Cell fates in nephrogenesis and spermatogenesis

Anne Raatikainen-Ahokas

Division of Physiology and Neuroscience
Department of Biosciences
Faculty of Biological and Environmental Sciences
and
Institute of Biotechnology
and
Helsinki Graduate School in Biotechnology and Molecular Biology
University of Helsinki

ACADEMIC DISSERTATION

To be presented for public examination
with the permission of
the Faculty of Biological and Environmental Sciences of
the University of Helsinki
in Lecture hall 7
B-building, Latokartanonkaari 7, Helsinki
on the 9th of May 2014
at 12 o’clock noon.

Helsinki 2014
Cover picture: Kidney rudiments grown in organ culture for one day (left), 4 days (middle) or 4 days with 50 ng/ml of BMP4 protein (right).
...and then nothing but the reeds' soft thin whispering.

Kenneth Grahame. Wind in the Willows
# Table of contents

Table of contents
List of original publications
Abstract
Abbreviations

1 REVIEW OF THE LITERATURE

1.1 Kidney development

1.1.1 Specification of the kidney morphogenetic field

1.1.2 Patterning along the anterior-posterior axis

1.1.2.1 Wolffian duct and pronephros

1.1.2.2 Mesonephros

1.1.3 Specification of the metanephric mesenchyme

1.1.4 Cell lineages in the metanephric mesenchyme

1.1.5 Ureteric bud outgrowth

1.1.5.1 Restriction of Gdnf expression

1.1.6 Cap condensation of the mesenchyme

1.1.7 Cap condensate as the nephron progenitor pool

1.1.7.1 Regulation of survival and proliferation of nephron progenitors

1.1.7.1.1 FGFs/EGF family

1.1.7.1.2 BMP7

1.1.7.2 Differentiation of the nephron

1.1.7.3 Maintenance of the nephron progenitor pool

1.1.7.3.1 Compartments within the cap condensate

1.1.7.3.2 Balance between self-propagation and differentiation

1.1.8 Embryonic stromal cells

1.1.8.1 Stromal impact on ureteric branching

1.1.8.2 Modulation of the cap condensate function by the stroma

1.1.9 Programmed cell death

1.1.9.1 Cell death in kidney development

1.1.9.2 Regulators of metanephric cell death

1.1.10 Ureteric branching morphogenesis

1.1.11 Ureter maturation

1.1.12 Cessation of nephrogenesis

1.2 Spermatogenesis

1.2.1 Germ cell specification

1.2.2 Primordial germ cells

1.2.3 Postnatal establishment of the spermatogonial stem cell population

1.2.4 Steady-state spermatogenesis in adulthood

1.2.4.1 Spermatogonial stem cells

1.2.4.2 Spermatogonial stem cell niche

1.2.4.3 Proliferation and maintenance of SSCs

1.2.4.4 Signals for the differentiation of spermatogonia

1.2.5 Cell death in the male germ cell lineage

1.2.5.1 Primordial germ cells

1.2.5.2 The first wave of spermatogenesis

1.2.5.3 Adult mice

1.2.5.4 Spermiogenesis

2 AIMS OF THIS STUDY
3 MATERIALS AND METHODS .................................................................................................................... 36
  3.1 Organ culture ........................................................................................................................................... 36
  3.2 Immunohistochemistry ........................................................................................................................ 36
  3.3 TUNEL staining ........................................................................................................................................ 36
  3.4 In situ hybridization ............................................................................................................................ 37
  3.5 Histology .................................................................................................................................................... 37

4 RESULTS AND DISCUSSION ...................................................................................................................... 38
  4.1 Programmed cell death in kidney development .................................................................................. 38
    4.1.1 Mesonephric regression coincides with initial development of the metanephric kidney (unpublished results) ............................................................................................................ 38
    4.1.2 PCD in embryonic kidneys cultured in vitro (I, III and unpublished results) ........................ 38
    4.1.3 Artefactual in situ hybridization signal from pBluescript vector cloning site is associated with apoptosis (III) .......................................................................................................................... 42
  4.2 Effects of BMP4 on development of the metanephric kidney ......................................................... 42
    4.2.1 BMP4 inhibits ureteric budding from the Wolffian duct (I) ......................................................... 44
    4.2.2 Effect of BMP4 on ureteric branching morphogenesis (I) ......................................................... 46
      4.2.2.1 rhBMP4 inhibits ureteric branching ..................................................................................... 46
      4.2.2.2 BMP4 - a physiological regulator of UBM? ..................................................................... 46
    4.2.3 Influence of BMP4 on fates of the metanephric mesenchyme (I) .......................................... 48
      4.2.3.1 rhBMP4 has adverse effects on nephrogenic cells ............................................................ 48
      4.2.3.2 BMP4 is a growth factor for stromal cells ....................................................................... 50
    4.2.4 BMP4 treatment reveals an anterior-posterior axis of the metanephric kidney (I) ............... 50
    4.2.5 BMP4 recruits the smooth muscle layer around the ureter (I) ............................................. 51
  4.3 Influence of GDNF dose on the cell fates of undifferentiated spermatogonia ................................ 52
    4.3.1 GDNF is essential for the proliferation and maintenance of undifferentiated spermatogonia (II) .............................................................................................................................................. 52
    4.3.2 Spermatogonia in clusters, unable to differentiate, are removed by apoptosis (II) .............. 54

5 CONCLUDING REMARKS ............................................................................................................................ 56
Acknowledgements ........................................................................................................................................ 57
References ....................................................................................................................................................... 58
List of original publications


In addition, unpublished data are presented.

Author’s contribution to the studies included in the thesis

I  The author conducted the organ culture experiments, immunohistochemical stainings, *in situ* hybridizations and TUNEL analysis. The author planned the experiments with embryonic kidney explants, modified staining techniques for whole mounts and wrote the article.

II  The author participated in the histological characterization of the testicular phenotype of transgenic GDNF mice and performed TUNEL analysis.

III  The author did *in situ* hybridizations, TUNEL analysis and the colocalization of apoptotic cells with the signal. The author planned the experiments and wrote the article together with TI who supervised this work.

Article II has been used in the following theses:
Abstract

Embryonic cells undergo sequential specification processes to generate multiple cell types of mature organs. Some cells retain pluripotency. They serve as stem or progenitor cells, and provide both new stem cells (self-renewal) and offspring for differentiation. The fate of some cells is to die by programmed cell death. In this thesis, the cell fates in nephrogenesis and spermatogenesis were studied.

During kidney organogenesis, an outgrowth of the Wolffian duct, the ureteric bud, induces condensation of the metanephric mesenchyme into a cap condensate, the progenitor cell population that forms the epithelium of all future nephrons. The cap condensate is surrounded by stromal cells. The developmental fates of these cells that also surround the ureter and nascent nephrons, i.e. the kidney stroma, are poorly understood.

Bone morphogenetic protein 4 (BMP4) inhibited the outgrowth of the ureteric bud from the Wolffian duct in organ culture. It also had an inhibitory effect on subsequent ureteric branching. The branching defect primarily reflected the effect of BMP4 on the mesenchymal components of the kidney. BMP4 promotes the recruitment of mesenchymal cells around the ureter and their differentiation into smooth muscle. This periureteric cell population likely has a regulatory function in subsequent ureteric growth and differentiation.

The exogenous BMP4 also disrupted the cap condensates in kidney explants and large amounts of mesenchymal cells underwent apoptosis. BMP4 maintained the isolated metanephric mesenchymes while suppressing the nephrogenic potential, suggesting that BMP4 acts as a survival/differentiation factor for the stromal progenitors. The stromal cells are apparently essential for the formation and maintenance of the cap condensate.

In some organs, such as the testis, the maintenance of stem cells throughout the life span is essential to the normal function, e.g. the formation of sperm cells. Spermatogonia with stem cell activity (SSCs) are among the undifferentiated spermatogonia located at the basement membrane of the seminiferous tubule. Daughters of SSCs both replenish the stem cell pool and enter the differentiation pathway into spermatozoa.

Glial cell line-derived neurotrophic factor (GDNF), essential for ureteric branching morphogenesis, is also crucial to the self-renewal of the SSCs. Haploinsufficiency of the Gdnf gene in Gdnf+/- mice caused segmental exhaustion of stem cells, resulting in germ cell loss in old mice. In mice overexpressing GDNF in the testis, spermatogenesis was arrested and large clusters of spermatogonia accumulated in prepubertal animals. Thus, high GDNF concentration promotes the propagation of undifferentiated spermatogonia, whereas low GDNF levels allow SCCs to differentiate in excess and make them prone to depletion.

In conclusion, signalling molecules, such as BMP4 and GDNF, affect the cell fates both in nephrogenesis and spermatogenesis by maintaining the precursor cells and promoting their differentiation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
</tr>
<tr>
<td>CAKUT</td>
<td>congenital anomalies of the kidney and urinary tract</td>
</tr>
<tr>
<td>CC</td>
<td>cap condensate</td>
</tr>
<tr>
<td>E</td>
<td>embryonic day</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>fibroblast growth factor receptor</td>
</tr>
<tr>
<td>GDNF</td>
<td>glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GFRα1</td>
<td>GDNF family receptor alpha 1</td>
</tr>
<tr>
<td>IM</td>
<td>intermediate mesoderm</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MeM</td>
<td>mesonephric mesenchyme</td>
</tr>
<tr>
<td>MET</td>
<td>mesenchyme-to-epithelium transition</td>
</tr>
<tr>
<td>MM</td>
<td>metanephric mesenchyme</td>
</tr>
<tr>
<td>NZ</td>
<td>nephrogenic zone</td>
</tr>
<tr>
<td>P</td>
<td>postnatal day</td>
</tr>
<tr>
<td>p75 NTR</td>
<td>p75 neurotrophin receptor</td>
</tr>
<tr>
<td>PCD</td>
<td>programmed cell death</td>
</tr>
<tr>
<td>PGC</td>
<td>primordial germ cell</td>
</tr>
<tr>
<td>PLCγ</td>
<td>phospholipase C gamma</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>Ret</td>
<td>rearranged during transfection</td>
</tr>
<tr>
<td>rhBMP4</td>
<td>recombinant human BMP4</td>
</tr>
<tr>
<td>RTK</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SMA</td>
<td>smooth muscle alpha-actin</td>
</tr>
<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
</tr>
<tr>
<td>SSC</td>
<td>spermatogonial stem cell</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase dUTP nick end labelling</td>
</tr>
<tr>
<td>UB</td>
<td>ureteric bud</td>
</tr>
<tr>
<td>UBM</td>
<td>ureteric branching morphogenesis</td>
</tr>
<tr>
<td>WD</td>
<td>Wolffian duct</td>
</tr>
</tbody>
</table>
1. REVIEW OF THE LITERATURE

1.1 Kidney development

A cell’s fate is what the cell will become if development continues undisturbed [1]. The developmental fates of mammalian cells are generated by sequential inductive interactions with surrounding tissues that progressively restrict the choices available. The commitment to a certain fate can be seen as a two-step process: the cell is said to be specified when it has received enough information to follow its developmental pathway under neutral conditions - neutral in this context meaning the conditions without conflicting instructive signals that could direct the cell to an abnormal fate [1, 2]. Specification is the result of a network of transcription factors, some of which are lineage-specific and the others effectors of extracellular signalling molecules [3]. For the survival and realization of their fate, the specified cells need permissive interactions within their neighbourhood. When a cell is able to differentiate according to its fate, even in a non-supporting environment, it is said to be determined [2].

The group of cells that will form the kidneys, the kidney morphogenetic field, is specified shortly after gastrulation. In mammals, three subsequent renal organs (pronephros, mesonephros and metanephros) are formed. The kidney field must be first compartmentalized. Best understood is the specification of the metanephric mesenchyme (MM) of the permanent kidney. An outgrowth of the nephric duct, the ureteric bud (UB), induces the MM to form a cap condensate (CC, also called the cap mesenchyme), which contains progenitors for secretory nephron epithelia (FIG. 1 B–E). Interaction with the UB tip induces some cells of the CC to form the functional unit of the kidney, the nephron (nephronogenesis). The continuing growth and branching of the UB and the functioning of the CC makes the iterative induction of thousands of nephrons possible during less than a fortnight.

FIGURE 1. The intermediate mesoderm: its origin and derivatives. (A) Schematic picture representing the intermediate mesoderm in relation to the surrounding tissues. (B–E) The temporal and spatial succession of renal organs along the anterior-posterior axis [4]. Abbreviations: PM, paraxial mesoderm; BMP, bone morphogenetic protein; AS, antagonistic signals; D↔V, dorsal–ventral; A↔P, anterior–posterior. Figure reproduced with permission of Development.
1.1.1 Specification of the kidney morphogenetic field

Both kidneys and gonads arise from the intermediate mesoderm (IM), a strip of tissue between the axial and lateral plate mesoderm (FIG. 1 A). Mesodermal cell fates are partially determined by the level at which cells traverse the primitive streak: the cells of the anterior streak become the paraxial mesoderm and the last cells to pass form the lateral plate mesoderm and extraembryonic tissue. During migration or shortly after, the surrounding tissues impart signals to the nascent mesoderm for mediolateral patterning [5]. The medial structures, possibly activin from the dorsal neural tube, induce the IM-specific genes *LIM class homeobox (Lhx1, also known as *Lim1*) and *paired box 2 (Pax2)* [5, 6]. Concurrently, bone morphogenetic protein 4 (BMP4), expressed by the lateral plate and surface ectoderm, has opposing concentration-dependent impact on the mesodermal identity: a high level of BMP signalling promotes the lateral plate and low levels the IM fate [7, 8].

The kidney morphogenetic field has a characteristic gene expression profile [4]. The transcription factors Odd-skipped related 1 (*Osr1, also Odd1*) and *Lhx1* are essential for early nephric specification [9, 10]. Expressed in the IM before any kidney structures have emerged, they are reused for subsequent differentiation processes in urogenital development. *Lhx1* is essential not only for the formation of the pronephric duct and its derivatives [11] but also for the patterning of nascent nephrons [12]. Accordingly, *Osr1* in the nephric mesenchyme functions as a transcriptional repressor, maintaining the precursor state until it is finally restricted into the nephron progenitors of the metanephros and down-regulated during epithelialization [13–16].

*Pax8*, soon accompanied by *Pax2*, is expressed in the future pronephric duct [4, 17, 18]. *Pax2;Pax8*−/− double-mutant mice have no nephric structures, including pronephric ducts or tubules [19]. *Pax2/8* seem to be able to direct cells to nephric epithelial lineages and impart competence to the cells of both the nephric duct and the nephron to undergo mesenchyme-to-epithelium transition (MET), which is at the heart of all nephrogenesis [18].

1.1.2 Patterning along the anterior-posterior axis

1.1.2.1 Wolffian duct and pronephros

Even though axial signals are active along the entire IM, kidney-specific genes are activated only posterior to the sixth somite. This correlates with the anterior border of expression of the homeobox genes belonging to paralogous group 4 (*Hox4*) [5, 6]. Thus, the kidney morphogenetic field constitutes cells that receive the right mediolateral signals and anterior-posterior information provided by the Hox code. The pronephros is formed at the location where the appropriate signals meet [4].

The first morphological sign of kidney development is the primordium of the pronephric duct on the eight embryonic day (E8.0) [19]. This compaction of cells extends caudally along the IM and undergoes epithelialization to form a tubule [20]. Extension of the nephric duct, also called the Wolffian duct (WD), is achieved in mammals by cell proliferation and caudal migration of the duct progenitor cells [15, 21].

The development of pronephric tubules is unique in the succession of kidneys in that they are formed *de novo* without preceding condensation of the mesenchyme and without induction by the nephric duct [1]. However, the molecular pathways directing the pronephros development seem to be essentially the same as in the metanephros [22]. From the biological
standpoint, the three successive kidneys can be seen as a holonephros, one organ adopting
different phenotypes, depending on the animal class and the developmental stage of an
individual [23] (TABLE 1).

