and/or (3) the recruitment of a stem/progenitor population from a non-epithelial compartment of the kidney (Little 2011).

The existence of stem or progenitor cells within the postnatal kidney has been questioned by many studies over the last few decades. Renal stem cells have been proposed to exist in the papilla, the proximal tubules, or the interstitium. However, the effect of these proposed cells on repairing at damaged kidney was probably due to a pro-reparative humoral response rather than any stem cell activity. (Little 2011). The most convincing data on plausible endogenous transdifferentiation within the kidney was demonstrated in podocyte repair. Here, the cells of Bowman's capsule acquired podocyte markers and migrated onto the vascular tuft to replace the podocytes in response to injury. (Appel *et al*. 2009, Ronconi *et al*. 2009).

2.5 Expression Profiling in the Developing Kidney

Although kidney organogenesis has long been studied as a model organ system, the underlying molecular mechanisms that control development are still partially unknown. Many key regulators have been defined by traditional developmental genetic tools as described above. However, much remains to be clarified. The gene expression patterns in any developing organ are very complex in their nature. Thus, any expression profiling needs to be done in both a spatial and a temporal context. Microarray-based approaches provide an opportunity to

monitor the spatial expression patterns of individual as well as classes of genes over time. This data serves as source material for functional genomics. This chapter reviews a few of the important steps when unravelling the global gene expression patterns involved in kidney development.

In 2001, a first temporal analysis of global gene expression patterns during rat kidney development was published. It covered microarray analyses of rat whole kidneys at four developmental stages and three postnatal stages (Stuart *et al*. 2001). Similar analysis was done to the mouse kidney two years later. It also included analyses of the isolated UB and the MM at a single point in time (Schwab *et al*. 2003). The rat kidney analysis was later completed with temporal analyses of the *in vitro* cultured, separated UB and MM (induced with eSC). These analyses covered four culture time points; 0, 1, 2 and 5 days for the UB and 0, 24, 72, and 120 hours for the MM (Stuart *et al*. 2003).

A more comprehensive spatial and temporal analysis of mouse kidney development was published in 2005. Here, the analysis covered all the developmental stages of the whole kidney from the day E10.5 to 1 day after birth at 24- hour intervals. Additionally, at E15.5 the isolated (FACS) UB and MM cells were included in the analysis (Challen *et al*. 2005). Later, microarray analyses were utilized to study the global effects of different mutations on kidney development. Combining manual microdissection and laser capture dissection techniques and FACS isolation from fluorescently tagged transgenic animals with microarray analysis provided more and more spatially defined data sets (Brunskill *et al*. 2008, Potter *et al*. 2007, Schwab *et al*. 2006, Takasato *et al*. 2004). Recently, a consortium published the Genitourinary Database Molecular Anatomy Project (GUDMAP) database, which included microarrays on micro-anatomic renal subcomponent structures. (Harding *et al*. 2011, McMahon *et al*. 2008).

The epigenetic regulation has arisen next to the genetic regulation of the developmental processes. The role of non-coding RNAs, chromatin modification, and DNA methylation and acetylation are gathering interest among developmental biologists. To uncover these phenomena during development will be the next phase of functional genomics. A powerful aid to realize the goals comes from Next Generation sequencing (NextGen) (Wang *et al*. 2009). These new sequencing techniques enable to study transcriptional regulation e.g. via reviling of the numerous binding sites of transcription factors (Motamedi *et al*. 2014). Just recently, single-cell specific transcriptional programs and RNA processing patterns have been analyzed in embryonic kidneys (Brunskill *et al*.

2014). Thus, invariably accumulating and elaborating bioinformatics data provides a rich resource for the functional studies.

3 Outline of the present study

The first aim was to screen for differences in the gene expression between the uninduced and the experimentally induced, isolated metanephric mesenchymes. The advantage in using the *ex vivo* induction is that the induced cells develop temporally more parallel or in synchrony compared to *in vivo*, where several different morphogenetic stages overlap, creating a blur for gene expression data.

The second aim was to develop methods for the functional screening of the candidate genes obtained from the microarray analyses. The goal was to utilize the RNAi in cultured tissue efficiently. The traditional dissociation and reaggregation of the metanephric mesenchymes would be used, but the dissociation performed prior to the induction of nephrogenesis if the apoptosis can be prevented in uninduced MM. The research problems involved in this thesis were the following:

1. Which genes are expressed dynamically during experimental nephrogenesis, and can we study their functions in a cost-effective, but reliable, manner?
2. How well does the experimental induction assays mimic the outcome of *in vivo* nephrogenesis and fulfil the kinetics of experimental nephrogenesis? Is it possible to return the UB into the system?
3. Is there a way to preserve nephrogenic competence when the organization of progenitors is disturbed by dissociating and reaggregating the MM prior to the induction?
4. Is it possible to add to, remove, or manipulate cells within the initial progenitor population?
5. For future experiments, which interesting genes and signalling pathways are most dramatically changed during experimental nephrogenesis?

4 Material and methods

Detailed description of the material and methods used in this study are found in the original publication I and the manuscript II. However, a concise decription of the essential tissue manipulation and culture techniques is presented below.

Table 1. Methods used in this study.