<table>
<thead>
<tr>
<th></th>
<th>Pronephros</th>
<th>Mesonephros</th>
<th>Metanephros</th>
</tr>
</thead>
<tbody>
<tr>
<td>lamprey and hagfish</td>
<td>Adult</td>
<td>Embryo/Larva</td>
<td>Adult</td>
</tr>
<tr>
<td>other fishes</td>
<td>Embryo/Larva</td>
<td>Adult</td>
<td>Adult</td>
</tr>
<tr>
<td>amphibians</td>
<td>Rudimentary</td>
<td>Embryo</td>
<td>Adult</td>
</tr>
<tr>
<td>reptiles</td>
<td>Rudimentary</td>
<td>Embryo</td>
<td>Adult</td>
</tr>
<tr>
<td>birds</td>
<td>Rudimentary</td>
<td>Embryo</td>
<td>Adult</td>
</tr>
<tr>
<td>mammals</td>
<td></td>
<td>Embryo</td>
<td>Adult</td>
</tr>
</tbody>
</table>

TABLE 1. Different kidneys of vertebrates at different developmental stages [1, 24, 25].

1.1.2.2 Mesonephros

The mesonephros in amniotes is an embryonic organ, the structure and function of which
vary among species. The mouse mesonephric tubules begin to form at E9.5 from the cranial
direction [26] and reach the final number of 18–26, of which only the 2–6 cranial tubules are
connected to the WD [27]. Rudimentary glomeruli imply that the mouse mesonephros is
never an efficient excretory organ [26]. In contrast, the mesonephri of sheep, pigs and humans
are elaborate and function for an extensive period during embryonic development [27, 28].

In addition to the kidneys, the IM gives rise to the adrenals and gonadal tissue inseparable
from mesonephric development. This connection is highlighted by the incorporation of the
cranial mesonephric tubules into a rete testis and epididymis, while the WD serves as a vas
deferens [27]. The cranial mesonephric tubules are most likely formed by molecular
mechanisms different from those in the caudal tubules, which are specifically lost, e.g. in
Wilms' tumour 1 (Wt1) and Osr1 mutants [13, 27, 29–31].

1.1.3 Specification of the metanephric mesenchyme

The nephric mesenchyme runs parallel to the WD. Until E10.75 this structure is continuous
from the mesonephric area to the posterior trunk [32]. Nevertheless, before the physical
separation of the mesonephric mesenchyme (MeM) and the MM, there are distinct changes in
the gene expression between these two compartments. Of note, the specification of MM
precedes, and is not dependent on, subsequent contact with the ureter [33, 34].

Genes expressed in the nephric mesenchyme can be divided into three classes. Some of the
genes, such as Osr1, Pax2 and Wt1, are expressed in both the meso- and metanephric areas by
the time of MM specification. Other genes, central to further metanephric development, such
as glial cell line-derived neurotrophic factor (Gdnf) and sine oculis homeobox 2 (Six2), are
specifically downregulated at the MeM and restricted to the MM by E10.5 [31].

The transcription factors of the Hox11 paralogous group are expressed exclusively in the MM
[31]. The triple-mutant mice of Hoxa11;Hoxc11;Hoxd11 are born without metanephric kidneys
[35]. The partial conversion of the mesonephric tubules into a metanephric phenotype by the
ectopic expression of Hoxd11, further supports the role of Hox11 paralogues behind MM
identity [31]. Nevertheless, no (re)activation of Gdnf in the mesonephric area could be
detected. Thus, Hoxd11 expression seemed not to be sufficient for a full metanephric
programme [31].
Several lines of experimental evidence indicate an evolutionarily conserved Hox11-Pax-Eya regulatory network crucial to metanephric development. *Hox11* triple-mutant mice express *Pax2* and *eyes absent homologue 1* (*Eya1*) in the MM area but no Gdnf or Six2 [35]. In addition, *Eya1*−/− mice exclusively lack metanephric kidneys [36]. Eya1 is a transcriptional co-activator that cannot bind DNA on its own, but is transported to the nucleus by the Six family of transcription factors [37]. Accordingly, *Six1;Six4* double-mutant mice have no metanephric kidneys or MM-specific gene expression [38]. The homologues of *Pax, Eya* and *Six* regulate eye development in *Drosophila* [39] and the agenesis of the metanephros in *Eya1;Six1;Pax2* triple-heterozygotes recapitulates this interaction [37]. *Pax2*−/− mice have no Gdnf expression in the MM [18, 40] and Pax2 activates Gdnf transcription in both cell and organ culture [40, 41]. Furthermore, the Hox11, Pax2 and Eya1 proteins physically interact with each other and upregulate Six2 and Gdnf expression [40, 42].

Growth/differentiation factor 11 (GDF11, also BMP11) may activate Gdnf expression by an independent pathway [43]. *Gdf11*−/− mice have homeotic changes in the axial skeleton and over half of these animals display renal agenesis, due to the loss of initial Gdnf expression [43, 44].

### 1.1.4 Cell lineages in the metanephric mesenchyme

The MM is a mosaic of cells [45] (TABLE 2). Temporal fate mapping of *Osr1*+ cells has shown that most cell types in the metanephric kidney arise from the IM [15]. The first restriction in fate options is the separation of the nephric duct rudiment around E8.0. From this stage on, these two lineages, the duct epithelium and the nephric mesenchyme, do not mix [15, 45–47].

In addition to the ureter and the mesenchyme converting into nephrons, the embryonic stroma constitutes the third major cell population in kidney development. The traditional view saw the stromal cells as a part of the MM left without inductive signals [48]. Nevertheless, recent studies have shown that at E10.5 when the MM is established, the stromal progenitors, expressing the transcription factor *forkhead box D1* (*FoxD1*), form a cell population distinct from the nephrogenic cells. Hence, the nephrogenic and the stromal progenitors are separated before the contact with the UB [15, 45].

The endothelial cell lineage diverges from the *Osr1*+ IM cell pool before E10 [15]. Angioblasts are first detected adjacent to the ureter and around the nephrogenic cells shortly after the ureter ingrowth [41, 49]. The origin of the mesangial cells that support the vessels of the glomerular tuft has been a subject of controversy [50]. They invade the future glomerular region in the wake of endothelial cells but display an *Osr1* expression profile more like the cells of stromal origin [15, 50].

Neural precursors, most likely neuroblasts from the neural crest, are present in the metanephric kidney rudiment at E11 [51]. Embryonic macrophages infiltrate the metanephric stroma at E11.5 [52, 53]. A recent report shows that macrophages, representing 2–5% of resident cells at E15.5, have a trophic effect on kidney development [53].
### Cell lineage

<table>
<thead>
<tr>
<th>Cell lineage</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>nephrogenic cells</strong></td>
<td>IM [15]</td>
</tr>
<tr>
<td>⇒ Bowman’s capsule, podocytes</td>
<td></td>
</tr>
<tr>
<td>⇒ secretory epithelia of the nephron</td>
<td></td>
</tr>
<tr>
<td><strong>stromal progenitors</strong></td>
<td>IM [15], paraxial mesoderm [54], neural crest [55]</td>
</tr>
<tr>
<td>⇒ interstitial cells/peritubular fibroblasts</td>
<td></td>
</tr>
<tr>
<td>⇒ pericytes</td>
<td></td>
</tr>
<tr>
<td>⇒ mesangial cells ?</td>
<td></td>
</tr>
<tr>
<td>⇒ smooth muscle cells (SMCs)</td>
<td></td>
</tr>
<tr>
<td>⇒ kidney capsule</td>
<td></td>
</tr>
<tr>
<td><strong>endothelial precursors</strong></td>
<td>IM [15]</td>
</tr>
<tr>
<td>⇒ endothelium</td>
<td></td>
</tr>
<tr>
<td>⇒ mesangial cells</td>
<td></td>
</tr>
<tr>
<td><strong>neuroblasts</strong></td>
<td>neural crest [51]</td>
</tr>
<tr>
<td>⇒ neurons</td>
<td></td>
</tr>
<tr>
<td><strong>leukocytes</strong></td>
<td>yolk sac [53]</td>
</tr>
<tr>
<td>⇒ macrophages</td>
<td></td>
</tr>
<tr>
<td>⇒ dendritic cells</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 2.** The *metanephric mesenchyme is a mixture of prespecified cells with different fates* [32].

### 1.1.5 Ureteric bud outgrowth

By E9.5 the WD reaches the urogenital sinus, the primordium of the bladder and urethra. The first sign of metanephric kidney development can be seen at E10 when the duct epithelium becomes pseudostratified at a specific location at the mid-hindlimb level, a phenomenon associated with high cell density and upcoming epithelial outgrowth. The signals mediating this step are currently unknown [56].

GDNF is the foremost regulator of UB outgrowth [56, 57]. Not only targeted deletion of *Gdnf* and its receptors *Ret* (*rearranged during transfection*) and *GDNF family receptor alpha 1 (Gfra1)*, but also the deletions of several other factors affecting the GDNF-Ret signalling axis cause high incidence of renal agenesis [58–61]. Nevertheless, half of the Ret-deficient embryos form UBs and some have rudimentary kidneys [58]. Thus, there must be other factors promoting ureteric budding, such as fibroblast growth factor 10 (FGF10) expressed in MM from E10.5 onwards [61].

GDNF is a distant member of the transforming growth factor beta (TGFβ) superfamily, but unlike the other family members, the ligands of the GDNF subfamily (GDNF, neurturin, persephin and artemin) signal through a receptor tyrosine kinase (RTK), Ret, first identified as an orphan receptor [62, 63]. The interaction of GDNF with Ret is dependent on a glycosylphosphatidylinositol (GPI)-linked coreceptor GFRα1 [60, 63] (FIG. 2). GDNF is a mitogen acting on the cells of the ureteric tip, but the exact mechanism of the bud-promoting effect is not known [64]. The bud formation is associated with the extensive cell rearrangements within the UB epithelium [65] and many GDNF downstream genes are associated with cell migration [66]. It is conceivable that GDNF guides the directed growth of the UB tips, even though the Gdnf expression pattern is too diffuse to be the sole chemoattractive cue [64].

Before the onset of metanephric development, *Ret* is expressed throughout the WD [67]. *Gdnf*, on the other hand, is expressed at E9.5 in the nephric mesenchyme along most of the length of the embryo [68, 69]. In the axolotl (*Ambystoma mexicanum*), the interaction of GDNF with the GPI-linked protein (GFRα1) guides the growth of the nephric duct [70], even though in mammals Ret activity is not needed for the development of the WD [58, 65].
FIGURE 2. Major cellular pathways activated by Ret. Binding of GDNF is dependent on the GPI-anchored GFRα1 receptor. Ligand binding leads to dimerization of Ret and intracellular tyrosine kinase activity. Autophosphorylated tyrosine residues on Ret serve as docking sites for signalling proteins with Src homology 2 (SH2) or phosphotyrosine-binding (PTB) domains [71].

Y1062 is the key residue in Ret signalling, because a mutation at this single site recapitulates the kidney agenesis phenotype of Ret<sup>−/−</sup> mice [72]. Phosphorylation of Y1062 leads to activation of both phosphoinositide-3 kinase (PI3K)/Akt and Ras/mitogen activated protein kinase (MAPK), which are essential for UB outgrowth and branching, in addition to other intracellular signalling pathways [71, 73, 74]. In contrast, phosphorylation of Y1015 recruits phospholipase C gamma (PLCγ) [71]. Abrogating signalling via RetY1015 causes complex anomalies in the development of the kidney and urinary tract [72]. Defects in RetY1015 mutants are at least partly due to enhanced MAPK activation [75].

The signalling mechanisms of GDNF in spermatogonial proliferation and survival include Ras and the PI3K/Akt pathway and Src family kinases [76–79]. The GDNF effects on cell fates are realized through the activation of expression of genes such as Bcl6b, Etv4, Etv5 (Erm) and Lhx1 [66, 78, 80]. Adapted from [81, 82].
During subsequent development, Gdnf expression is progressively restricted to the MM [31, 68, 69]. After the onset of ureteric branching, Ret is expressed only in the UB tips [67]. Gfra1, on the other hand, is also expressed in the condensing MM and early nephrons [57]. Even though GDNF is able to signal through interaction with GFRα1 and the neural cell adhesion molecule (NCAM), exogenous GDNF does not affect MM in vitro [57, 83, 84]. The finding that the Drosophila Gfrα homologue, Drosophila melanogaster GDNF receptor-like (DmGfrl), does not seem to function as a coreceptor for Drosophila Ret, suggests that the interaction of GFRα with NCAM is evolutionarily older than that with Ret [85].

1.1.5.1 Restriction of Gdnf expression

The WD is able to respond to GDNF signals by forming buds throughout its caudal portion at the time of metanephric initiation [40, 57, 86]. Studies of multiplex ureter systems have led to the hypothesis that the proper restriction of GDNF activity is essential for normal urogenital development [69].

In addition to various defects in the skeleton and prominent hydrocephalus, the spontaneous mouse mutant congenital hydrocephalus displays additional mesonephric tubules and duplex kidneys and ureters [87]. The genetic defect behind the congenital hydrocephalus phenotype is a point mutation in the forkhead box C1 gene (FoxC1, previously Mf1) [88]. FoxC1 and the closely related FoxC2 are essential for consolidating the lineage decisions between the paraxial mesoderm and the IM [89]. The WD of FoxC1 mutants runs more medially than normal and the additional mesonephric tubules reach the 23rd somite level in contrast to the 16th in the wild type. The expression pattern of Gdnf is anteriorly extended, supporting the growth of the ectopic buds. Nevertheless, these anomalies are only penetrant in certain mouse strains, pointing to modifying genetic factors [69].

Slit2 is a large secreted glycoprotein best known for providing chemorepulsive cues for axons and directing cell migration [90]. Mice with targeted deletion of Slit2 or one of its receptors, Robo2 (homologues of Drosophila slit and roundabout), have multiplex kidneys and the Gdnf expression is maintained in the nephric mesenchyme more anteriorly than in the wild type [68]. In contrast to the role of the Slit-Robo pathway in cell migration, tracing of cells expressing Gdnf did not show movement [68]. Recently, a very similar phenotype was reported in mice with Ret mutated at tyrosine (Y) 1015 [75].

1.1.6 Cap condensation of the mesenchyme

UB outgrowth is followed by condensation of the MM as a cap around the ureteric tip [23, 91]. This primary condensate is 4 or 5 cell layers thick and constitutes about 10000 Six2+ cells at E11.5 [92]. The current view is that these cells provide the progenitor cells of the nephric epithelia for all nephrons to be formed.

The condensation process of MM is associated with cell proliferation and tight packaging of cells with changes in the composition of the extracellular matrix (ECM) [23, 84, 93, 94]. This condensation correlates with the upregulation of many genes essential for subsequent metanephric development, including Pax2 [95], Wt1 [29], Eya1 [37], Gdnf [57], sal-like 1 (Sall1) [96], Bmp7 [97] and p75 neurotrophin receptor (p75 NTR) [98].

The interaction between integrin α8β1 in condensing mesenchymal cells and the ECM protein nephronectin, produced by the ureter, is needed for the proper upregulation of Gdnf and the ureteric outgrowth [99, 100]. Targeted deletion of the transcription factor Sall1 leads to
The kinesin protein Kif26b, which promotes cell adhesion in the condensing mesenchyme, possibly via integrin α8, is a downstream target of Sall1 [101, 102].

The signals regulating the condensation process are unknown [103]. Factors secreted by the UB are definitely required, but none of the molecules subsequently involved in the proliferation and survival of CC cells or in inductive epithelial-mesenchymal signalling are irreplaceable for the initial condensation [e.g. 104–108]. Thus, this process is most likely regulated by highly redundant factors.

TGFβ signalling plays a role in the condensation of CC. Targeted deletion of an intracellular mediator of TGFβ family signals, Smad4, leads to the defective recruitment of nephrogenic Bmp7+ cells, initially dispersed in the MM, around the tip of the growing ureter [45]. Less compact CCs are also seen in those mice deficient in crosveinless 2 (Cv2). This BMP-binding protein may participate in creating the microenvironment with high BMP7 activity adjacent to the UB surface [109]. Accordingly, the inhibition of c-Jun N-terminal kinase (JNK), downstream of the BMP and Wnt signals, caused failure in the condensation of the nephrogenic cells in vitro [17].

Wt1-deficient mice lack caudal mesonephric tubules, metanephric kidneys and gonads [29, 30]. In Wt1−/− mice, the MM dies by apoptosis after the UB fails to outgrow, although the MM is initially specified [29, 34, 41]. Some progress has been made in elucidating the molecular mechanisms behind the renal agenesis phenotype of Wt1−/− mice. Vascular endothelial growth factor A (Vegfa), expressed in the condensed mesenchyme, is one of the Wt1 target genes [41, 110]. A recent study has indicated that the signals from the endothelial cells expressing Flk1, a VEGF-A receptor, are essential for the maintenance of high Pax2 activity and Gdnf expression in the CC during early kidney development [41]. Furthermore, e.g. Bmp7 and Sall1 have been identified as the direct transcriptional targets of Wt1, and nephrogenic progenitors in kidney explants treated with a morpholino, knocking down Wt1 expression, were unable to undergo condensation [110].

1.1.7 Cap condensate as the nephron progenitor pool

Recent advances in cell-fate studies have led to the recognition of the CC as a progenitor cell population for the nephron epithelia. A stem cell must self-renew indefinitely or for a prolonged time, and it must produce at least one highly differentiated progeny. The CC contains multi-potent progenitor cells, because the descendants of the Six2+ cells contribute to all epithelial cell types of the nephron [92]. The same result has been achieved by fate-mapping of the progeny of CC cells expressing Bmp7 or CBP/p300-interacting transactivator 1 (Cited1) [45, 47]. During kidney development, Six2+ cells undergo a 15.6-fold increase. Based on pulse-labelling experiments, the Six2 progenitor pool is sustained by self-maintenance, i.e. this cell population is not replenished from Six2- cells [92].

The cells of the CC are residing in an environment that supports their function as progenitor cells, i.e. the nephrogenic niche [111]. The stem cell niche, originally described for haematopoietic cells, refers to a special tissue architecture and growth factor milieu maintaining the self-renewal capacity of the stem cells [112, 113]. In the case of nephron progenitors, the niche environment is constructed by the physical support and signalling molecules produced by the ureteric tip and stromal cells on the other side of the cap (and possibly by more differentiated nephric structures).
1.1.7.1 Regulation of survival and proliferation of nephron progenitors

The MM separated from the UB dies under standard culture conditions [114, 115]. This death was inhibited by exogenous epidermal growth factor (EGF), thus evoking the idea of survival signalling mediated through RTKs [114, 116]. Inductive signals were also able to support the isolated MM [114]. Hence, kidney induction\(^1\) has been seen as a two-step process: the MM is first rescued from the cell death inherent to it by signals from the UB and, subsequently, some nephrogenic cells are induced to undergo nephronogenesis [117, 118].

1.1.7.1.1 FGFs/EGF family

FGFs are considered as the major survival molecules for the MM, based on findings that basic FGF (FGF2) is able to promote mesenchyme survival \textit{in vitro} [97, 117, 119, 120]. The inactivation of \textit{fibroblast growth factor receptor 1} \((Fgfr1)\) and \(Fgfr2\) in the MM leads to hypoproliferation of the mesenchyme and kidney agenesis resulting from failed UB outgrowth [121]. Nevertheless, targeted deletion of \(Fgf2\) does not cause gross embryonic defects [122].

Mice deficient in \(Fgf20\) have slightly smaller kidneys than the wild type, but double-mutants of \(Fgf9\) and \(Fgf20\) have kidney agenesis [108]. The mutant MM is small and poorly condensed cells around the UB tip display high levels of apoptotic cell death, indicating a role for these FGFs in the establishment of the progenitor cell pool. The kidneys of compound heterozygotes, \(Fgf9;Fgf20^{+/-}\), have regions of premature differentiation [108]. FGF9 and FGF20 are not only needed for the survival and proliferation of the nephron progenitors, but they also promote the undifferentiated state. Accordingly, FGF9 maintained the MM as competent to respond to inductive signals \textit{in vitro} [108].

FGF20 is exclusively expressed in the CC and functions in an autocrine manner, whereas FGF9 acts mostly from the UB. BMP7 synergized the FGF9 effect \textit{in vitro} while enabling cohesion between the progenitor cells. FGF9/20 and BMP7 may act together to build the niche for the nephron progenitors [108].

In cultured cells from the nephrogenic zone (NZ), RTK signalling through the EGF and FGF receptors increased nephron progenitor survival and proliferation and induced the expression of \(Fgf9\) [123]. Since EGF itself is expressed postnatally and has adverse effects on nephrogenic cells, the relevant EGF family ligands are unknown, but \textit{e.g.} amphiregulin is expressed in the nephrogenic mesenchyme [124, 125]. Nevertheless, the lack of a kidney phenotype in a triple-mutant of EGF receptor ligands \(Egf;Tgfα;amphiregulin^{-/-}\), suggests functional redundancy with FGFs [126]. The dependence of nephron progenitors on survival signals may change in time, based on the late onset of phenotype, \textit{e.g.} in \(Bmp7^{-/-}\) mice, and the more severe effect of RTK inhibition in late nephrogenesis [105, 123].

1.1.7.1.2 BMP7

\(Bmp7^{-/-}\) mice have a kidney phenotype suggestive of a role in progenitor cell survival. At birth, the kidneys of \(Bmp7^{-/-}\) mice are severely dysplastic [104, 105]. The initial development seems normal, but the nephrogenic mesenchyme is progressively lost by apoptotic cell death from E14.5 onwards. Nevertheless, the lack of BMP7 functioning does not prevent nephronogenesis [105].

\(^1\) For historical reasons, the term kidney induction refers to the interaction leading to nephron formation.
Bmp7 is first expressed in the ureter and subsequently also in the nephrogenic mesenchyme. The regulation of Bmp7 is poorly understood and, even though Bmp7 expression in the CC is not turned on by BMP7 from the UB, it is dependent on ureter signalling, possibly on Wnts [97, 127]. BMP7 has only limited survival effect on MM in vitro [128]. BMP7 induced the proliferation of Six2+ nephron progenitors in the primary culture of NZ cells. This proliferative effect of BMP7 is mediated through JNK (but not p38 MAPK) and transcription factors Jun and ATF2. Thus, Bmp7 seems to maintain the nephrogenic progenitors by regulating their proliferation [129].

1.1.7.2 Differentiation of the nephron

Kidney development is one of the few biological systems in which cells of mesenchymal origin epithelialize (undergo MET) [130]. A small cell aggregate of 6–8 cells is formed at a precise position just behind the ureteric tip at the medial edge of the CC. This pretubular aggregate lies in contact with the basal lamina of the ureter [93]. These cells proliferate and gradually acquire an epithelial phenotype. The lumen is formed inside the renal vesicle. Differential gene expression at this stage reflects future patterning events, and the connection between the renal vesicle and the ureter is established [46]. A cleft is formed at the proximal end of the vesicle (comma-shaped body), wherein the renal corpuscle develops. A second cleft at the distal end transforms the developing nephron into an S-shaped body. During subsequent development, the nephron is further patterned along the proximodistal axis [4].

The paracrine Wnt9b signal from the UB upregulates the expression of Wnt4, the autoregulatory factor behind MET, in cells of the future pretubular aggregate [107, 131, 132]. Surprisingly, the expression of constitutively active Notch1 in the CC also causes MET without Wnt4 or Wnt9b functioning [130]. Thus, Wnt9b is a physiological factor that provides a signal to unleash the potential to form nephron epithelia in certain cells of the CC. This interaction is mediated through the canonical Wnt signalling pathway by β-catenin stabilization [107, 127, 133]. FGF8 is an autocrine factor supporting the survival of tubule progenitors. Quite surprisingly, Fgf8−/− mice, which have hardly any nephric structures, also lack nephrogenic progenitor cells. FGF8, expressed in the future nephrons from the pretubular aggregate stage, must signal to the progenitor cell compartment, and similar interactions may continue between the more mature nephric structures and the CC [134, 135]. Nevertheless, the mechanism is unclear, because FGF8 is not able to support the progenitor cells in vitro [123].

1.1.7.3 Maintenance of the nephron progenitor pool

1.1.7.3.1 Compartments within the cap condensate

In addition to nourishment and physical support, the niche must provide signals for self-proliferation and differentiation. The maintenance of progenitor cells demands not only long-lasting proliferative capacity but also careful balancing with differentiation to protect stem cells from exhaustion.

Pretubular aggregates are always formed at particular locations. The expression patterns of several transcription factors also show that the CC is not a homogenous population of cells [14]. In addition to regional specification around the perimeter of the UB, the CC also has a dimension from the ureter basement membrane to the outer border facing the embryonic
stroma. The continual growth and branching of the ureter further increase the dynamics of the niche [111].

Mugford et al. (2008) divided the CC into three compartments, all positive for Osr1, Pax2 and Six2 [14]. Most of the transcription factors studied are expressed diffusely in more than one compartment, which can be separated by marker combinations (FIG. 3).

**FIGURE 3.** The nephrogenic niche. Cap condensates with specific compartments are in contact with ureteric tip cells and the stromal progenitors in the nephrogenic zone. Adapted from [14, 136].

The induced mesenchyme is located on the medullary side of the UB and is delineated by Wnt4 expression, indicating commitment to MET. Consequently, only the inner and outer capping mesenchyme, expressing both Six2 and Cited1, can be regarded as a true progenitor compartment.

Six2 is expressed specifically in the CC and early pretubular aggregates and is gradually downregulated as the nephrogenic cells differentiate [92]. In contrast, Cited1 is sharply shut down at the border of the Wnt4-expressing domain, thus Wnt4 and Cited1 expression seems to be mutually exclusive. Cited1 is expressed at E10.5 in a few cells of the MM, but Cited1+ cells accumulate in the CC between E10.5 and E12.5, possibly reflecting the establishment process of the CC [14]. Cited1 is a transcriptional co-activator that interacts with Smad4 and β-catenin, enhancing TGFβ/BMP effects while repressing canonical Wnt signals [137].

1.1.7.3.2 Balance between self-propagation and differentiation

Six2 maintains the progenitor cells in an undifferentiated, proliferative state. Targeted deletion of Six2 causes the premature differentiation of the CC all around the ureteric tip,
depletion of nephrogenic progenitors and cessation of kidney development [138]. This ectopic nephronogenesis is Wnt9b-dependent and highlights the fact that the entire CC has access to the Wnt9b produced by the UB [92].

Wnt9b is preferentially expressed on the medial side of the ureteric tip, and the high local concentration of Wnt9b may direct the formation of pretubular aggregate. Nevertheless, several Wnt9b target genes are expressed in the uninduced CC and are activated by canonical Wnt signalling through β-catenin [133]. A recent study outlined how Six2 and Wnt9b signalling together regulate genes for nephronogenesis [127]. In progenitor cells, Six2 binds to DNA with T-cell-specific transcription factor (Tcf) and represses the binding of β-catenin to the regulatory sequences of Wnt4 and Fgf8. In differentiating nephron progenitors, high β-catenin and low Six2 levels activate these genes, leading to MET. For other Wnt target genes, there must be different regulatory mechanisms with other interaction partners [127].

1.1.8 Embryonic stromal cells

The tips of the branching ureter reach the kidney periphery at E13.5 [93]. From this stage on, nephron induction is limited to the NZ, a narrow strip of kidney cortex just underneath the kidney capsule. The NZ can be seen as a series of nephrogenic units spaced regularly side by side along the periphery of the kidney [136]. A nephrogenic unit consists of the ureteric tip, the CC and the surrounding stroma (FIG. 3).

Even though prominent during embryonic development, consisting of nearly 40% of the cells in the NZ (E17.5) [129], the nature and functions of the kidney stroma have remained elusive. The cells expressing the transcription factor FoxD1 are considered to be the progenitor cells of the stromal lineage [15, 48, 139, 140]. They populate the NZ surrounding the CCs, i.e. the stroma of the NZ, throughout nephrogenesis. Underneath the NZ, these cells differentiate into the embryonic stroma, clear cytoplasmic, spindle-shaped cells embedded within the prominent ECM [48, 94, 141].

The signals regulating the specification of the stromal progenitors are unknown. Their origin has also been a subject of debate. Their common lineage with nephrogenic cells is supported by the fate mapping of Osr1+ cells, even though Osr1 expression in the IM is not exclusive [7, 15, 89]. The neural crest may be a source of some stromal cells of the meso- and metanephros [55, 141]. A recent fate-mapping study in chickens pointed towards the paraxial mesoderm as a source of capsular and stromal fibroblasts, vascular smooth muscle, pericytes and mesangial cells [54].

The FoxD1+ stromal lineage is detected at E11.5 as a highly concentrated caplike structure immediately anterior to the MM. These cells surround the kidney by E13.5 and integrate into the cortex. This process is disrupted in Hoxa10;Hoxc10;Hoxd10 triple-mutants, which have hypoplastic kidneys with distinct patterning defects at the posterior region of the kidney [139]. In addition to expression of Hox10 genes in the stromal progenitors, the association of the defects with the stroma is substantiated by phenotypic similarity to FoxD1−/− mice (see later) [139, 140, 142]. In Hox10 triple-mutants, FoxD1+ cells could be detected only in the anterior periphery of the kidneys, and UB branching and nephronogenesis were disrupted in the areas without stromal cells. Consequently, the integration of the stromal progenitors is a prerequisite for ureteric branching morphogenesis (UBM) and proper gene expression in the CC [139].
1.1.8.1 Stromal impact on ureteric branching

Retinoic acid (RA) is essential for kidney morphogenesis. RA functions through nuclear retinoic acid receptors (RARs), which are potent transcriptional activators in RA binding [143]. Rara;Rarb2 double-mutant mice have dysplastic kidneys with only a few nephrons and severely impaired ureteric branching. Kidneys of newborn Rara;Rarb2-/- mice display a thick subcapsular stromal layer and no NZ [143]. The rescue of the phenotype by forced expression of Ret in the UB of Rara;Rarb2-/- mice suggests that the primary cause behind the defects in ureteric branching and stromal patterning is the loss of Ret expression [144].

RA is produced from dietary retinol (vitamin A) by retinaldehyde dehydrogenase 2 (Raldh2) in the stromal cells of the NZ [136]. Rara and Rarb2 are coexpressed in stromal cells but also in the ureter [145]. The expression of dominant-negative RAR in the ureter recapitulates the defects of Rara;Rarb2-/- mutants. Thus, RA acts directly on UB cells [145]. The local RA production in the stroma of the NZ maintains Ret expression and directs the development of the kidney to the outward direction [48, 144].

1.1.8.2 Modulation of the cap condensate function by the stroma

Neutralizing antibody against the cell surface disialoganglioside G03 inhibited conversion of the nephrogenic mesenchyme to the nephron epithelia [146]. Since G03 is only expressed in the stroma, this effect must be mediated through the interaction between the stromal cells and the CC. The distinct expression pattern of G03 only in a subpopulation of stromal cells at E11 suggested that the stromal and nephrogenic precursors are separated by this early time point [146].

FoxD1, pre-B-cell leukaemia homeobox 1 (Pbx1) and Pod1 are all expressed in the mesenchymal component of the kidney. The phenotypes of mouse lines with targeted deletion of these transcription factors exhibit pointed similarities: the mutant kidneys are hypoplastic with reduced ureteric branching and disorganized branching patterns. The expression of Ret is not restricted to the tip region. The stromal progenitor cells are specified, but further differentiation is defective, and distinct cortical and medullary compartments are poorly defined. Large condensates of nephrogenic mesenchyme are formed and the development of nephrons is delayed [140, 147–149].

FoxD1 is only expressed in the stroma, Pbx1 and Pod1 in both the stroma and nephrogenic mesenchyme, but the expression in CCs is downregulated by MET [140, 142, 147, 148]. Based on the accumulation of CC cells and delayed nephronogenesis, these transcription factors may regulate processes that prevent stromal signals from repressing CC differentiation [148]. No direct genetic interactions have been shown between FoxD1, Pbx1 and Pod1, indicating that multiple signalling pathways are active at the interphase between the stroma and the nephrogenic mesenchyme [147, 148]. The role of stromal cells in modulating nephronogenesis is also supported by certain in vitro results [128, 150].

FoxD1-/- kidneys are fused and poorly detached from the body wall, features suggested as resulting from defective development of the kidney capsule [140, 142]. Cells of improperly differentiated capsules emit aberrant BMP4 signals to the NZ and disrupt the normal patterning in CCs [142]. The marked similarity between the phenotypes of the FoxD1 and Pod1 mutants, even though these genes are expressed in complementary regions: FoxD1 mainly in the stromal progenitors and Pod1 in the more differentiated embryonic and
medullary stromata, implies that loss of proper stromal compartments may lead to defects in branching and differentiation [140, 142, 147, 149].

Subsequently, embryonic stromal cells differentiate into mature interstitial-cell types, which although sparse, play multitudes of roles in kidney physiology and pathology [151]. The decline of the prominent embryonic stroma to relatively sparse cells of the mature interstitium includes a low proliferation index compared with the nephrogenic structures and the cell death among stromal cells [116, 141, 152].