Method	Original article
Affymetrix GeneChip arrays	II
Bioinformatics	II
Dissociation and Reaggregation assay	I
Embryo staging and dissection	I-II
Fluorescence-activated cell sorting (FACS)	I
Hematoxylin-eosin staining	I
Immunohistochemistry	I
Knock-down efficiency in mK4 cell line	I
Organ culture	I-II
Real-time Quantitative polymerase chain reaction (qPCR)	II
Recombination of MM and UB	I
RNA extraction	II
RNA in situ hybridization, sections	I
RNAi, shRNA cloning	I
Time lapse imaging	I
Transfilter Induction	I-II
Transgenic mouse lines	I-II
Retrovirus infection assay	I
Retrovirus production and concentrating	I

Dissociation of the metanephric mesenchyme, its reaggregation, and recombination with the ureteric bud

The metanephric kidneys were dissected out from E11.5 mice embryos. The UBs were teased out with the aid of enzymatic treatment and the MMs were collected. If the isoalted MM were applied for dissociation and reaggregation expresiments, they were dissociated with Collagenase III and gentle pipeting, reaggregated by gentle (1380g) centrifugation in the presence of hrBMP7 and hrFGF2. The pellet

was allowed to firm up in Eppendorf tube for 1-2 hours before placing on top of the Nuclepore membrane. The reaggregates were cultured for 24 hours in the presence of hrBMP7 and hrFGF2 . The MM was induced with a small piece of embryonic spinal cord (eSC) in a trans-filter set-up and sub cultured for up to nine days without hrBMP7 or hrFGF2.

For embryonic kidney reconstitution, UBs which were shortly (30 minutes) treated with hrGDNF were aggregated with the MM pellet. The reconstructed UB/MM organoids were cultured for 4-8 days.

The Foxd1+ cells were purified from the dissociated MM by fluorescence-activated cell sorting and the purified cells or the rest of the MM was reaggregated and induced with eSC as described above.

The retroviruses were used as approximately twelve to twenty times concentrates. The retroviruses were introduced directly into the freshly dissociated MM cells prior to their reaggregation. The reaggregation was subsequently performed as described above.

5 Results

5.1 Experimentally Induced Metanephric Mesenchyme Differentiates into Well-segmented Nephrons (I)

The embryonic kidney has served as a model system for studying the mechanisms of embryonic inductive signalling for five decades. The kidney can also be dissected out and the MM separated to address the details of induction and subsequent nephrogenesis *ex vivo*. The eSC serves as a robust tubule inducer, apparently since it expresses a panel of Wnts that provide key tubule inductive signals. (Saxén 1987).

The value of an experimental kidney tubule induction and nephrogenesis model depends on how well it reflects the processes *in vivo*. To address this issue in more detail, we have taken advantage of a panel of nephron-segment -specific markers (Table 2) to study the level of differentiation in eSC- induced and subcultured MM explants.

First, we have used the classical transfilter set-up where the mouse embryonic kidney was dissected out at E11.5 and the UB was further removed with the aid of enzymatic treatment (Figure 9, Step 1). The isolated intact MM (iMM) was conjugated with eSC in transfilter position and cultured for nine days to induce nephrogenesis experimentally (Figure 9, Step 2a). As expected, differentiation of Pecam1^{+} endothelial cells and Pax2^{+} epithelial tubules histologically resembling the proximal and the distal tubules took place. Renal corpuscle-like structures were also detected in the histological sections (Supplemental Figure 1 in (I)).

Next, we have studied more precisely the differentiation level of eSC-induced nephric tubules. The presence of segment-specific ion-channel-expression in the intact MM explants induced with eSC and subcultured is shown in Table 2 and in Figure 2 in (I). These data show that experimental induction of the nephron progenitor/stem cell containing MM with eSC generates nephric tubules having a number of features of the functional segments of the mature kidney *in vivo*.

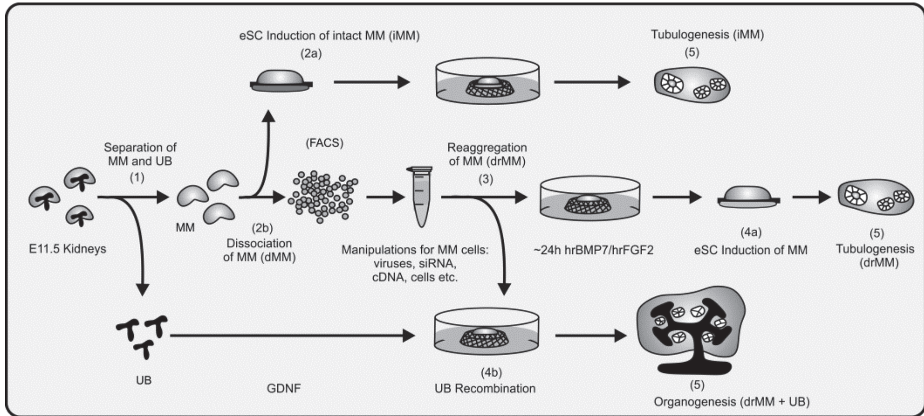

Fig. 9. Schematic presentation of the experimental set-ups developed to address kidney development *ex vivo*.

5.2 Competence of Metanephric Mesenchyme to Form Segmented Nephrons is Maintained after Dissociation and Reaggregation of the Progenitor Cells (I)

Currently, the intact embryonic kidney is not readily accessible for studying nephrogenesis in a reproducible, tissue- and cell-specific manner with functional genomic tools because of the poor penetration of gene manipulative agents (Lee *et al*. 2008). To obtain that capability, we have selected an approach where the isolated iMM was dissociated further into single cells (Figure 9, Step 2b). However, the caveat of such an approach is that the MM undergoes apoptosis unless the tubulogenesis process is rapidly induced (Koseki *et al*. 1992, Saxén 1987). We have solved this problem by supplying the dissociated MM (dMM) cell suspension with hrBMP7 and hrFGF2 (Figure 9, Step 3). These growth factors (GF) have been shown to inhibit apoptosis, retain nephrogenesis capability, and promote stromal cell expansion in an uninduced MM (Dudley *et al*. 1999, Koseki *et al*. 1992).

The dissociated and reaggregated MMs (drMM) were first cultured in the presence of the GFs for 24 hours and then induced with eSC (in absence of GFs) and subcultured. The outcome was then compared to that obtained with control explants not at all treated with these GFs prior to the induction. Significantly more $Pax2^{+}$ foci were present in the GF- treated drMMs than in the controls after subsequent culture indicating that more nephron progenitors survived in GF

treated explants the 24 hours period as uninduced (Figure 3 in (I)). Thus, hrBMP7/hrFGF2 promotes survival and nephrogenesis competence in the drMM as well.