### 1.1.9 Programmed cell death

The fate of some cells is to die soon after cell division. During animal development, cell death commonly occurs, most likely because it represents a developmental cassette that is easily coopted for evolutionary novelties [153, 154]. Cell death in the nematode *Caenorhabditis elegans* is an invariable destiny determined by cell lineage, but in higher animals cell fate, including death, is regulated by interactions between neighbouring cells [155]. Moreover, most cells seem to be dependent on signals from other cells to avoid the activation of the death programme [156].

The name programmed cell death (PCD) refers to the genetic regulation of the process [155, 157]. Even though the term itself was coined to describe cell death during insect metamorphosis, very little is known about the factors regulating life-and-death decisions in developmental contexts, except in certain special cases [157]. Apoptosis is a specific type of cell death with characteristic morphological features including cell shrinkage, nuclear condensation and fragmentation followed by phagocytosis of the resulting apoptotic bodies [158]. The degradation of cellular DNA into 180–200 base-pair fragments is considered a hallmark of apoptotic death [159]. Even though there are alternative modes of death that are also under genetic regulation [160], most cell-death events, e.g. in kidney development (and in spermatogenesis), seem to be apoptosis [114, 115, 161, 162]. Thus the terms PCD and apoptosis are used interchangeably in this study.

PCD can be initiated by an extrinsic pathway when a death receptor, most notably the Fas receptor (Fas) or tumour necrosis factor receptor (TNFR), is bound by its respective ligand (Fas ligand (FasL) or tumour necrosis factor alpha (TNFα)). Conserved death domain motifs mediate the recruitment of death-inducing signalling complex (DISC) to the intracellular tail of the death receptor, leading to the activation of caspase 8 and subsequent cell death. In the intrinsic or mitochondrial apoptosis pathway, extracellular proapoptotic stress is mediated through changes in the balance of B-cell lymphoma 2 (Bcl2) family proteins and leads to mitochondrial changes initiating the cascade of caspase protease activation through caspase 9 [159].

#### 1.1.9.1 Cell death in kidney development

Large-scale cell death has been reported in metanephric development [114, 116]. Even though the amount of dying cells has been re-evaluated [163], death is a notable destiny of cells with nephric fate. Dead cells are removed by macrophages or neighbouring mesenchymal cells acting as facultative phagocytes [53, 116, 164].

PCD is detected in all compartments of the developing kidney, most likely having differing function and regulation in each of these. The regression of the mesonephros in amniotes is a prime example of phylogenetic death [155, 165]. A vestigial organ is removed in a timetable
and spatial sequence dependent on species-specific programming. An analogous process may also occur inside the metanephric kidney, where nephrons from the initial rounds of induction are assumed to be repositioned at the corticomedullary border by kidney growth and development of the medulla, but may instead be transient and disintegrate [28, 49, 166].

The factors regulating mesonephric regression are unknown, but a recent study indicates the involvement of Ret signalling in this process. Mice with mutated Y1015 on Ret, Ret\textsuperscript{Y1015F}, the docking site for PLC\textgreek{g}, have additional ureters and mesonephric tubules spanning the region between the meso- and metanephros and even incorporating into multiplex metanephric kidneys [72, 75]. Reduced Gdnf doses rescued the Ret\textsuperscript{Y1015F} ureter phenotype, while the MeM still persisted, indicating that Y1015- and PLC\textgreek{g}-mediated restriction of MAPK activity are essential for the timely elimination of mesonephric tissue [75].

Morphogenetic death sculpts developing structures by deleting cells [165]. In the nascent nephron, cell death is detected especially in the proximal region of the S-shaped bodies where the glomerulus develops [116, 167]. Apoptosis among endothelial cells is involved in lumen formation within the glomerular capillaries [168]. The development of the kidney medulla and the remodelling of the pelvis include morphogenetic cell death, which is poorly characterized [116].

Prominent cell death in the NZ may play a role in promoting the differentiation of the CCs and stroma (histiogenetic death) [116, 165]. Most PCD in the NZ occurs in the stromal compartment [116, 163]. Cortical stromal cells die preferentially in close proximity to the developing nephric structures [114, 116, 152, 169]. The limited survival signalling is believed to adjust the interacting cell populations [156]. PCD may match the number of cap cells to the ureteric tip [116] or the removal of `excess´ cells may facilitate the inductive signalling between the tip and the mesenchyme [170].

1.1.9.2 Regulators of metanephric cell death

<table>
<thead>
<tr>
<th>THE Bcl2 PROTEIN FAMILY</th>
</tr>
</thead>
<tbody>
<tr>
<td>PROAPOPTOTIC MEMBERS</td>
</tr>
<tr>
<td>BH3 only-proteins</td>
</tr>
<tr>
<td>Bim</td>
</tr>
<tr>
<td>Bid</td>
</tr>
<tr>
<td>Bik</td>
</tr>
<tr>
<td>Bad</td>
</tr>
<tr>
<td>Puma</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ANTIAPOPTOTIC MEMBERS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bcl2</td>
</tr>
<tr>
<td>BclxL</td>
</tr>
<tr>
<td>Bclw</td>
</tr>
<tr>
<td>Mcl1</td>
</tr>
</tbody>
</table>

**Bak/Bax dimers induce permeabilization of the outer mitochondrial membrane and the initiation of the caspase cascade**

*TABLE 3. Some members of the Bcl2 family. The BH3 only-proteins are believed to bind to antiapoptotic family members and release effector proteins (most notably Bak and Bax) to promote apoptosis [171, 172].*
The survival of vertebrate cells is usually not determined by single factors but by a network of influences integrated in the balance between pro- and antiapoptotic Bcl2 family members (TABLE 3). Mammalian cells usually express multiple members of the Bcl2 family, providing redundancy [173]. Bcl2 functioning has been extensively studied in kidney development, because in addition to impaired lymphatic systems and pigmentation in the hair, Bcl2\(^{-/-}\) mice have defects in kidney development and function (polycystic kidney disease) [174].

At birth, Bcl2\(^{-/-}\) mice have hypoplastic kidneys with narrow NZs. This phenotype is partially explained by increased cell death in the nephrogenic mesenchyme at E12 [174, 175, 176]. Bcl2\(^{-/-}\) mice are indistinguishable from the wild type, even though there is a three-fold increase in apoptosis. Therefore, the developing kidney is able to compensate for a substantial amount of cell death in the mesenchymal component. The nephron number seems not to be linearly correlated with the amount of nephron progenitors but is dependent on a critical threshold [174].

Recent studies have emphasized the role of transcriptional and posttranscriptional regulation of BH3 only-proteins in cell-death decisions [171]. The kidney phenotype of Bcl2\(^{-/-}\) mice can be ameliorated by the removal of one allele of a proapoptotic factor, Bim [177]. Bim has also been indicated as a target of micro-RNA regulation. Conditional deletion of an RNA-processing enzyme, Dicer, in Six2+ cells, led to the apoptotic loss of nephrogenic progenitors [178].

PCD in the ureter is a rare event, at least during the early stages of kidney development [116, 179]. This has been attributed to Pax2 functioning. Haploinsufficiency of Pax2 leads to hypoplastic kidneys with reduced numbers of nephrons, considered as a direct consequence of excess apoptosis in the ureter lineage [179–181]. Proper suppression of apoptosis in the UB may be essential for efficient ureteric branching and high nephron number. The effect of Pax2 may be mediated by the transcriptional activation of neuronal apoptosis inhibitory protein (NAIP), an endogenous caspase inhibitor expressed in the embryonic ureter [182]. During the later stages of kidney development, PCD is more prominent in the collecting system, especially in the developing papillae [116, 167].

Bcl2 is expressed in the CC, ureter and developing nephrons [111, 167, 183]. The kidney phenotype of Bcl2\(^{-/-}\) mice can be partially rescued by the overexpression of Bcl2 in the ureter [184]. Nevertheless, Bcl2 overexpression also enhances ureteric branching in the wild type [180, 184]. The interaction of Bcl2 with a focal adhesion protein, paxillin, may promote adhesion-independent survival and facilitate UB cell migration or the morphogenesis of nascent nephrons [185–187]. Proteins in apoptotic pathways can also function in other cellular processes.

Even though PCD in the embryonic kidney better fits the execution by the intrinsic pathway [169], extrinsic cell death may be involved on certain occasions [164]. TNF\(\alpha\), expressed in the metanephric kidney from E11, caused increased apoptosis in vitro and inhibited ureteric branching and early stages of nephron differentiation [52]. A wide variety of adult kidney cells constitutively express either Fas or FasL and this system is further activated in states of inflammation and injury [188], but no Fas expression was detected in the embryonic kidney of the mouse [189].
1.1.10 Ureteric branching morphogenesis

Iterative branching of the ureter in the NZ drives kidney growth [93]. The CC provides not only progenitor cells but also factors stimulating ureteric branching. GDNF also plays a major role in the ureteric branching morphogenesis (UBM) after the initial outgrowth (reviewed by Costantini and Shakya (2006) and references therein [64]). Wnt11 secreted from the ureteric tips upregulates Gdnf. GDNF-Ret signalling, in turn, maintains Wnt11 expression, thus establishing the positive feedback loop ensuring robust GDNF production and continuous branching of the ureter [106].

Targeted deletion of Sprouty 1 (Spry1), an endogenous inhibitor of RTK signalling in the UB lineage, leads to hypersensitive WD and a massive number of ectopic buds. Removal of a single Gdnf allele reversed this phenotype, emphasizing the importance of the balance between positive input and negative feedback regulation to ensure proper branching [190]. Nevertheless, the double-mutants Gdnf;Spry1-/- have near-normal kidneys in the majority of animals; thus, other growth factors operating through RTK signalling are involved in the UBM [61, 191].

Mice with targeted deletion of Fgfr2 in the ureter lineage have hypodysplastic kidneys with reduced UBM [192]. Those mice null for Fgf10, a gene essential for pulmonary branching morphogenesis, have small kidneys [193, 194]. Removal of even a single copy of Fgf10 in Gdnf;Spry1-/- mice resulted in complete failure of ureteric outgrowth, indicating cooperation between FGF10 and GDNF in UBM [61]. Fgf7-/- mice also have markedly reduced collecting duct systems [195]. This effect becomes visible after the initial stages of development (E16.5); thus FGF7, produced by stromal cells, is needed for the full extent of ureteric growth [195–197]. Furthermore, hepatocyte growth factor (HGF, scatter factor), expressed in the MM, stimulates ureteric growth and branching [198, 199].

UBM results from interaction between the ureter epithelium and MM. Quite surprisingly, dissected UBs are able to branch in a suitable ECM (Matrigel) with GDNF stimulation, indicating that the branching and elongation are intrinsic properties of the ureter [200]. Nevertheless, the heterologous recombination of UBs with lung mesenchyme led to lung-type branching morphogenesis [201], and Gdnf;Spry1-/- kidneys have defects in the branching pattern [191]. Thus, local expression of mesenchyme-derived factors, either promoting or inhibiting ureteric growth and branching, determines the branching pattern [56]. Several members of the TGFβ superfamily have been proposed as these negative regulators [202].

1.1.11 Ureter maturation

The excretion system needs a proper outflow tract. Before the first nephrons begin to function, the ureter, originally sprouting from the WD, is relocated to the base of the bladder by ureter maturation. The distal part of the WD, the common nephric duct, is removed by a caudal-to-rostral wave of PCD, possibly induced by RA from the urogenital sinus region [203–205]. This locates the distal end of the ureter along the urogenital sinus epithelium. Subsequent remodelling of the ureter base and growth of the bladder anlagen positions the ureter orifice in an anterior location, where the ureterovesical junction can be fully functional [204, 205]. Defects in this process lead to congenital anomalies of the kidney and urinary tract (CAKUT)-type disorders [206].

Ret signalling is involved in distal ureteric morphogenesis. Ret expression, regulated by RA, is needed for the normal integration of the ureter to the bladder [203, 204]. Cell death in the
common nephric duct, on the other hand, is dependent on the developmental downregulation of Ret transcription in the WD and attenuation of the receptor activity by the receptor protein tyrosine phosphatases RPTPσ and LAR, encoded by the genes protein tyrosine phosphatase, receptor type S (Ptprs) and F (Ptprf) [205].

1.1.12 Cessation of nephrogenesis

Iterative branching of the ureter and subsequent nephron induction continue until the loss of the CCs at P3 when the progenitor population is depleted by an abrupt differentiation wave [207]. This may be caused by changes in the signalling environment surrounding the CCs or by passive exhaustion of the nephron progenitors [111]. Indeed, the cell number in the CC is smaller at later stages in development [92]. The sudden end of nephronogenesis could imply a physiological trigger, even though the timing of cessation is species-specific [207]. Nevertheless, the development of nephric tubules continues in rats until 4 weeks post partum before adult kidney shape and functioning are attained [151].

After this perinatal period, no new nephrons are formed in mammals. Regeneration after injury is achieved by the proliferation of an existing nephron epithelium or scattered tubular progenitors. In contrast, nephron neogenesis can occur in some other vertebrates [reviewed by 25].

1.2 Spermatogenesis

The functioning of some organs, such as the testis, is based on the stem cell population maintained throughout the lifetime of an animal. The male germ cells have several features that make them a good model in stem cell biology. The differentiation pathway of these cells, including syncytial divisions and distinctive morphology, is very well documented. The functional transplantation assay, in which the presence of stem cells can be proven by the ability of mixed populations of germ cells to re-establish normal spermatogenesis in recipient mice, is the undisputed test for stem cells [208]. Caenorhabditis and Drosophila gonads with simple structures have provided a molecular dissection of the germline stem cell niche, inspiring mammalian research in which the temporal and spatial relationships are more associative [209].

1.2.1 Germ cell specification

Segregation of the germ cell lineage as primordial germ cells (PGCs) is one of the first cell fate decisions occurring during gastrulation. In mammals, germ cells are specified from the epiblast, pluripotent cells giving rise to all tissues of the embryo proper, by inductive signalling between the extraembryonic ectoderm and endoderm [210].

Based on gene ablation studies, BMPs are fundamental to the generation of PGCs. Bmp4−/− mice have no PGCs [211] and BMP4, expressed in the extraembryonic ectoderm, induces PGC fate [211, 212]. Inhibitory signals emitted from the anterior visceral endoderm limit PGC induction to the most proximal posterior epiblast. Bmp8b controls the development of the anterior visceral endoderm and restricts inhibitory signalling, whereas BMP2 augments the BMP4 effect [212]. The specification of germ cells seems to be sensitive to the BMP dose, because Bmp4−/+ mice have reduced PGC founder populations [211].

PGCs undergo fundamental internal changes. Somatic programming, as in mesoderm induction and Hox gene expression, must be repressed. Simultaneously, germ cell-specific
genes, some of them associated with pluripotency (e.g. Nanog and Oct4/POU5f1) are upregulated. Extensive epigenetic reprogramming is also essential for the functioning of these cells in gametogenesis and in the propagation of genetic information to the next generation [210].

1.2.2 Primordial germ cells

The first sign of gonad development is the thickening of the coelomic epithelium at E10.5, in close association with the prominent mesonephros [213]. PGCs that colonize the gonadal area between E10.5 and 11.5, are attracted by chemotactic cues, possibly stromal cell-derived factor 1 (SDF1, or chemokine (C-X-C motif) ligand 12 (CXCL12)) and/or Kit ligand (KitL), also known as stem cell factor (SCF) [214]. PGCs are enclosed inside the condensing seminiferous cords with pre-Sertoli cells, which most likely originate from the coelomic epithelium [215]. After a burst of mitotic activity, PGCs, now called gonocytes, enter mitotic quiescence at E13.5 in males. The entry of germ cells into meiosis is regulated by RA, but in males the RA-degrading enzyme CYP26B1, produced by pre-Sertoli cells, prevents the initiation of meiosis until puberty [214].

1.2.3 Postnatal establishment of the spermatogonial stem cell population

In the mouse, spermatogenesis begins on the first postnatal days (P) and the first spermatozoa are ready some 30 days later, depending on the mouse strain [216]. Between P3 and P5, gonocytes migrate inside the seminiferous cords from the central position to the basement membrane [217]. Subsequently, some of these differentiate directly into A1 spermatogonia and initiate spermatogenic progression. This first wave of spermatogenesis is inefficient and subject to large amounts of cell death [e.g. 162], but it does produce mature sperm at P40–P41 [218]. Simultaneously, the other gonocytes establish the undifferentiated spermatogonia population responsible for the self-renewal of spermatogonial stem cells (SSCs) and the production of differentiating offspring throughout adult life [218, 219]. During this period, proliferation must be favoured over differentiation. The number of SSCs is indeed increased 39-fold during the development from neonate to adult [220].

A functional stem cell niche is established during this neonatal period. The Sertoli cells acquire adult phenotype at P11 and cease dividing shortly after [221]. Maturing Sertoli cells establish intercellular occluding junctions, creating the blood-testis barrier between P10 and P16 in the mouse [222]. Henceforward, the seminiferous epithelium has two compartments: the basal compartment with spermatogonia and the adluminal compartment in which the spermatocytes undergo meiosis and differentiate further into spermatids and finally acquire the mature phenotype as spermatozoa (FIG. 4).

One intriguing feature of SSCs (and the haematopoietic stem cells) is homing, the ability to migrate to the environment where they are maintained, i.e. the niche. In transplantation experiments, the germ cells injected into the lumen of the seminiferous tubule attach to the Sertoli cells, traverse the blood-testis barrier and localize themselves into the basement membrane. Contacts with ECM proteins are postulated to play a major role in both the migration and retention of stem cells in the basal lamina, e.g. integrin β1-deficient SSCs have reduced transplantation efficiency [223]. The niche must guide the migration of spermatogonia [224]. A recent study identified CXCL12 as a chemotactic cue for the SSCs [225].
1.2.4 Steady-state spermatogenesis in adulthood

The testis is fully mature at 8 weeks post natum. In adult mice, the differentiation of spermatogonia proceeds in a highly ordered sequence, such that cohorts of differentiating germ cells (usually four in fixed combinations) are supported by a single Sertoli cell. Based on these specific germ cell associations, the seminiferous epithelium can be classified into stages. The spermatogenic wave is a succession of stages along the seminiferous tubule [228].

Studies of mammalian SSCs are mostly done during the expansion of the stem cell population (in prepubertal animals or after transplantation) or during postinsult regeneration [229].
Nevertheless, the establishment of the SSC pool and its maintenance are most likely regulated by different processes [230].

### 1.2.4.1 Spermatogonial stem cells

There is no unequivocal marker for SSCs. Therefore, their exact identity or localization is not known. SSCs are believed to be part of single type A spermatogonia (Asingles) residing along the basement membrane of the seminiferous tubule. During division, some daughters of Asingles remain as stem cells, while others, connected by intercellular bridges, give rise to undifferentiated spermatogonia, from a pair of two (A\textsubscript{paired}) to chains of 4, 8, 16 and sometimes 32 (A\textsubscript{aligned}), also called the progenitor spermatogonia [231–233]. Nevertheless, based on recent studies, it is apparent that the spermatogonial population with stem cell activity is more dynamic [226, 234] (FIG. 4).

Nakagawa et al. (2007) distinguished between the actual stem cell population (about 2000 cells per adult mouse testis) and potential stem cells able to self-renew after breakage of the intercellular bridges during damage or experimental manipulation [234]. Further lineage analysis has shown gradual changes in gene expression among undifferentiated spermatogonia that are correlated with their stem cell potential. The distribution of self-renewal capacity among the undifferentiated spermatogonia with different chain lengths provides plasticity for the differentiation pathway [226]. Whether aligned type A spermatogonia function as stem cells during steady-state spermatogenesis remains to be elucidated [232].

### 1.2.4.2 Spermatogonial stem cell niche

‘Niche’ refers to the milieu that promotes the self-renewal of stem cells. Consequently, the idea that differentiation is directed outside this location is inherent to the term. Undifferentiated spermatogonia are located unevenly along the periphery of the seminiferous tubule, preferentially in areas adjacent to the vasculature and interstitial tissue. When differentiating, they are seen to move away and become more evenly distributed [235]. Nevertheless, the available niches are also correlated with the number of Sertoli cells, not the contact area with the vasculature or interstitial tissue [236]. On the other hand, the cyclic changes in the production of growth factors by Sertoli cells may provide a temporal niche for stem cells favouring self-renewal in certain specific stages of the seminiferous epithelium cycle [237].

Mammalian spermatogenesis is regulated by paracrine factors inside the seminiferous epithelium and those with longer range from the interstitium and circulation. Testosterone from Leydig cells and follicle-stimulating hormone (FSH) secreted by the anterior pituitary are essential for qualitatively and quantitatively normal spermatogenesis. These hormones function mainly through the somatic cells of the niche [230]. Production of the factors essential for the proliferation and differentiation of spermatogonia, GDNF and SCF (KitL), respectively, is regulated in Sertoli cells by FSH [238, 239].

The Sertoli cells are recognized as major regulators coordinating various inputs and secreting paracrine factors for the germ cells. Nevertheless, multiple somatic cell types contribute to the niche environment, even though they are not in direct contact with the SSCs. Among them are the peritubular myoid cells surrounding the seminiferous tubules and various cell types resident in the interstitial tissue, e.g. Leydig cells; mesenchymal and immune cells and macrophages; in addition to the endothelium of small vessels and nerves [224] (FIG. 5).
The cyclic nature of sperm production demands that the niche factors change according to the seminiferous epithelium cycle. Sertoli cells express several gene products stage-dependently [e.g. 237, 244]. This can be seen in Sertoli cells at late embryonic age, implying an intrinsic capacity for cycling [245]. On the other hand, mouse Sertoli cells can support the differentiation of rat germ cells, requiring twice the time in mice [246]. Most likely, both the Sertoli cells and the spermatogonia have an internal `rhythm` but they must synchronize with each other for proper functioning of the seminiferous epithelium.

1.2.4.3 Proliferation and maintenance of SSCs

SSCs are constantly lost by either differentiation or death [234], and they must be replaced for spermatogenesis to continue. A recent study demonstrated the constant turnover of the stem cell pool. The cell fates follow a neutral drift, i.e. the stem cells are lost or maintained in a stochastic manner without relevance to past division history [229].

GDNF has been established as the major regulator of SSC self-renewal in vivo and in vitro (see Results and Discussion for details). In the presence of GDNF, mouse SSCs can be maintained and propagated in vitro on a feeder cell layer in a chemically defined medium supplemented
with FGF2 and, with the SSCs from most mouse lines, soluble GFRα1 [247]. SSCs are usually enriched from testis cell suspensions by cell surface antigens, usually thymocyte differentiation antigen 1 (Thy1) or GFRα1 [248, 249]. Based on transplantation assays, the stem cell activity is maintained over months and the SSC numbers increase exponentially [247, 250].

Even though GDNF, expressed by the Sertoli cells [e.g. 244], is crucial to the proliferation of undifferentiated spermatogonia, there must be other niche factors regulating the appropriate balance between self-renewal and differentiation [224]. Colony stimulating factor 1 (CSF1) enhances the stem cell expansion but not the overall cell proliferation in SSC culture. CSF1, produced by Leydig and peritubular myoid cells, may act upon a subset of spermatogonia expressing CSF receptor 1 and direct them to self-renewal in concert with GDNF [241].

In addition to extrinsic signalling molecules, the cell-fate decisions of SSCs are dependent on intrinsic factors. Promyelocytic leukaemia zinc finger (Plzf) is a transcriptional repressor expressed in all undifferentiated spermatogonia [237, 251, 252]. In mice with deletion of Zfp145, the gene coding for Plzf, or in natural mutant luxoid, the initial increase in the proliferation of spermatogonia is followed by the exhaustion of SSCs, leading to atrophic tubules with Sertoli cells only [252, 253]. Nanos2, a posttranscriptional regulator, is expressed predominantly in the A singles and A paired that harbour the stem cell activity. Nanos2 deficiency induced in postnatal mice led to gradual loss of stem cells. Nanos2 overexpression, on the other hand, suppressed the differentiation and led to the accumulation of Plzf+ spermatogonia [227, 254]. Therefore, both Nanos2 and Plzf maintain the undifferentiated state in spermatogonia.

### 1.2.4.4 Signals for the differentiation of spermatogonia

During stages VII and VIII of the seminiferous epithelium cycle, almost all A aligned differentiate into A1 spermatogonia [232]. This transition implies commitment to meiosis and can occur in the undifferentiated spermatogonia of variable chain length with the probability of increasing in longer chains [226]. Surprisingly, the fate decision of a single cell is apparently stochastic and not dependent on the lineage or environmental cues. Nevertheless, cells adopt the fates of self-renewal versus differentiation at population levels with fixed probability under certain culture conditions, such as growth factor supplementation [255]. Environmental cues can change the odds between the developmental outcomes.

The commitment of stem cells to the differentiation pathway is difficult to study, because this transition lacks definitive markers and the differentiating germ cells die in standard culture [224, 255]. Neuregulin 1 and 3 (NRG3), members of the EGF superfamily, stimulated the proliferation and the meiotic entry of spermatogonia, and neuregulin 1, especially in combination with GDNF, promoted the formation of aligned A spermatogonia in vitro [242, 256].

Initiation of meiosis is regulated by RA. Mice maintained on a vitamin A-deficient diet display spermatogenic arrest and the accumulation of undifferentiated spermatogonia [257]. RA replacement restores synchronized spermatogenesis in these mice [243, 258]. The effect of RA on meiotic initiation may be mediated by production of neuregulin 1 in Sertoli cells [242]. During steady-state spermatogenesis, the regulation of RA signalling may drive the cyclic differentiation of spermatogonia. The differentiating germ cells seem to regulate the local RA levels to which Sertoli cells respond by pacing their rhythm [243].
A classical marker for differentiating spermatogonia, RTK Kit, is a receptor for SCF/KitL. Kit is expressed in the late A aligned spermatogonia onwards, and it regulates the survival and proliferation of the differentiating A1 spermatogonia [259]. Kit/KitL is also essential for PGC development and Kit mutant mice, white spotting (W), are virtually devoid of all germ cells and used as recipient mice in SSC transplantations [247, 259].

BMP family members play a role in germ cell differentiation. BMP4 is highly expressed in early postnatal Sertoli cells and its receptor ALK3 in the spermatogonia of comparable age. In vitro BMP4 exposure induces the expression of Kit, indicating the differentiation of germ cells [259, 260]. This is in agreement with the decrease in SSC numbers during short-term culture of spermatogonia with exogenous BMP4 [261]. Both Bmp4+/- mice and hypomorphic Bmp4 mutants without proper protein maturation have spermatogenic defects [262, 263].

Most Bmp8b-deficient mice are sterile. BMP8b activity seems to be necessary for both the initiation and maintenance of spermatogenesis [240]. Bmp8b is expressed transiently in spermatogonia during early puberty and at high levels in round spermatids in adults. The primary spermatocytes are the first germ cell population to be depleted in Bmp8b+/- mice. High levels of BMP8b produced by the round spermatids at stages VI and VII most likely signal as a paracrine factor to Sertoli cells and the younger generations of germ cells [240].

1.2.5 Cell death in the male germ cell lineage

It has been estimated that only 25% of the calculated number of early spermatocytes are derived from A1 spermatogonia, and only a fraction of the hypothetical SSC descendants reach maturity [264]. The large population of undifferentiated spermatogonia provides plasticity in the case of stochastic fluctuations or accidental depletions in the stem cell population. Apoptosis of male germ cells seems to be regulated by the balance between the anti- and proapoptotic Bcl2 family members and is relatively inflexible in dependence on certain family members [265]. The narrow margin between life and death is most likely a prerequisite for the mass production of high-fidelity haploid genomes. It may also protect parent animals against the adverse effects of early germ cells which, besides being motile, can revert to pluripotency [266–268].

1.2.5.1 Primordial germ cells

SCF is essential for PGC survival from their specification onwards. The migration of PGCs is partially guided by SCF expressed in the surrounding tissues. Substantial numbers of PGCs are left along the route or go astray. They are removed by Bax-mediated cell death outside the range of the SCF survival signal [266].

Rapid proliferation of the PGCs is accompanied by an apoptotic wave around E13.5 [161]. The survival of PGCs during this stage is dependent on the balance between Bclx and Bax. The reduced number of PGCs in hypomorphic Bclx mutants indicates Bclx as the principal cell survival molecule after the arrival of germ cells in the gonads [265].

1.2.5.2 The first wave of spermatogenesis

Cell death is especially prominent during the first wave of spermatogenesis and is observed in mice from the second to the fourth week, peaking at the third week of postnatal life [162, 269]. These death events occur mostly among the differentiating A spermatogonia and to a lesser extent in primary spermatocytes but, in contrast to adults, frequent apoptosis is
detected in pachytene spermatocytes at stages VII and VIII of rat seminiferous epithelium [270]. Germ cells die as clones and are removed by the Sertoli cells [271].

Neurons, haematopoietic systems and spermatogenesis are especially sensitive to the levels of the Bcl2 family proteins [172]. The crucial step in apoptosis by the intrinsic pathway is Bak or Bax homodimerization to form pores in the mitochondrial outer membrane and the subsequent activation of the caspase cascade [173]. Even though the double-mutant Bak;Bax<sup>−/−</sup> mice have multiple developmental defects [272], mere Bax<sup>−/−</sup> mutants exhibit limited hyperplasia of neuronal and lymphoid tissue and disrupted spermatogenesis [273].

Bax deficiency leads to atrophic testes and sterility in adults. These changes are preceded by reduced apoptotic death and the expansion of the spermatogonial population [273, 274]. Similar changes are seen in mice overexpressing Bcl2 or BclxL, the long-splice variant of Bclx [269, 275]. The inhibition of apoptosis during the first round of spermatogenesis leads to a defect in testis maturation and a failure of steady-state spermatogenesis [269, 274].

The early apoptotic wave may adjust the amount of germ cells to the Sertoli cells, as in adult mice. Nevertheless, the hormonal immaturity of prepubertal animals seems to heighten the amount of cell death, because this early apoptosis can be reduced by testosterone injections [269]. The supporting structures are also immature: the peak of spermatocyte apoptosis occurs in those tubules without a patent blood-testis barrier [276]. The first wave of spermatogenesis also differs from the later spermatogenesis, because the seminiferous epithelium lacks the more mature germ cells. Spermatocytes in the immature testis are left without survival signals, such as BMP8a/b from round spermatids [240, 270, 277]. Most likely, germ cells are needed for the maturation of the somatic environment, which is not yet ready to support quantitatively normal differentiation.

### 1.2.5.3 Adult mice

Germ cells are very sensitive to apoptotic death (e.g. the inhibition of protein synthesis induces germ cell death), especially in particular stages [278]. This stage-specificity of cell death, e.g. at stages I, XII and XIV in rats, can be observed in both natural death events and in the apoptosis induced by extrinsic factors [278–280]. At these specific stages, cells from all associated germ cell types are dying, indicating the regulatory role for Sertoli cells in germ cell PCD [278]. This synchronized cell death of multiple germ cell types may be one reason why the loss of certain cohorts of germ cells often leads to the depletion of the entire seminiferous epithelium, frequently in patches [281].

Sertoli cells can only nurse a species-specific number of germ cells through the differentiation process [282]. In the adult testis, the undifferentiated spermatogonia are not evenly distributed, but adjustment of the germ cell number to the Sertoli cell capacity is achieved by the apoptotic elimination of A<sub>2</sub>, A<sub>3</sub> and A<sub>4</sub> spermatogonia as clones [231, 264]. The apoptotic death of germ cells in adults coincides with critical transition steps: mitotic peaks in A<sub>2</sub>–A<sub>4</sub> spermatogonia and chromatin rearrangements, with the risk of genetic defects, in premeiotic spermatocytes [281]. The DNA repair system halts the cell cycling, but the prolonged arrest leads to cell death. This may be caused by a decline in prosurvival protein levels, the accumulation of p53 and the transcriptional activation of its proapoptotic target genes (e.g. Fas and Bax) [283].
The expression of Bcl2 family proteins in the testis is developmentally regulated. Bcl2 seems not to play a role in male germ cells [269]. Bclw is essential for the survival of the apoptosis-reluctant, postmitotic Sertoli cells [284, 285]. The expression of proapoptotic Bax coincides with the early peak of germ cell apoptosis, especially in pachytene spermatocytes [269, 270]. Upregulation of Bax expression seems to sensitize the differentiating spermatogonia to apoptosis, while the subsequent Bax downregulation and the expression of prosurvival family members counteracts this effect. Factors regulating germ cell differentiation, such as SCF, may convey their survival effect by the balance between the pro- and antiapoptotic Bcl2 family members [280].