In the drMM induced with eSC and then subcultured, the $Pax2^{+}$ and $Pecam1^{+}$ and the presence of renal corpuscle-like structures were similar to iMM as described above (Supplemental Figure 1 in (I)). Further, the major nephron segment-specific, ion-chanel marker genes had become induced in drMM (Table 2). At protein level Six2, Foxd1, Pax2, Nephrin, Aquaporin1, and NCC representing the tubulogenic mesenchyme, interstitial mesenchyme, renal epithelium, podocyte, proximal and distal tubules, respectively, were also induced in the drMM reconstructions (Supplemental Figure 2 in (I)).

In conclusion, following a complete dissociation of the MM tissue into single cells – in the presence of BMP7/FGF2 – the cells maintained their capability to assemble into a well-segmented nephron in response to tubule induction.

Table 2. Segment specific markers in experimentally induced intact or dis/re MM explants.

Gene Probe	Segment	iMM	drMM	Reference
Slc34a1	PT	Present	Present	Magagnin *et al*. 1993
Slc14a2	DTL	Present	Absent	Olives *et al*. 1996
Slc12a1	TAL	Present	Present	Kaplan *et al*. 1996
ClcnKb	TAL/DCT	Present	Present	Kobayashi *et al*. 2001
Slc12a3	DCT	Present	Present	Campean *et al*. 2001, Loffing *et al*. 2001
Podxl	Podocytes	Present	Present	Kerjaschki *et al*. 1984

5.3 The Dissociated and Reaggregated Metanephric Mesenchyme Will Interact with the Recombined Ureteric Bud (I)

Given the capacity of the kidney nephron forming cells and the stromal cells to reassmble and reconstitute the nephrogenesis, we have asked whether the reciprocal and sequential tissue interactions with the UB could be recovered in the conjugates as well. To target this question, freshly separated UB was pre-treated with GDNF (Lin *et al*. 2001) and subjected to the MM cells during the reaggregation step (Figure 9, Step 4b). The tissue conjugates were cultured for nine days and the degree of kidney organogenesis was evaluated (Figure 6 in (I)).

Interestingly, the UB had reintegrated within the reaggregated MM since the UB had branched extensively during culture. The UB also had retained the

potential for tubule induction since the early nephron marker, $Pax2^+$ MM cells around the UB tips, was present. $Pax2^+$ RV-like structures and some UB connecting tubules had formed as well. A juxtamedullary-like region also was $Pax2^+$ and the RC-like structures $Nephrin^+$. Thus, the induced drMM recovered the reciprocal signalling with the UB to some extent, suggesting that the set-up allows targeting of the UB molecular biology as well.

5.4 Cell-fate Tracking in Chimeric Dissociated and Reaggregated Kidney Culture – a Single Nephron is not Clonal of Its Origin (I)

We have used our established technique of dissociation and reaggregation of the MM progenitor cells to identify the cellular foundation for nephrogenesis via producing chimeras. For this purpose, we used MMs collected from stage-matched CD-1-GFP^+(Hadjantonakis *et al*. 1998) and wild-type CD-1 embryos. The MMs were dissociated, mixed in 1:6 ratios, then reaggregated and induced with wild-type eSC. The ratio between GFP^+ and wild-type mesenchymes was set to 1:6 to ensure sufficient sized GFP^+ population for trakcing the cells. Too big GFP^+ cell population would technically "saturate" the specimen, making it difficult to trace individual cells. It should be noted that GFP^+ in this CD-1GFP^+ mouse model is directed by a constitutively active β-actin/CMV promoter thus, GFP being expressed in all cells of the mouse (Hadjantonakis *et al*. 1998). This appreach provides a standardized, gene loci-independent set-up for mapping cell fates.

As expected, the GFP^+ MM cells within the cultured chimeric explants had the capacity to incorporate with the epithelial tubules and among the interstitial mesenchyme (Foxd1), proximal tubules (AQ1), distal tubules (Slc12a1) and podocytes (Nephrin), as illustrated with antibody mediated localization (Figure 4 in (I)). It is noteworthy as well that we failed to find nephrons that were composed exclusively of GFP^+ cells. Thus, individual nephrons are unlikely derived from a single induced founder cell, but rather from a cluster of induced founder cells, which is in accordance with the data *in vivo* (Mugford *et al*. 2008). We have thus suggested that the chimeric MM serves as a faithful approach fo examining the details of renal cell fate control mechanisms.

5.5 Cell Sorting and Depletion in Dissociated and Reaggregated Kidney Culture (I)

The capacity to dissociate the MM into single cell suspension and still obtain well-segmented nephrons was considered as a technique also allowing for the sorting and extraction of specific cells from the MM. We demonstrated this possibility by utilizing Foxd1cre$^+$; floxed Rosa26tdTomato$^+$ mice (Humphreys *et al*. 2010, Madisen *et al*. 2010). In these mice, the stromal progenitors were labelled with a fluorescent tag according to *Foxd1* expression. Fluorescent activated cell sorting (FACS) was used to sort out the *Foxd1*$^+$ cells from the dMM.

The separated *Foxd1*$^+$ cells were cultured as homotypic cell aggregates in the presence of hrBMP7/hrFGF2 for 24 hours and then contacted with eSC. In these conditions, the *Foxd1*$^+$ cells survived, but the Pax2 and Pecam1 marker expressions remained weak (Figure 5 in (I)). When the freshly sorted cells were re-conjugated with the rest of the MM cells and processed as above, a robust expression of tubulogenesis markers was noted. These data indicate that the developed set-ups provide new ways to study the roles of specific progenitor cell types, their interactions, and expressed factors during nephrogenesis.

5.6 Efficient Viral Transduction in Dissociated Metanephric Mesenchyme (I)

Although engineered recombinant viruses have provided useful tools for introducing genes and shRNAs into cultured cells for functional studies, their poor penetration into tissues makes them less useful for studying organs in culture (Lee *et al*. 2008). In line with this view, we have shown that the GFP$^+$ retroviruses are poor at infecting freshly separated iMM tissue, and indeed only a few superficial cells were GFP$^+$ after infection. On the other hand, if the dMM cells are transduced with GFP$^+$ retroviruses, robust transduction is obtained (Figure 7 in (I)).

We also have followed the fate of the GFP retrovirus-infected MM cells in the reaggregates to ascertain whether transduction would compromise their nephrogenic capacity. Immunohistochemical staining with antibodies for nephric segment markers indicated that GFP$^+$ cells are capable of integrating into Pax2$^+$ renal tubules as well as RC-like structures, based on Nephrin$^+$; proximal tubules, based on Aq1$^+$; distal tubules, based on NCC$^+$. Thus, retrovirus-transduced

nephron progenitor cells are able to differentiate into distinct segments of a nephron and RC-like structures (Figure 8 in (I)).

Having shown that GFP^+ control retrovirus-transduced MM cells remain capable for nephrogenesis, we asked whether that trasduction can be used to target the functions of nephrogenesis controlling genes. To address this question, we selected *Lhx1* as a proof-of-principle gene since the *Lhx1*-deficient nephron progenitor cells initiate differentiation but then degenerate later (Kobayashi *et al*. 2005, Kopan *et al*. 2007, Shawlot & Behringer 1995, Tsang *et al*. 2000). The dMM cells were transduced with *GFP-Lhx1-shRNA* (*GFP-Lhx1*KD) retroviruses, which silenced *Lhx1* expression by 69±8,2% from the control values in the model (mK4) cells. These retroviruses were introduced to the dMM cells in the presence of hrBMP7/hrFGF2. The MM cells were reaggregated and cultured for 24 hours, after which tubulogenesis was induced with eSC.

In line with earlier *in vivo* findings (Kobayashi *et al*. 2005, Kopan *et al*. 2007) and in comparison to the control GFP^+ retrovirus the *GFP-Lhx1*KD retrovirus-infected and reaggregated GFP^+ Lhx1-KD cells failed to integrate into the epithelialized nephrons (Figure 9 in (I)). While the nephron tubular development was devoid of $GFP\text{-}Lhx1KD^+$ cells, some GFP^+ cells were sufficient to incorporate the RC-like structures in line with the *in vivo* data (Kobayashi *et al*. 2005, Kopan *et al*. 2007, Nakai *et al*. 2003).

In conclusion, viral infection *per se* does not disturb the competence of nephron progenitor cells, while shRNA silencing of a critical gene can compromise this activity. Thus, the viral vector –based shRNA- or cDNA approaches should enable us to address the functional roles of essential and non-redundant genes for maintaining the capacity of nephron progenitors for nephrogenesis *ex vivo*.

5.7 Genome-Wide Time-Course Analysis of Experimentally Induced Metanephric Mesenchyme (II)

We performed a genome-wide mRNA abundance analyses at multiple time points (0, 12, 24, 48, 72 and 96 hours) to study the gene expression patterns after eSC induction. Altogether, 3593 genes (4968 probe sets) were detected and showed significant change during the *ex vivo* culture.

The relatively largest changes in gene expressions were detected at 12, 24, and 72 hours; fewer changes were observed at 48 and 96 hours. At the time points 12, 24, and 72 hours, the number of increased and decreased genes were almost

similar, whereas at 48 hours and 96 hours, most of the differentially expressed genes were upregulated compared to 0 hours (Figure 1 in (II)). Interestingly, more genes showed dramatic upregulation at 48, 72, and 96 hours (fold-change > 20) than at 12 and 24 hours, where the total number of differentially expressed genes was greater (see Table 1 in II). Altogether, 104 genes had a dramatic fold-change larger than 10 times during one of the time points compared with 0 hours (see Supplemental Table 1 in (II)). The rest of the fold-changes of ~3000 genes are available upon request.

5.8 Gene Expression Patterns vs. Kinetics of Induction and Early Nephrogenesis (II)

Using double filter between the metanephric mesenchyme and the inducer tissue (eSC) prolongs the induction period for 12 hours, which reflects the time required for the inducing message from eSC to pass through the second filter (Saxen & Lehtonen 1978). Thus, in the experimental set-up utilized here, the five periods of early nephrogenesis (Figure 5) are expected to shift as follows: Period I (Establishment of eSC/MM contact) lasting until 24 hours; Period II (Active induction) lasting until 36 hours; Period III (Completion of induction, PTA) lasting until 48 hours; Period IV (Polarization of induced cells, RV and lumen formation) lasting until 84 hours; and Period V (Elongation, segregation) from that time onward (Figure 10). This sequence seemed to fit nicely with the most represented gene expression patterns of dynamic genes, which peak at 48 and 72 hours. Morphologically the cultured explants seemed to follow the sequence of induction periods suggested by Saxén (Saxén 1987) (Figures 10 and 11). Altogether, 1672 genes from a total of 1848 genes faced dramatic changes at these time points.