The surplus germ cells in Bax−/− mice undergo Bax-independent apoptotic cell death at late puberty when these cells enter the adluminal compartment. This indicates that PCD in germ cells is regulated by different mechanisms along their differentiation pathway [274]. Cell death in spermatocytes has special features and can involve the extrinsic apoptotic pathway via the Fas/FasL system [286].

### 1.2.5.4 Spermiogenesis

In the course of spermiogenesis, a germ cell undergoes cellular rearrangements, e.g. the removal of excess cytoplasm and cellular organelles as residual bodies that have morphological features resembling apoptotic bodies. The activation of the apoptotic process in a special cytoplasmic compartment of an elongating spermatid leads to the formation of a residual body and functional, healthy spermatozoa [287]. At the molecular level, the function of apoptosis-related protein in the TGFβ signalling pathway (ARTS) (coded by the Sept4 locus), possibly regulating caspase activity through the antagonism of inhibitor of apoptosis proteins (IAPs), is essential for spermiogenesis. Sept4−/− mice display a defective removal of residual cytoplasm [288].
2 AIMS OF THIS STUDY

- To determine the roles of programmed cell death in kidney morphogenesis and the factors regulating cell fate, survival and death during meso- and metanephric kidney development (I, III and unpublished results).
- To evaluate the function of GDNF in the testis and, specifically, the impact of the GDNF dosage on the cell fates of undifferentiated spermatogonia (II).

Due to the lengthy period between the publication of the original articles and the composition of this thesis, certain subsequent advances related to our findings are reviewed with some length in Results and Discussion.
3 MATERIALS AND METHODS

3.1 Organ culture

Embryonic tissues were dissected from embryos of Sprague-Dawley rats or CBA x NMRI mice. The day after overnight mating (rats) or the appearance of a vaginal plug (mice) was counted as day zero of embryogenesis (E0). The embryonic development in rat kidneys was considered to be 2 days behind that of mice (E11 in mice, corresponding to E13 in rats). All experimentation with laboratory animals was approved by local authorities.

Kidney rudiments were grown in Trowell-type organ culture on Nucleopore filters (Costar, Corning Inc., Corning, NY, USA) in an air-medium interface. Eagle’s Minimum Essential medium (Gibco/Life Technologies Corp., Carlsbad, CA, USA) supplemented with 10% fetal calf serum was used as a basic medium and changed every second day.

The MMs were dissected free from the UB tissue after pancreatin-trypsin treatment with needles under a stereomicroscope. Mesenchymes considered ‘young’ and ‘old’ were classified according to UB morphology from litters with the same gestation period: ‘young’ representing bud (E11) and ‘old’ T-bud stage (E11.5 →).

Medium containing recombinant human BMP4 (rhBMP4) (R&D Systems Inc., Minneapolis, MN, USA) was changed every second day. In the experiments with a peptide inhibitor of caspase activity (z-Val-Ala-Asp(OMe)-CH2F) (zVAD-fmk) (Enzyme System Products Inc., Livermore, CA, USA), the medium was changed at 24-h intervals.

3.2 Immunohistochemistry

Immunohistochemical stainings were performed in cultivated explants as previously described [51]. Briefly, the samples, fixed in ice-cold methanol for 10 min, were incubated with primary antibodies overnight at 4 °C, washed three times for 2 h with phosphate-buffered saline (PBS) before overnight incubation with the respective fluorochrome-conjugated secondary antibodies. After the washes, the samples were mounted in ImmuMount (ThermoFisher Scientific Inc., Waltham, MA, USA). The primary antibodies are listed in TABLE 4.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>source</th>
<th>reference</th>
<th>used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>pan-cytokeratin</td>
<td>Sigma</td>
<td>[118]</td>
<td>l</td>
</tr>
<tr>
<td>EHS-laminin</td>
<td>Sigma</td>
<td>[289]</td>
<td>l</td>
</tr>
<tr>
<td>α-smooth muscle actin</td>
<td>Boehringer Mannheim Biochemicals</td>
<td>[290]</td>
<td>l</td>
</tr>
<tr>
<td>anti-rat low-affinity neurotrophin receptor (p75)</td>
<td>Boehringer Mannheim Biochemicals</td>
<td>[98]</td>
<td>l, unpub.</td>
</tr>
</tbody>
</table>

TABLE 4. Primary antibodies used in immunohistochemistry.

3.3 TUNEL staining

Terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) [292] was performed according to the manufacturer’s instructions for the ApopTag in situ Apoptosis Detection Kit (Oncor Inc., Gaithersburg, MD, USA). The samples were fixed for 10 min in 66%
ethanol + 33% acetic acid and 30 min in 4% paraformaldehyde (PFA). In whole-mount samples, the incubation time with TdT enzyme was extended for 2 h at 37 °C, followed by an overnight wash at 4 °C, as described in [293]. Detection of labelled cells was done with a fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibody, incubated overnight, followed by three 2-h washes with PBS. The structures were visualized by immunostaining of the samples after the TdT reaction. The specificity of the detection method was tested by omitting the TdT enzyme in some control reactions.

Double detection of apoptosis and radioactive in situ hybridization in sections was done by performing TUNEL staining after the radioactive in situ hybridization procedure. Briefly, the developed and washed slides were treated with the equilibrium buffer of the Apoptag kit. The TdT reagent was applied to the samples and a plastic coverslip was placed on top. The TdT reaction was performed at 28–30 °C for 3-h to avoid melting of the emulsion.

### 3.4 In situ hybridization

Whole-mount in situ hybridization was performed according to Wilkinson (1993) and described in (I) [294]. The cultivated kidneys were fixed in ice-cold methanol for 2 min and then in 4% PFA overnight. The proteinase K treatment (10 μg/ml) was 40 s at room temperature.

Radioactive in situ hybridization of the sections was performed according to Wilkinson and Green (1990) with some modifications, as described in (III) in detail [295]. The probes for in situ hybridizations are listed in TABLE 5.

<table>
<thead>
<tr>
<th>Probe</th>
<th>origin</th>
<th>reference</th>
<th>used in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp4</td>
<td>P. Sharpe</td>
<td>[296]</td>
<td>I</td>
</tr>
<tr>
<td>Bmp7</td>
<td>J. M. Wozney</td>
<td>[297]</td>
<td>I</td>
</tr>
<tr>
<td>ALK3 (Bmpr-IA)</td>
<td>D. Huylebroeck</td>
<td>[298]</td>
<td>I</td>
</tr>
<tr>
<td>ALK6 (Bmpr-IB)</td>
<td>D. Huylebroeck</td>
<td>[298]</td>
<td>I</td>
</tr>
<tr>
<td>Gdnf</td>
<td>P. Suvanto</td>
<td>[299]</td>
<td>I</td>
</tr>
<tr>
<td>Ret</td>
<td>V. Pachnis</td>
<td>[67]</td>
<td>I</td>
</tr>
<tr>
<td>Pax2</td>
<td>G. Dressler</td>
<td>[300]</td>
<td>I</td>
</tr>
<tr>
<td>Wt1</td>
<td>J. Kreidberg</td>
<td>[301]</td>
<td>I</td>
</tr>
<tr>
<td>Fas</td>
<td>S. Nagata</td>
<td>[302]</td>
<td>III</td>
</tr>
<tr>
<td>Fasl</td>
<td>S. Nagata</td>
<td>[303]</td>
<td>III</td>
</tr>
<tr>
<td>ΔKS+</td>
<td>T. Immonen</td>
<td></td>
<td>III</td>
</tr>
</tbody>
</table>

**TABLE 5.** Probes used for in situ hybridizations.

### 3.5 Histology

The dissected testes were fixed in Bouin’s fixative or 4% PFA. For examination of histology, the sections were stained with haematoxylin-eosin.
4. RESULTS AND DISCUSSION

4.1 Programmed cell death in kidney development

4.1.1 Mesonephric regression coincides with initial development of the metanephric kidney (unpublished results)

Cells undergoing programmed cell death were detected by TUNEL staining of paraffin sections of the urogenital area and developing metanephric kidneys of various embryonic ages from both rat and mouse. The distribution of TUNEL+ cells, *e.g.* in the stroma of the NZ and in close proximity to the developing nephric structures, was in accordance with previous studies by Koseki *et al.* (1992) and Coles *et al.* (1993) and colocalized with typical nuclear changes of the apoptotic cells, *i.e.* condensation of the nucleus and fragmentation of the cells to the apoptotic bodies [114, 116, 159].

In the caudal mesonephric region, prominent apoptotic cell death was detected in sections of E13 rats (unpublished results, data not shown). This represents the beginning of the mesonephric regression, as reported in E10.5 mice [26, 75, 304] and E13 rat [305, 306]. The spatial and temporal correlation of mesonephric PCD with the delimitation of the MM, which has a diffuse anterior border at this stage (our observations and [27]), suggests that the mesonephric regression may play a morphogenetic role in sculpting the metanephric rudiment.

4.1.2 PCD in embryonic kidneys cultured *in vitro* (I, III and unpublished results)

*In vitro* cultured and whole-mount immunostained kidney rudiments were three-dimensional (although flattened and compressed under the coverslip) and gave a `stacked` impression. The relatively rare cell-death events can be interpreted in the context of structures, if suitable markers are available. The temporal changes in the distribution of PCD in the embryonic kidneys cultured *in vitro* were studied by whole-mount TUNEL staining combined with markers for ureteric epithelium (*Dolichos biflorus* lectin) and CC (p75 NTR) [98, 291].

In the metanephric rat kidney rudiments (E13) cultured for 25 h, PCD was very rare in CCs and occurred mostly in cells at the kidney periphery (I, Fig. 4 I–L; FIG. 6). Contact with UB and subsequent condensation seems to cause an internal change in the threshold of nephrogenic cells to undergo PCD. A band of apoptotic cells surrounded the CCs and traversed the mesenchyme between the kidney rudiment and the WD. The periductal mesenchyme itself was markedly devoid of apoptotic cell death.
RESULTS AND DISCUSSION

FIGURE 6. An E13 rat kidney explant cultured in vitro for 24 h. Apoptotic cells, detected by TUNEL staining (on the left, the ureteric epithelium visualized with Dolichos biflorus lectin), surround the cap condensates (immunostained with antibody against p75 NTR on the right) and pass the mesenchyme (indicated by arrows) between the kidney proper and the mesenchyme around the Wolffian duct. Scale bar = 400 µm.

PCD during the initial stages of metanephric development has received little focus. A confocal microscopy analysis by Foley and Bard (2002) reported substantial amounts of apoptosis in both the stromal area and stem cells at E11.5 of mice [163]. Nevertheless, the cell populations were identified only by morphological criteria. Based on recent studies, cells dying at the kidney periphery may not be stromal progenitors but nephrogenic cells not recruited to the condensate [15, 45, 139]. The death of these cells may facilitate the formation of the primary condensate, the integration of stromal cells or the interactions between the CC and stroma [128, 136, 139, 142, 147–149]. PCD among the peripheral cells may also demarcate the kidney rudiment and ‘loosen’ the surrounding mesenchyme – a process defective in kidneys of FoxD1 and Hox10 triple-mutant mice unable to detach from the body wall [139, 140, 142]. The complex phenotype of angiotensin type 2 receptor (Agtr2) null mice was associated with the attenuated apoptosis of undifferentiated mesenchyme around the metanephros and urinary tract [307].
RESULTS AND DISCUSSION

After 48 h, apoptotic cell death was detected among the embryonic stroma between the deep branches of the ureter (FIG. 7). To confirm this apoptosis as a normal developmental event, we did TUNEL stainings on the paraffin-embedded sections of rat E15 kidneys. Abundant apoptotic cells were observed in the nascent medullary area, subsequently accommodating the elongating tubules and collecting ducts (III, Fig. 3 B–D, F). Accordingly, the peak apoptotic index in mouse kidneys was reported at the corresponding age (E13.5), the cell deaths occurring mostly in the developing medulla [152]. Some dying cells in the medulla region may derive from the disintegrating nephric structures formed by the first rounds of nephron induction [28, 166].

![FIGURE 7](image)

**FIGURE 7.** An E13 rat kidney cultivated for 3 days in vitro. The apoptotic cells were stained with TUNEL labelling (green) and the ureteric epithelium was visualized by Dolichos biflorus lectin (red). Scale bar = 250 µm.

The experimental prevention of cell death could help to elucidate the roles of PCD during development. For this, we performed a series of preliminary experiments with the pan-caspase inhibitor zVAD-fmk. In our hands, 100 µM zVAD-fmk reduced the apoptosis among the stromal cell population in a 3-day culture of E11 mouse kidneys (FIG. 8 A–D). However, the dimethyl sulphoxide (DMSO) used as a solvent for the inhibitor caused some adverse effects on UB branching (when the controls with the 1% DMSO solvent were compared with samples cultured in basic medium) and consequently this line of experimentation was abandoned.
FIGURE 8. Examples of experiments with caspase inhibitor zVAD-fmk (A and C control (DMSO) and B and D 100 µM zVAD-fmk in DMSO). In 3-day culture of mouse kidneys, zVAD-fmk decreased apoptotic cell death (detected by TUNEL staining) among the stromal cell population (white asterisk), even though death among cells surrounding the nephrogenic mesenchyme was still prominent (ureteric epithelium was visualized by Dolichos biflorus lectin in A and B). Scale bar = 400 µm.

This same experimental approach was used in three investigations by other groups. The results reported may reflect the developmental stage-specific responses of kidney rudiments to the inhibition of apoptosis, in addition to differences in the culture conditions. Araki et al. (1999, 2003) found that the caspase inhibitors Ac-IETD-CHO (caspase 8) and Ac-DEVD-CHO (caspase 3/7) reduced the UBM and inhibited the differentiation of nephrons in E12 kidneys in vitro [169, 170]. The authors suggested that the removal of undifferentiated mesenchymal cells is essential for the interaction between the UB and MM [169, 170]. After a preculture period, the inhibitor had a smaller adverse effect on branching [170]. In studies by Dziarmaga et al. (2003) and Clark et al. (2004), the treatment of E13.5 kidneys with zVAD-fmk increased ureteric branching and nephron number, and this effect was attributed to the inhibition of apoptosis in the ureter [179, 181]. In vitro culture conditions were suggested to have caused a considerable amount of additional apoptotic cell death in these studies [181]. Nevertheless, the explants of E11 kidneys, such as ours, are much smaller and subject to lesser adverse effects of culture than E13.5 kidney rudiments. Daily in vivo administration of zVAD-fmk by Clark et al. (2004) to pregnant females while embryos were E10.5–E17.5 reduced apoptosis in kidneys by 50% but caused no obvious malformations [181].
4.1.3. Artefactual in situ hybridization signal from pBluescript vector cloning site is associated with apoptosis (III)

During our in situ hybridization experiments, the signals with certain probes were artefacts. The signal was derived from a stretch of polylinker sequence incorporated into the multiple cloning site of several vectors from Stratagene/Agilent Technologies (La Jolla, CA, USA) (III, Fig. 1). This hybridization signal displayed rat-specific distribution at the same locations as the prominent PCD (III, Fig. 2 and Fig. 3 A–C). By combining TUNEL staining with radioactive in situ hybridization, we were able to colocalize the artefactual signal with TUNEL+ cells, preferentially to those with large diffuse nuclei, most likely representing the early stages of cell death (III, Fig. 3 D–F).

Short sequences of vector polylinker incorporated into in vitro-transcribed probes were not considered to interfere with the specificity of hybridization. Accordingly, our study revealed a real threat to the reliability of in situ hybridization, because the sequence identified can be part of either the sense or antisense probe, depending on the direction of the insert and the orientation of the multiple cloning site in the vector variety used (such as Bluescript SK vs. KS). When incorporated into the antisense probe, a combination of real and artefactual signals can be created that is impossible to recognize by standard controls (see Millar et al. (1994) and Blödorn et al. (1998) and references herein [308, 309]).

Artefactual signals with probes transcribed from vectors harbouring the same cloning site have been reported twice previously, but have not been associated with apoptosis. One of the affected tissues is rat testis, in which the residual bodies engulfed by the Sertoli cells showed intense staining [308]. Most interestingly, the residual bodies are formed in the spermatids by a subcellular apoptotic process [287]. The artefactual signal was specifically located in stages IX and X of the seminiferous epithelium cycle when the residual bodies were being degraded, but still contained RNA [308]. In another report, the artefactual signal was detected in rat and porcine neurons [309]. The regions in the multiple cloning site causing the artefact partially overlapped the sequence identified by us (III, Fig. 1 B) [308, 309]. The identity of the target nucleic acid has remained unknown.