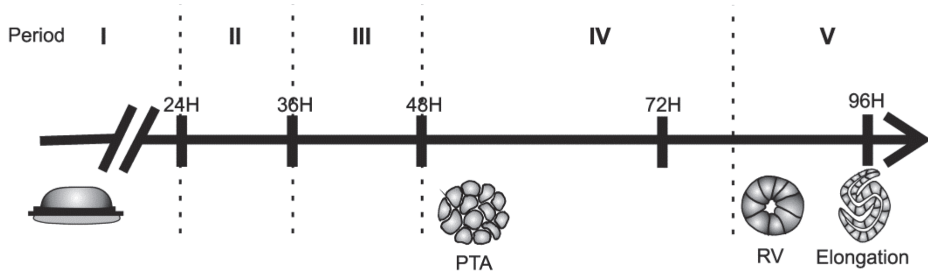

Fig. 10. Sequence of induction periods with "the double filter" set-up according to Saxen, 1987

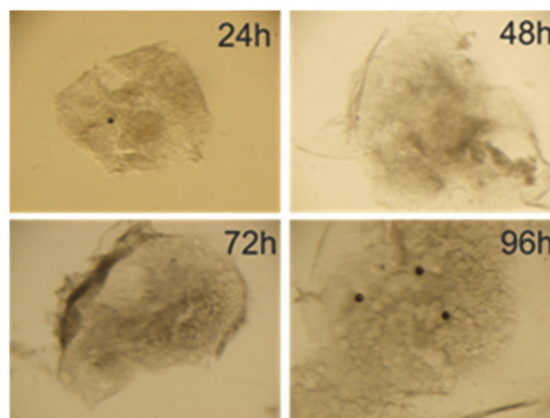

Fig. 11. Whole mount images of MMs induced with eSC and subcultured for the indicated time. At 24 and 48 hours, no morphological changes are seen. At 72 hours, translucent spots are visible indicating PTA formation. At 96 hours, already elongating tubules are visible.

To explore the genes showing a similar expression pattern, the ~3000 dynamic genes were grouped into six clusters (Figure 12) by performing a 'soft clustering' analysis (Kumar & E Futschik 2007). The number of genes in the clusters ranged from 56 to 907 genes and 5 main profiles could be identified from these clusters: Up-regulated profile (Figure 12, Cluster3); up-transiently profile (Figure 12, Cluster1); M-shaped profiles (Figure 12, Cluster2 and Cluster5); down-regulated profiles (Figure 12, Cluster4); and a W-shaped profile (Figure 12, Cluster6). The major biological activities represented by each cluster were indicated by a gene

ontology (GO) analysis (Table 2 in (II)). The top ten enriched GO biological processes were selected for each cluster.

Cluster1genes had a profile that was first slowly up-regulated, then rapidly up-regulated to their maximal activity from 48 hours to 72 hours. The 124 genes in this cluster were enriched mainly in the *development, differentiation* and *morphogenesis* related processes. Also genes related to a *"negative regulation of cell proliferation"* were enriched in Cluster1. The enriched GO biological processes with such an expression profile fit well within the periods of experimental nephrogenesis used here (Figure 10). From 48 hours to 72 hours, one could assume that the PTAs were going through a morphogenetic change into the RVs. It was also expected that cell proliferation would be down-regulated during these morphogenetic changes. Genes falling into Cluster1 included, e.g. the *Notch1, Notch2* with their ligands *Dll1* and *Jag1*, and *Lhx1*, all of which are involved in the proximal-distal polarization of RV and thus expected to be upregulated from 48 to 72 hours (Table 3).

Cluster2 included a large number of genes with M-shaped expression profiles having maximal activities between 12 and 24 hours and at 72 hours. In addition, there was dramatic minimal activity at 48 hours. The most enriched genes here can be annotated into those GO biological processes involved in the *regulation of transcription/gene expression, regulation of macromolecule biosynthesis,* and *regulation of cellular biosynthesis.*

Cluster3 with an up-regulated profile consisted of genes having GO biological processes related to immune and inflammatory responses.

Cluster4 with a down-regulated profile was composed of genes related to skeletal and blood system morphogenesis. Genes falling into Cluster4 included, e.g. *Six1,* which is required for correct expression of *Pax2, Six2* and *Eya1*(Xu *et al*. 2003). The down-regulation of *Six1* might indicate the continuing loss of progenitor cells during *ex vivo* culture (Table 3).

Cluster5 with a much milder "M-shaped" profile included genes involved in the *positive regulation of transcription/gene expression* and *macromolecule biosynthesis*. The profiles of Cluster2 and Cluster5 shared a similar shape, but there was a difference in the intensity of fluctuation. An example of Cluster5 genes is *Smad1*, member of Bmp signalling during kidney development (Vrljicak *et al*. 2004). *Bmp2* also follows the same expression pattern (Table 3). BMP/Smad signalling is leading to apoptosis in early MM progenitors but in later stages BMP/Smad signalling promotes the MM cells to enter the MET (Brown *et al*. 2013, Motamedi *et al*. 2014).

Cluster6 included genes with “W-shaped” expression profile. These genes are mainly involved in *protein modification, their metabolism, transport* and *catabolism*. Also *Wnt receptor signalling pathway* genes were strongly represented in this cluster, for example, *Dvl2, Frizzled-4* and *-6*, and a β-catenin regulating Wnt antagonist *Apc* (Yang *et al*. 2006). Notable is the aspect that all these genes are down-regulated in relation to uninduced situation (Table 3).

Taken together, when comparing the periods of induction in the transfilter set-up suggested by Saxén (Saxén 1987) and the GO biological processes enriched in each expression profile, similar fashions were seen. The dramatic changes in gene expression levels take place temporally during phases of dramatic morphological changes.

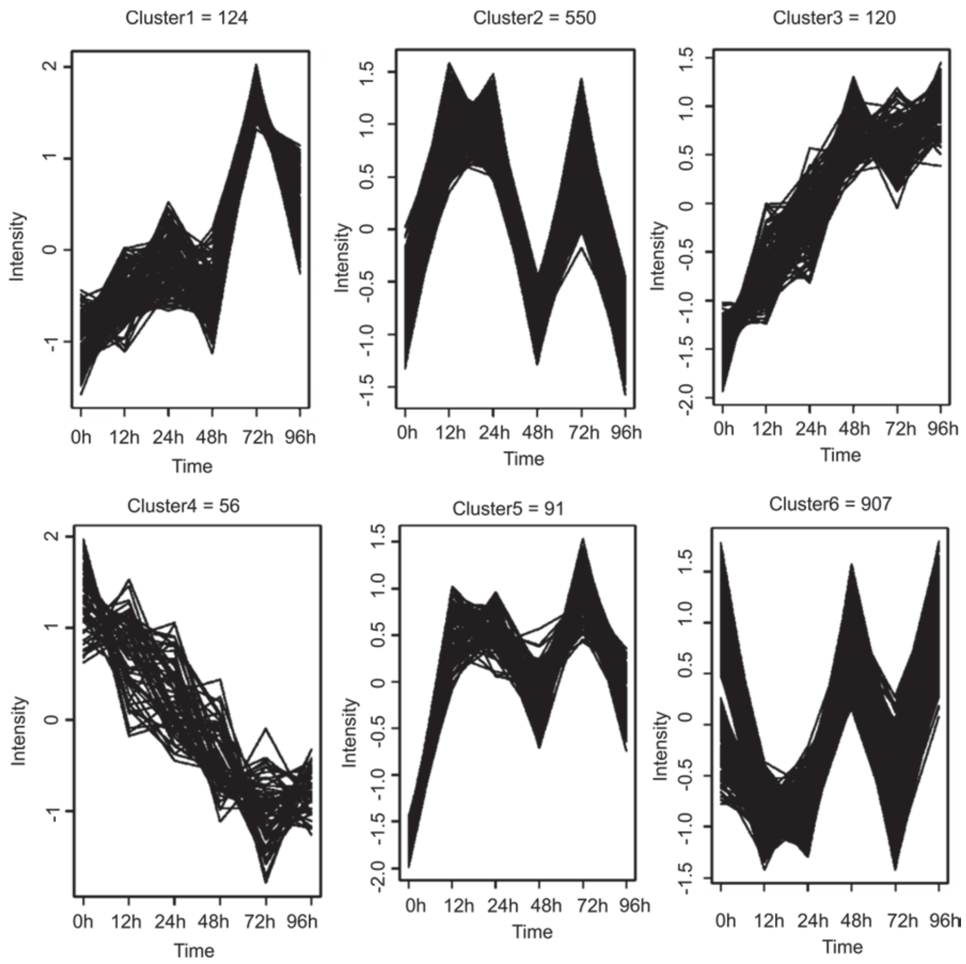

Fig. 12. Clustering of dynamic genes according to the expression pattern.

Table 3. Examples of genes with fold changes following expression pattern clustering criteria.

Gene	12h	24h	48h	72h	96h	Cluster
Notch1	2.44	3.25	2.52	4.78	3.31	1
Notch2	1.90	2.46	1.21	2.71	1.24	1
Dll1	1.57	3.50	2.07	7.13	3.37	1
Jag1	1.83	4.25	2.28	8.82	3.57	1
Lhx1	4.09	26.02	17.04	60.27	24.23	1
Six1	-1.48	-3.23	-4.14	-4.65	-4.25	4
Smad1	2.18	2.22	1.49	2.34	1.55	5
Bmp2	2.04	5.71	4.02	6.95	4.04	5
Dvl2	-5.42	-4.62	-1.22	-4.95	-1.05	6
Fzd4	-3.08	-2.94	1.08	-2.21	1.27	6
Fzd6	-2.09	-1.91	1.50	-1.62	2.15	6
Apc	-2.50	-2.46	-1.00	-2.64	1.02	6

5.9 Examples of Dramatically Changed Genes in Developing Metanephric Mesenchyme

Lgals3 (lectin, galactose binding, soluble 3, or galectin-3) was the gene that increased the most; fold-change increased to more than 100 at 48 hours and decreased to ~81 at 72 hours, and finally increased again to ~159 at 96 hours. The most decreased gene was *Hbb-bh1* (hemoglobin Z, beta-like embryonic chain), which was down regulated about 118 fold-changes at 72 hours and 41 fold-changes at 96 hours.

The expression levels of *Lgals3, Lhx1* and *Nfix* were further analysed using quantitative real time PCR. The expression level at 0 hours was set to be a basal level, or baseline, and the expression levels at other time points were compared against 0 hours. These results are seen in Figure 5 in (II). The charts present the time- related variation of gene expression in eSC induced kidney mesenchymes at time points 12h, 24h, 48h, 72h, 96h. As the un-induced kidney-mesenchyme (0h) was used as a baseline, it had the value of 1. The column presents the range of measured values and the vertical line presents the range for the 95 % confidence interval. *Lhx1* was an exception, because it was not detected at all at 0 hours. Thus, the expression value at 12 hours was used as the baseline. All the genes showed similar up- and down-regulation trends in both qPCR and microarray. Only in *Lgals3* or Galectin-3 was there a difference in timing of the maximum expression: qPCR analysis suggests the 72 hours, whereas microarray analysis

suggested the 96 hours. More biological replicates should be analysed in qPCR analysis to decrease the variation.

5.10 Members of Wnt/PCP and Ca^{2+}-dependend Wnt Pathways are Represented in Dynamic Genes During Nephron Induction *In vitro* (II)

According to the microarray data, the canonical Wnt-signalling target TCF/Lef is up-regulated at 24 hours after eSC contact, suppressed at 48 hours and 96 hours, thus suggesting initially some canonical signalling during the early steps of induction (PTA formation) and subsequent suppression of canonical Wnt-signalling after the RV stage. At the same time, members of the Wnt/PCP pathway (*Syndecan 3, Dishevelled, Mapk8/JNK, Makpk9/Jun*) showed an opposite transcription profile; up-regulated at 48 hours, then rapidly down-regulated and again up-regulated at 96 hours. However, *RhoA* and *Rock,* likewise members of the wnt/PCP pathway, showed an opposite expression pattern (Figure 6 in (II)). In addition, members of the Ca^{2+}-dependent Wnt pathway (*Plcb, CamK2g, Tak, Nfat*) followed a transcription pattern similar to PCP-pathway members; up-regulated at 48 hours, then rapidly down-regulated and again up-regulated at 96 hours. Again two members of Ca^{2+}-pathway, *Calcineurin* and *Nlk* showed an opposite expression pattern on the RNA level (Figure 7 in (II)).