4.2 Effects of BMP4 on development of the metanephric kidney

We approached the regulation of cell fates and PCD during metanephric kidney development by studying the effects of BMP4, expressed in embryonic kidney from E11.5 onwards [310] in organ culture. The early mortality of Bmp4 null animals [311] precluded the transgenic approach to organogenesis. Previous studies showed that BMP4 mediates epithelial-mesenchymal interactions and regulates cell differentiation, as does PCD, e.g. during tooth organogenesis [312, 313].
RESULTS AND DISCUSSION

FIGURE 9. BMP signalling pathways. BMPs bind as dimers to type I serine/threonine kinase receptors that serve as high-affinity anchoring receptors. BMPs have different affinities for individual type I BMP receptors, but the in vivo interactions in cellular contexts are currently inadequately known [314]. In vitro binding assays suggest that BMP2 and BMP4 both bind ALK3 (BMPR-IA) and ALK6 (BMPR-IB) type I receptors, whereas the other essential BMP in kidney development, BMP7, also signals through activin receptor ALK2 (ActRI) [314–316].

The best characterized signalling pathway for BMPs is transcriptional regulation by Smads. ALK3 and ALK6 activation leads to the phosphorylation of Smad 1, 5 and 8 (receptor-associated, R-Smad). Most BMPs activate R-Smads without selectivity. R-Smads complex with the common mediator Smad4, also conveying signals from other TGFβ family receptors. In BMP activation, Smad dimers accumulate in the nucleus and regulate transcription. The tissue specificity of BMP signals is believed to arise from accessory transcription factors (Co) and the epigenetic state of the responding cell [314, 317].

BMPs also signal through MAP kinases. TGFβ-activated kinase (TAK) binds to the intracellular tail of BMP type I receptor in a complex with X-linked inhibitor of apoptosis protein (XIAP) and TAB and in receptor activation phosphorylates JNK and p38 MAPK [318]. Adapted from [319].
4.2.1  BMP4 inhibits ureteric budding from the Wolffian duct (I)

At the earliest developmental stage we studied, E10 mouse urogenital rudiment cultured *in vitro*, recombinant human BMP4 protein (rhBMP4) at a final concentration of 75 ng/ml arrested UB outgrowth at the bud stage in 43% of the explants. The rest (57%) showed very limited ureteric growth and branching during the 3-day culture period. In all controls, UB grew and in most explants branched extensively (I, Fig. 3; Table 1).

The developmental mechanism behind this phenomenon was not understood by us, but subsequent progress in BMP4 research, especially the findings of Miyazaki *et al.* (2000) and Michos *et al.* (2007), showed that BMP4 is essential for the regulation of UB outgrowth at proper locations [320, 321]. The budding site not only impacts kidney development but also ureteric maturation. CAKUT is a common class of human congenital malformations, including metanephric defects such as hypo/dysplastic kidneys, gross malformations of the urinary outflow tract and various forms of multiplex structures [206]. CAKUT has been associated with defective ureterovesical junctions at either a more distal position in the bladder or non-remodelled locations in WD derivatives, causing obstruction of urine flow [322].

All Bmp4 null mice die before E9.5 [311]. The phenotype of heterozygous Bmp4 mice has been extensively studied. In all, 12–53% of Bmp4+/- mice, depending on the study, exhibit CAKUT-type defects in the inbred C57Bl/6 background [320, 323, 324]. The most common finding is hydronephrosis/ureter associated with an abnormally caudal ureterovesical junction (TABLE 6). The UB was in the anterior location in most Bmp4+/- mice [320]. Unlike some other mouse models of CAKUT, Bmp4+/- mice do not have additional UBs from the WD (some Bmp4+/- mice have bifid ureters, *i.e.* two ureters with a common caudal part, originating from an additional bud from the UB stalk, see later) [320]. In contrast, 50% of the induced Bmp4 null embryos have one additional bud anterior to the bud at the normal location [325].

The activity of BMPs is regulated by numerous extracellular binding proteins [314]. Targeted deletion of Gremlin 1 (Grem1), a BMP antagonist with a high affinity for BMP2 and BMP4, causes arrested UB outgrowth and kidney agenesis [304]. At E11, Bmp4 is expressed in the mesenchyme surrounding the WD and nascent UB [310, 321, 326]. UB outgrowth in Grem1/- mice can be restored not only by lowering the Bmp4 dose (Bmp4+/-) but also, quite surprisingly, in the double-mutants Grem1;Bmp7-/- [321, 325]. The reduction in overall BMP activity is needed at the location where the UB will form.

The mechanism by which BMP4 inhibits UB outgrowth is unresolved. BMP4 can abolish GDNF activity without changing its expression pattern [40, 320, 321]. Deletion of ALK3 or Smad4 in the UB lineage causes no additional UBs, even though a minority (5%) of mice overexpressing ALK3 in UB have kidney aplasia or severe dysgenesis [45, 327, 328] (FIG. 9). BMP4 likely binds to another receptor on the WD or the effect is mediated through the periductal mesenchyme, a cell population whose nature and functions are inadequately known. Low levels of phosphorylated Smad1 (pSmad1) in the ventral mesenchyme overlap the Grem1 expression at the site of UB outgrowth [321]. Bmp4 itself seems to be regulated by Gata2, a transcription factor, the expression of which is controlled in the urogenital region by three separate enhancers, each active in a spatially restricted compartment [329–331].
Defective signalling in periductal mesenchyme

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bmp</strong>+/−</td>
<td>one anteriorly located UB, hypo/dysplastic kidneys, hydroureters, abnormal ureterovesical junction, additional bud from UB trunk in some mice, duplex kidneys, bifid ureters</td>
</tr>
<tr>
<td><strong>Bmp4</strong>+/−</td>
<td>one anterior additional UB</td>
</tr>
<tr>
<td><strong>Gata2 hypomorph</strong></td>
<td>anteriorly located UB, abnormal ureterovesical junction, hydroureters/nephrosis</td>
</tr>
<tr>
<td><strong>Grem1</strong>−/−</td>
<td>no UB outgrowth</td>
</tr>
<tr>
<td><strong>GREM1 in vitro</strong></td>
<td>additional UBs</td>
</tr>
<tr>
<td><strong>Fgfr2</strong>−/−</td>
<td>anterior additional UB, hydroureters, duplex kidneys</td>
</tr>
<tr>
<td><strong>Agtr2</strong>−/−</td>
<td>hydroureters/nephrosis, ureter obstruction, hypo/dysplastic kidneys, renal aplasia</td>
</tr>
</tbody>
</table>

Defect in GDNF expression/activity

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GDNF in vitro</strong></td>
<td>UBs along caudal WD</td>
</tr>
<tr>
<td><strong>Hox7b-Gdnf</strong></td>
<td>UBs throughout the WD-derived structures</td>
</tr>
<tr>
<td><strong>Gdnf protein in vitro on GDF11</strong>−/−</td>
<td>more UBs than in the wild type, ureter hypersensitive to GDNF effect</td>
</tr>
<tr>
<td><strong>FoxC1</strong>−/− congenital hydrocephalus mice</td>
<td>additional UBs, multiplex kidneys, multiple ureters, one of which is properly remodelled, ectopic mesonephric tubules</td>
</tr>
<tr>
<td><strong>Slit2</strong>−/−, <strong>Robo2</strong>−/−</td>
<td>additional UBs, multiplex kidneys, hydroureters, no ureter maturation</td>
</tr>
</tbody>
</table>

Defect in Ret signalling and/or ureteric differentiation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RetY1015F</strong></td>
<td>additional UBs, multiplex kidneys, multiple hydroureters, no ureter maturation, ectopic mesonephric tubules</td>
</tr>
<tr>
<td><strong>Ret</strong>−/−</td>
<td>renal agenesis, severe hypo/dysplasia, no ureter maturation in some animals</td>
</tr>
<tr>
<td><strong>Rara;Rarb2</strong>−/−</td>
<td>renal hypoplasia, hydroureters/nephrosis, no ureter maturation</td>
</tr>
<tr>
<td><strong>Ptprs</strong>−/−;<strong>Ptprf</strong>∆P/∆P</td>
<td>duplex systems in some animals, no ureter maturation</td>
</tr>
<tr>
<td><strong>Spry</strong>−/−</td>
<td>additional ureters, multiplex kidneys, hydroureters, no ureter maturation</td>
</tr>
<tr>
<td><strong>GATA3</strong>−/−</td>
<td>additional UBs, duplex kidneys, hypo/dysplastic kidneys, hydroureters, renal aplasia</td>
</tr>
<tr>
<td><strong>Hox7b-Jagged1</strong></td>
<td>hypoplastic kidneys, hydroureters, renal aplasia</td>
</tr>
</tbody>
</table>

**TABLE 6.** Some mouse models with Congenital Anomalies of the Kidney and Urinary Tract (CAKUT). The right location of a single bud is achieved by a complex interplay between the Wolffian duct (e.g. changes making the epithelium unstable or subject to augmented MAPK signalling), the proper expression of trophic factors in the metanephric mesenchyme and the activity of modulating factors in the periductal mesenchyme.
4.2.2 Effect of BMP4 on ureteric branching morphogenesis (I)

4.2.2.1 rhBMP4 inhibits ureteric branching

In our study, rhBMP4 reduced the number of UB tips of E11 mouse kidney explants in a dose-dependent manner: the effect was clearly seen at a concentration of 50 ng/ml and was more pronounced at 100 ng/ml (I, Fig. 1 B; Fig. 2 G–I). The treatment of E12 kidneys with 100 ng/ml of rhBMP4 caused only minor branching defects (data not shown). The rhBMP4 also influenced the UB stalk growth by making the ureter shorter but thicker (I, Fig. 1 C, D). These results are in agreement with studies by other groups showing that BMP4 (or BMP2, a protein with 94% homology with BMP4) reduces ureteric growth and branching [73, 202, 324, 326, 335–337].

Kidney organ culture is a complex system to study. The observed effect on ureteric branching can be caused directly by the ureteric epithelium, mediated through changes in the MM or both. We studied the distribution of signalling receptors binding BMP4 [315, 316] by in situ hybridization. The type I BMP receptor ALK3 was ubiquitously expressed in both ureteric epithelium and the MM and upregulated in the CCs (I, Fig. 4 A), whereas the ALK6 receptor was restricted to the ureter (I, Fig. 4 B) [298, 320].

A study by Bush et al. (2004) showed that BMP4 and some other members of the TGFβ family can directly influence the growth of the ureteric epithelium. In UB cultures, BMP2, BMP4, activin and TGFβ1 inhibited growth and branching of the UB [202]. Studies with conditional ALK3 mutants were in accordance with direct inhibitory function for BMPs on UBM. Expression of constitutively active ALK3 in the ureter reduces ureteric branching [327]. Accordingly, mice deficient in ALK3 in the UB lineage have additional ureteric branches in the first- and second-order branch generations. This is followed by severe reduction of UBM [328].

Miyazaki et al. (2000) proposed that BMP4 promotes ureteric elongation [320]. Bmp4-/- mice have short UB stalks and stems of the first branches at E11.5, and BMP4 increased the growth of the ureter in glycosaminoglycan-striped kidney explants [198, 320]. A stimulating effect on ureteric growth was also reported for BMP7, which has, in kidney cultures, either a growth-promoting effect at low concentration or inhibitory influence on UBM at higher concentrations [336]. Inhibitory, high BMP7 doses activate Smad1, but a stimulating effect at low concentrations is mediated by p38 MAPK activation [338, 339].

4.2.2.2 BMP4 - a physiological regulator of UBM?

Our in vitro experiments, as subsequent studies from other groups, showed an inhibitory effect of BMP4 on the UBM. Does this result reflect a physiological role of Bmp4 in kidney development?
Conclusions drawn from gain-of-function-type studies with BMP proteins *in vitro*, as in our experiments, are complicated by low receptor specificity of the BMPs [314]. Therefore, there is no sure knowledge about which factor contributes to the particular effect *in vivo*, except in reference to the expression patterns (TABLE 7) and phenotypes of transgenic mice. Even though BMP2/4 and BMP7 can use different receptors [336], Bmp4, expressed under the regulatory sequences of Bmp7, supports the normal development of the kidney [340]. The overall BMP activity may be more important than the actual identity of the signalling factor, at least in some locations [325].

<table>
<thead>
<tr>
<th>Bmp2</th>
<th>E11.5</th>
<th>E13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>the first pretubular aggregates</td>
<td>pretubular aggregates, comma- and S-shaped bodies (future distal tubule)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bmp4</th>
<th>E8.5-9.5</th>
<th>E10.5</th>
<th>E11.5</th>
<th>E13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>surface ectoderm, posterior mesoderm, neural crest</td>
<td>mesenchyme surrounding the Wolffian duct</td>
<td>mesenchyme around the Wolffian duct and the trunk of the ureteric bud, mesenchyme surrounding the metanephric mesenchyme</td>
<td>future podocytes, Bowman’s capsule, (comma-) and S-shaped bodies (proximal tubule), stromal mesenchyme associated with distal and proximal ureter</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bmp5</th>
<th>E13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stromal mesenchyme adjacent to the ureter and renal pelvis</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bmp7</th>
<th>E11.5</th>
<th>E13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ureter and scattered cells within the metanephric mesenchyme (condensing nephrogenic cells)</td>
<td>cap condensates and ureter, comma- and S-shaped bodies (distal tubule, presumptive and mature podocytes and Bowman’s capsule)</td>
</tr>
</tbody>
</table>

**TABLE 7.** Expression patterns of Bmp2, 4, 5 and 7 in the metanephric kidney [45, 310, 320].

In all, 4% of Bmp4+/− mice have an additional bud from the UB stalk leading to duplex kidneys and bifid ureters [320]. After the initial expression of Grem1 around the nascent UB, it is rapidly relocated to surround the ureteric tip, leaving the field around the ureteric stalk free for BMP4 activity. At this location, BMP4 can restrict branching from the UB.

Some lateral branching occurs during subsequent UBM [49, 201]. The periureteric stromal cells restrict bud formation in new stalks before they permanently differentiate [341]. Nevertheless, in spite of Bmp4 expression in the periureteric stroma, this effect seems not to be mediated by BMP4 itself [326, 341]. Grem1 protein in kidney culture caused only modest increase in ureteric branching, arguing against the major inhibitory role of BMPs in UBM [341]. Accordingly, Bmp4+/− kidneys (those without overt CAKUT phenotype) have normal ureteric branching pattern and, actually, display minor size reduction at P30 [324, 335]. If BMP4 levels are sufficient to direct ureteric outgrowth to the proper location, subsequent UBM is efficient. This indicates that Bmp4 is not a major physiological regulator of ureteric branching patterns.

A better candidate for inhibiting UB growth *in vivo* is Bmp2. It is expressed in the very first pretubular aggregates and subsequently transiently in renal vesicles, predominantly in the distal area [310, 342]. Expression pattern data suggest that Bmp2 involvement in the fusion between the early connecting segment and the ureter [46] and Bmp2;Smad4+/− mice have increased kidney size and branching of the ureter [343].
Smad pathway activation has been considered as an indication of BMP target cell populations. Smad expression is tissue-specific during embryonic development [45]. Nevertheless, immunohistochemical detection of pSmad proteins has given somewhat contradictory results, which are in conflict with reporter assays [45, 109, 142, 344] (TABLE 8) and the phenotypes of transgenic animals [104, 105, 328]. Ureteric tips, postulated to be the site of inhibitory BMP signals, have almost no Smad activation, whereas the UB trunk area shows both pSmad1 and pSmad2 staining and Id locus activation [45, 344]. Nevertheless, the UB-specific deletion of Smad4 does not cause any defects in kidney development, at least before E16.5 [45]. The effects of various TGFβ family members on UBM must be mediated through a noncanonical pathway or a mesenchymal component. Furthermore, the high concentrations of BMP4 protein in vitro caused ectopic activation of Smad-mediated BMP signalling at ureteric tips. Accordingly, the inhibitory effects of exogenous BMPs on UBM, as in our study, may not represent a physiological response [344].