Calcineurin is needed for proper nephron development and its inhibion in utero reduces the nephron number at birth. In addition, loss of the Calcineurin A-α subunit results in a reduced nephrogenic zone, kidney agenesis, and post-natal lethality (Godin *et al*. 1999, Tendron *et al*. 2003). Calcineurin inhibitors have been shown to disrupt the Wnt4 mediated MET (Burn *et al*. 2011).

Taken together, the JNK and Ca^{2+} signalling branches seem to be active on the later stages in the eSC induced MM model presented here. The canonical signalling branches are more active at the beginning when induction and MET occur via inducer- derived signals. This phenomenon, the down-regulation of canonical Wnt-signalling, has been shown also by others in different set-ups (Burn *et al*. 2011, Cizelsky *et al*. 2014, Gilbert *et al*. 2011, Karner *et al*. 2009, Lienkamp *et al*. 2012, McCoy *et al*. 2011).The activation of both PCP and Ca^{2+} dependent Wnt signalling pathways should be further studied for protein expression and functional level.

6 Discussion

6.1 A Genome-Wide Time Course Analysis of the Early Stages of Experimental Nephrogenesis

In a genome-wide characterization of the experimental nephrogenesis model, we found 3595 dynamically behaving genes across the time course that was analysed. From these, 104 genes changed at least ten times at least for one characterized time point. The dynamic genes could be diveded into six clusters according to their expression patterns.

The model-system – the transfilter induction of MM with eSC – aims to minimize a certain blur from gene expression analyses. The analyses are allocated to the MM cells only; the branching UB sharing several genes with the MM, is absent. In addition, the nephrogenic foci develop temporally almost parallel as a response to the robust eSC- mediated, global induction. In the naturally occurring, centrifugal sequence of ureter- driven induction, several differential stages of nephrogenesis exists at the same time. Thus, analyses of the experimental nephrogenesis model should provide sharper data about the dynamic genes particularly restricted to the MET.

The list of dynamic genes produced in the presented microarray set-up can serve as a reference resource for the MET operating genes. Obviuosly, keeping in mind that the gene expression profiles are characterizing nephrogenesis *in vitro*. There probably are differences in expression profiles between *in vitro* and *in vivo* situations. It would be interesting to compare the data produced in this study to the *in vivo* data provided previously e.g. by Challen *et al.* (Challen *et al*. 2005). It would be particularly interesting to compare our data to FACS purified mesenchyme-derived population. Of course, a slight blur would still occur in the gene expression data caused by the sequentially developing nephrons emerging parallel *in vivo*. However, such a blur could be overcome by using laser captured isolated nephrons at different developmental stages. However, in such a case, the data would then lack the interplay between the stroma and the developing nephrons (Das *et al.* 2013).

6.2 The Experimental Induction Model Follows the Known Kinetics and Well Reflects the Outcome of Nephrogenesis *In vivo*

The clustering of dynamic genes into six different expression profiles according to the microarray screening of the experimental nephrogenesis model, supports the temporal division of nephrogenesis into periods suggested already by Saxén (Saxén 1987). These gene expression profiles seem to peak at moments of critical cellular or morphogenetic changes. These assumptions are – of course – based only on the RNA-level patterns; additional regulation might occur at the protein expression level.

We have enhanced the resolution of inspection of the differentiation level that the experimentally induced nephrogenesis reaches. Our results further define the segmentation level which the eSC induced nephrons reache. In addition to that previously thought such nephron is expressing also markers for the loop of Henle. The panel of nephron segment-specific markers characterized here can serve as a reference guide when comparing treated specimen in experimental set-ups.

6.3 Competence of Metanephric Mesenchyme to Form Segmented Nephrons is Maintained after Dissociation and Reaggregation of the Progenitor Cells

Although the classical *in vitro* kidney culture and transfilter induction set-ups have been very useful for determining the molecular mechanism regulating the nephrogenesis, there are features that do limit their applicability with fast evolving molecular tools. Trials to introduce, e.g., RNAi vectors into the organ culture systems, have not been too successful (Davies *et al*. 2004, Durbeej *et al*. 1993, Rothenpieler & Dressler 1993, Sariola *et al*. 1991). The thickness and tightness of the tissues and sometimes a basement membrane that surrounds the organ explants severely restrict the penetration of e.g., siRNAs and viruses into the deeper layers of the organ (Lee *et al*. 2008). The dissociation and subsequent re-aggregation of the tissue into cell suspension would overcome the penetration problem. Indeed, many groups recently have published different kinds of dissociation and reaggregation protocols for kidney tissue (Ganeva *et al*. 2011, Joraku *et al*. 2009, Ranghini *et al*. 2013, Rosines *et al*. 2010, Unbekandt & Davies 2010). However, none of these set-ups could address the metanephric

mesenchyme prior to induction, or when induction was allowed at the same time as the manipulations that were occurring.

Our results show that with the aid of BMP7 and FGF2, the dissociated mesenchyme can be manipulated and reaggregated 24 hours prior to the induction. Without any experimental manipulations, the dissociated and reaggregated mesenchyme continues its development in similar fashion as the intact controls. This finding opens a window for the manipulations to occur well before the nephrogenetic processes are initiated. Thus, applying the approach presented in this thesis, the early steps of the nephron induction program can be addressed. Our results show that the dissociation and reaggregation model can be fulfilled with the recombination of the natural inducer, the ureteric bud. This again, enables studies that address the mutual interaction of the two natural counterparts during the nephrogenesis.

However, even though the metanephric mesenchyme was able to receive the signals from the ureteric bud, the branching morphogenesis of the recombined ureteric bud was restored, but somewhat disturbed. This aspect needs to be further investigated and optimized to reach the kidney organogenesis fully in the reported set-up.

6.4 Dissociation and Reaggregation of Uninduced Metanephric Mesenchyme Enable the Efficient Manipulation of the Progenitor Cells and Their Composition

We have demonstrated the applicability of the novel experimental approach in several respects. Our results show that the dissociation and reaggregation method enables the addition of cells among the initial progenitors. Thus, the system provides an excellent tool for testing the nephrogenic potential of different kinds of modified cells – e.g., IPS cells directed towards nephric lineage. If the added cells are able to incorporate the differentiated structures of the metanephric explant, then their nephrogenic potential could be considered to be well reached. We have also shown that the prolongation of an uninduced stage of dissociated metanephric mesenchymal cells enables a sorting of the progenitors with e.g., FACS. This process enables e.g., depletion of certain cell populations from the rest of the mesenchyme. Thus, the roles of different cell types can be more easily studied.

Finally, we show that dissociation and subsequent re-aggregation overcomes the transduction problem of organ explants. Via retroviral- mediated transduction,

cDNAs or shRNAs can be integrated into the genome of metanephric progenitors prior to their induction. Thus, genes activated at early stages of MET can now be efficiently addressed. For example, we show that the knock-down of the transcription factor *Lhx1*, which has an essential role in the early patterning of the PTA and the RV, leads to a phenotype resembling the phenotype in an *in vivo* knockout (Kobayashi *et al*. 2008, Kopan *et al*. 2007).

It is admitted that in spite of the dissociation and use of high titer (20x concentrated) retrovirus, the overall infection efficiency stays rather low; only around 2% to 3 % as estimated by FACS (data not shown). However, it is important that the infected cells can be traced in this system by using tagged constructs. Also, the cell population infected should be large enough to be recognized and traced. There are also benefits in terms of partial infection; the partial infection provides internal, uninfected control in the same explants at the same time. Of course, when other cells are still able to express e.g., the silenced gene, then the study of secreted proteins or wide range signalling genes become more difficult, if not impossible.

6.5 Aspects of Gene Expression Patterns For Further Study

The most dramatically changed gene appeared to be *Lgals3*, or *Galectin-3*. The dynamic expression pattern was shown also with qPCR analysis. Galectin-3 plays an important role in the growth and stability of polarized epithelial cells. It is also required for terminal differentiation of columnar epithelial cells during early embryogenesis. (Hughes 2001). Bullock *et al*. suggest a similar role for Galectin-3 in the morphogenesis of the UB – the precursor of collecting ducts – during early kidney development in an organ culture. According to their immunohistochemistry data, Galectin-3 expression is maximal in late fetal metanephric kidney, and expression falls off rapidly post-natally to very low levels in the adult kidney. However, the exogenous Galectin-3 seemed not to have any effect on isolated MM cultures induced with the spinal cord. (Bullock *et al*. 2001). It would be interesting to investigate the spatial expression of *Lgals3* in the eSC- induced MM, as it only has been reported as being expressed in ureteric tissue (Bullock *et al.* 2001).

According to the presented microarray data, members of both the Wnt/JNK- and Ca^{2+}-dependent Wnt signalling pathways are represented in the experimentally induced nephrogenesis with interesting expression profiles. The activation of both PCP and Ca^{2+} dependent Wnt signalling pathways and their

members should be further studied on protein expression and on a functional level. Here, the developed dissociation and re-aggregation technique can serve as an excellent tool to study the functional roles of these genes in the formation of the nephron.

7 Conclusions

In the studies included in this thesis, the classical organ culture techniques were used and further developed to be suitable for application to study gene functions during mammalian kidney development. The establishment of the strategy to dissociate and reaggregate the primary metanephric mesenchyme for the first time prior to its induction for nephrogenesis, enables focusing to the very early steps of nephrogenesis. The growth factor mediated extension of the nephrogenesis competence in the uninduced metanephric mesenchyme enables transduction of the dissociated cells with viruses and gives time for the virally mediated a cDNA or shRNA to become active in the uninduced cells. Moreover, the approach presented in this thesis allows specifically the nephron forming metanephric mesenchymal cells to be targeted to exclude the influence of the ureteric bud which expresses in part the same genes. However, as shown in the original article included in this thesis, the ureteric bud can be returned into the explant thus, enabling to target the functional genes in the control of ureteric bud development in the *in vitro* reconstituted kidney rudiment.

The tissue manipulation techniques developed during this thesis are intended mainly for functional screenings of genes considered as candidates for regulating the nephrogenesis. The candidate genes can be rapidly either overexpressed or silenced in the metanephric mesenchymal cells and the influences are readily seen in the three-dimensional, somewhat natural environment. The approaches can also be utilized when testing the nephrogenic potential of differentiated stem cells.

The transcriptional screenings are nowadays reaching even the single-cell level. However, the comprehensive temporal microarray screening of spinal cord induced metanephric mesenchyme serves as important reference resource, as the embryonic spinal cord induced nephrogenesis is widely used model in the research field of kidney development. It would be interesting to compare the expression data of spinal cord induced nephrogenesis to that of e.g. GSK3β inhibitor induced one, and of course, to the naturally occurring, ureteric induced one.

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Original articles

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Junttila S, Saarela U, Halt K, Manninen A, Pärssinen H, Lecca RM, Brändli AW, Sims-Lucas S, Skovorodkin I, and Vainio SJ (2014) Functional Genetic Targeting of the Embryonic kidney Progenitor Cells *Ex vivo.* JASN. In press.

II Junttila S, Halt K, Myllärinen T, Yan W, and Vainio SJ (2014) Identification of the expression patterns of dynamic genes in kidney mesenchyme induced with embryonic spinal cord. Manuscript.

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Original articles are not included in the electronic version of the dissertation.

1257. Lahti, Anniina (2014) Epidemiological study on trends and characteristics of suicide among children and adolescents in Finland

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