### Sites of active Smad-mediated signalling in metanephric kidney

<table>
<thead>
<tr>
<th>Time</th>
<th>Location Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E10.5</td>
<td>proximal pole of mesonephric tubules weak staining in the Wolffian duct</td>
<td>[344]</td>
</tr>
<tr>
<td>E11.5</td>
<td>scattered cells throughout the metanephric mesenchyme some cells in the ureteric bud</td>
<td>[45]</td>
</tr>
<tr>
<td>E12.5</td>
<td>trunk of the collecting duct islands of vasculature developing secretory nephrons</td>
<td>[344]</td>
</tr>
<tr>
<td></td>
<td>cortical and mature stroma</td>
<td>[45]</td>
</tr>
<tr>
<td>E17.5</td>
<td>trunks of the collecting ducts very small amount of single cells in the vicinity of</td>
<td>[344]</td>
</tr>
<tr>
<td></td>
<td>the cap condensate in the nephrogenic zone nascent proximal tubule, Bowman’s</td>
<td></td>
</tr>
<tr>
<td></td>
<td>capsule and glomerular capillary</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE 8.** Locations of Smad pathway activation in the developing kidney. TGFβ family signalling was detected by pSmad1/2 immunohistochemistry [45] or in vivo reporter assay (β-galactosidase under the BMP-responsive elements of the Id1 gene) [344].

### 4.2.3 Influence of BMP4 on fates of the metanephric mesenchyme (I)

#### 4.2.3.1 rhBMP4 has adverse effects on nephrogenic cells

The branching defects in the E11 mouse kidney rudiments, pretreated with 75 ng/ml of rhBMP4 for 22 h, were partially rescued after the rat E13 MM was applied to the posterior region, which was the most severely affected by the rhBMP4 treatment (I, Table 2). Rat nephrogenic cells (identified by staining with rat-specific p75 NTR antibody) developed into nephric structures during the 3-day culture in normal medium (FIG. 9). The grafted cells were able to organize a functional progenitor niche for themselves. This experiment, together with normal Ret expression at the ureteric tips (I, Fig. 2 G–I), implied that the ureter remained competent for inductive signalling and indicated rhBMP4 impact primarily on the mesenchymal component.
RESULTS AND DISCUSSION

FIGURE 9. Cells from the rat MM incorporated into the nephron-forming units in the posterior branches of the BMP4-treated kidneys during a 3-day culture in basic medium. The epithelia of the ureter and developing nephrons were visualized with anti-laminin antibody (A) and the grafted cells with a ratspecific antibody against p75 NTR (B). Scale bar = 300 µm.

In our experiments, rhBMP4 attenuated the nephronogenic potential of isolated mesenchymes induced by dorsal spinal cord or 15 mM LiCl [23, 33, 345] (I, Fig. 5 G–I; Table 3). This effect was more severe with long preincubation period with rhBMP4 before induction and if the mesenchymes were separated from the ‘early’ rudiments (E11, bud stage) (I, Table 3). Nevertheless, even high concentrations of rhBMP4 did not inhibit MET in the induced MM or kidney cultures (I, Table 3; e.g. Fig. 2 D), in accordance with results from other TGFβ family members in vitro [202, 346]. A similar effect was reported by Dudley et al. (1999) for BMP7, considered to be an essential factor for maintenance of the CC cells. Exogenous BMP7 inhibited both the differentiation of the nephrogenic cells in isolated mesenchymes and the nephronogenesis in the peripheral area of the kidney explants [128].

In kidneys cultured for 3 days with 75 ng/ml of rhBMP4, there were fewer Bmp7+ CCs than in control cultures (I, Fig. 2 E, F). The expression of Wt1, Pax2 and Gdnf in early condensate(s) was already downregulated after a 24-h exposure (I, Fig. 4 C–H). Apoptotic cell death was increased by rhBMP4 treatment, most prominently at the expanded peripheral zone around the CCs (I, Fig. 4 I–P).

Oxburgh et al. (2004) showed that TGFβ family signalling is essential for the condensation of the nephrogenic mesenchyme [45]. Impaired separation of the nephrogenic and stromal lineages in mice deficient in Smad4 in the nephrogenic cells led to an expanded cortical area with cells of immature character. These cells, subsequently removed by apoptosis, were assumed to be nephrogenic cells not properly recruited to the CC [45]. In our gain-of-function-type experiments, the condensation process was disturbed, which was seen as weak expression of CC-specific genes and morphologically ‘thin’ condensates (I, Fig. 4 C–P), the result corroborated by the results of Miyazaki et al. (2003) [347]. The excessive cell death at the periphery of the explants may remove the nephrogenic cells left outside the condensates. During subsequent development the BMP(7) effect on the CC is mediated through Smad-independent pathways [45, 129, 344].
4.2.3.2 **BMP4 is a growth factor for stromal cells**

In our experiments, rhBMP4 had a mild survival effect on isolated MMs. After 48 h of culture, the explants treated with 75 ng/ml rhBMP4 exhibited a rounded appearance, prominent apoptosis and maintenance of cells with undifferentiated morphology (I, Fig. 5 A–F). The anti-apoptotic effect on isolated mesenchymes has been reported for both BMP7 and BMP2/4. In these experiments, the survival effect of BMPs was inferior to that of FGF2 [97, 128, 347]. In kidney cultures, both BMP4 and BMP7 with FGF2 expanded the stromal cell population [128, 347]. Thus, both nephrogenic cells and stromal progenitors seem to be responsive to BMP signalling [45].

*Bmp4−/−* mice frequently have multicystic dysplastic kidneys, *i.e.* small kidneys with thin NZ and regions where nephrogenic structures are malformed or lost [320]. According to analysis by Miyazaki *et al.* (2000, 2003), the large amount of apoptotic death in the *FoxD1+* stroma preceded the development of dysplastic regions [320, 347]. The maintenance of cells in the NZ seems to demand the activity of both *Bmp7* and *Bmp4* [347]. The BMP effect promoting the growth of the stromal component may, in turn, support the nephrogenic cells [128]. The transgenic approach to BMP4 functioning has proven challenging. The induced inactivation of *Bmp4* causes embryonic mortality even after gastrulation [325, 326, 348], and *Bmp7−/−* mice succumb to the loss of a single *Bmp4* allele [325], indicating the physiologically significant interaction between these two BMP family members *in vivo*.

If BMP4 is a survival/differentiation factor for stromal cells in the NZ, what is its source? During early metanephric development (E10.5–11.5), the MM is surrounded by *Bmp4* expression [310, 321, 349]. CCs and the stroma in the NZ do not express *Bmp4* [310, 320]. Nevertheless, the developing kidney capsule has been reported as a site of *Bmp4* expression during normal development [320, 347], and in certain mutant mice [142, 152]. Nascent nephric structures also produce BMP4 [310, 319], known to signal in a concentration-dependent manner over some distance, [*e.g.* 7]. However, the phenotype of mutant mice with defective maturation of BMP4 protein does not support an essential role for long-distance signalling of BMP4 during kidney development [262, 350]. This is in accordance with *in vitro* results showing the BMP effect on nearby cells [128, 347].

4.2.4 **BMP4 treatment reveals an anterior-posterior axis of the metanephric kidney (I)**

Inhibition of the branching morphogenesis and nephronogenesis was clearly more pronounced in the posterior part of the kidney (I, Fig. 2 C, D; Fig. 7 B, C). This effect was associated with the downregulation of the CC markers *Wt1*, *Pax2* and *Gdnf*, preferentially in posterior structures (I, Fig. 4 C–H). Marked anterior-posterior asymmetry was also reported by Cain *et al.* (2005) and Cain and Bertram (2006) in E12.5 mouse kidneys cultured with 260 ng/ml of rhBMP4 [324, 335]. An extensive three-dimensional quantitative analysis showed that the posterior branching defects in rhBMP4-treated kidneys only amplified the asymmetry in the ureteric branching pattern of the normal mouse [324]. Time-lapse observations of cultured kidneys by Saxén and Wartiovaara (1966) showed distinct ureteric branching in the anterior and posterior area [23, 351] (reprinted in I, Fig. 7 A). Lin *et al.* (2003) estimated the ratio between the anterior and posterior branches to be as high as 2 [201]. This asymmetry can be seen from the earliest branching event onwards, when the anterior branch is longer than the posterior branch [324].
The asymmetric effect of rhBMP4 can result from the general anterior-to-posterior progression of the development, with the posterior regions lagging in developmental pace. Nevertheless, similar defects should have been common in the kidney organ culture experiments; this was not the case. On the other hand, defective branching in the posterior regions may reflect differences in the MM along the anterior-posterior axis; e.g. combinatorial expression of Hox genes may give positional identity to the mesenchymal component of the kidney [21].

Regional defects have been detected in two mouse mutants with deleted Hox genes. Hoxa11;Hoxd11/- double-mutants have deformed kidneys with no nephric structures in the ventral region [352]. Hox10 triple-mutants have aberrant UBM specifically in the posterior region, bearing some resemblance to our rhBMP4-treated kidneys. The underlying defect was postulated to be in the migration and/or integration of the stromal progenitors to the kidney cortex [139]. The rhBMP4 treatment in our experiments may disturb these processes, such as causing premature differentiation of the stromal progenitors.

### 4.2.5 BMP4 recruits the smooth muscle layer around the ureter (I)

In our experiments, exogenous BMP4 protein accelerated the development of smooth muscle alpha-actin (SMA)+ cell populations around the ureter. During a 4-day culture of E11 kidneys with 100 ng/ml rhBMP4, the distal ureter was surrounded by a thick layer of SMA+ cells in contrast to control cultures in which the development of a smooth muscle cell (SMC) layer was detected only after 7 days of culture (I, Fig. 6 D–I). This is in accordance with results of a study by Brenner-Anantharam et al. (2007) [326], whereas similar experiments by Miyazaki et al. (2003) with ureter grafts failed to show an increase in the number of SMCs [347]. Instead, in the latter study, cells of the periureteric mesenchyme accumulated around the BMP4-releasing beads and the migration assay further supported the BMP4 function as a chemotactic agent promoting mesenchymal condensation [347].

The mature ureter is surrounded by a muscular coat [353] that is first laid down around the ureter proximal to the kidney at E14.5 and proceeding distally [354]. The physiological role of BMP4 in this process is substantiated by the expression pattern of Bmp4 that sharpens from a diffuse, broad expression around the WD to surround the ureteric stalk after UB ingrowth (I, Fig. 6 A–C). The dependence of ureteric SMC development on the Bmp4 dose was shown by the analysis of transgenic mice by Miyazaki et al. (2003) and Wang et al. (2009): Bmp4+/- mice have fewer SMA+ cells around the ureter than the wild type [347] and in induced Bmp4-deficient mice, only a few disorganized SMCs surround the embryonic ureter [348]. In the latter study, beads releasing Grem1 locally disrupted the investment of the SMC layer, and the BMP-responsive pSmad+ cells were detected in the periureteric mesenchyme [348].

In the current view, sonic hedgehog, secreted by the ureteric epithelium, induces the proliferation of SMC precursors [354]. BMP4 is envisaged as functioning as an autocrine factor in SMC progenitors, and it upregulates genes such as Tshz3, which produces the SMC phenotype [353, 355]. BMP5, mutated in the mouse line short ear, having a hydroureter, is also expressed in the periureteric mesenchyme and may play a role in ureteric morphogenesis [356].

The periureteric mesenchyme, derived from the tail bud area, is a distinct cell population from the nephrogenic mesenchyme [326]. Tbx18, a transcription factor expressed in this mesenchyme flanking the distal ureter and the WD, is essential for the condensation of the
mesenchymal cells around the ureteric stalk. In addition to a hydroureter, Tbx18/- mice have markedly short ureters and defective urothelia [357]. Hence, the periureteric mesenchyme, in turn, regulates the development of the ureteric epithelium [357]. Accordingly, Brenner-Anantharam et al. (2007) showed that exogenous BMP4 converted the intrarenal collecting ducts to a ureteral phenotype and Bmp4 expression in the periureteric mesenchyme was required for the proper differentiation of the urothelium [326]. SMCs around the intrarenal collecting system have different ontogeny and their development is regulated by Wnt4 and Bmp4 expressed in the medullary stroma [152].

In conclusion, our in vitro studies and results from other groups suggest that BMP4 may be a pleiotrophic growth factor recurrently used in metanephric development. Bmp4, expressed in the periductal mesenchyme around the WD, is a major factor regulating ureteric outgrowth at the proper location. BMP4 promotes the condensation of the periureteric mesenchyme that forms the smooth muscle layer, providing peristalsis for urine transport. This cell population, in turn, influences the growth and differentiation of the ureter. In the NZ, Bmp4, perhaps together with Bmp7, may be a growth and differentiation factor for stromal cells that have a reciprocal relationship with the nephrogenic mesenchyme they are surrounding.

4.3 Influence of GDNF dose on the cell fates of undifferentiated spermatogonia

4.3.1 GDNF is essential for the proliferation and maintenance of undifferentiated spermatogonia (II)

In the second part of our study, we investigated the role of GDNF, a factor essential for kidney morphogenesis, in the testis. Two transgenic mouse lines were used to elucidate the effect of GDNF dosage on spermatogenesis: the mice overexpressing GDNF in the testis under human translation elongation factor-1α promoter and Gdnf+/- mice [59].

GDNF-overexpressing mice are infertile. They produce no progeny and no spermatozoa could be detected in the seminiferous tubules or epididymis of the adult males (II, Fig. 2 E; FIG. 10 B). Their testicular morphology is normal at birth, but within 3 weeks tubules with clusters emerge (II, Fig. 2 B). Clustered cells were classified as type A spermatogonia based on morphology, expression of the spermatogonial marker EE2 and lack of Kit receptor [259, 358] (II, Fig. 2 B, C; FIG. 10 C).

Mice heterozygous for Gdnf (Gdnf+/-) show various defects resulting from Gdnf haploinsufficiency [59]. Gdnf+/- mice are fertile. Nevertheless, the segmental disturbances in germ cell differentiation can be seen in testis histology from the fifth week of postnatal life onwards (II, Fig. 1 A; FIG. 10 D). Spermatogonia are the first to be depleted (II, Fig. 1 B), and the subsequent germ cell differentiation leads to the Sertoli cell-only phenotype in some seminiferous tubules of the older mice.
RESULTS AND DISCUSSION

FIGURE 10. Testis histology in sections stained with haematoxylin and eosin: A) All stages of germ cell differentiation, including spermatozoa, can be seen in seminiferous tubules of wild-type mice at 8 weeks of age. B) GDNF-overexpressing mice at same age (8 weeks) display atrophy of the seminiferous tubules with remnants of spermatogonial clusters and hyperplasia of the interstitial tissue. C) Close view of a spermatogonial cluster in a GDNF-overexpressing mouse (3 weeks of age). Pale nuclei with sparse heterochromatin indicate that cells in clusters are undifferentiated spermatogonia. D) Gdnf+/− mice at 7.5 weeks of age have chimeric testicular histology with both well-preserved tubules and atrophic tubules lacking spermatogonia. Scale bar is 100 µm in A and B; 33 µm in C and 200 µm in D. Photographs by courtesy of X. Meng/H. Sariola.

Our results have been corroborated and extended by studies from several groups. Yomogida et al. (2003) expressed GDNF in Sertoli cells by in vivo transfection, which led to the expansion of undifferentiated spermatogonia and formation of clusters similar to those in our study [359]. When transplanted, these germ cells maintained complete spermatogenesis in contrast to the spermatogonia overexpressing GDNF (II) that reproduced the spermatogonial clusters when transplanted into irradiated testes of recipient mice [359, 360].

Naughton et al. (2006) circumvented the perinatal mortality of Gdnf, Gfra1 and Ret null mutants, using whole-testis transplantation (at P0) under the skin of nude mice [251]. In only 7 days after the transplantation, the mutant testes showed complete lack of proliferating SSCs but normal levels of differentiating spermatogonia. Accordingly, GDNF is essential not only for SSC proliferation but also for the maintenance of the undifferentiated state in vivo. Identical phenotypes showed that the GDNF effect is mediated by signalling through GFRα1 and Ret.
RESULTS AND DISCUSSION

Mice with hypomorphic Ret allele acting in a dominant-negative manner also exhibited reduced numbers of germ cells and decreased proliferation of the early progenitors [361].

GDNF is thus crucial for the maintenance of the SSC population in prepubertal mice. Savitt et al. (2012) extended this finding, showing that GDNF is also an irreplaceable factor for spermatogonial proliferation and self-renewal in steady-state spermatogenesis [362]. Inhibition of Ret activity in adult mice led to loss of most undifferentiated spermatogonia between days 2 and 11 [362]. Accordingly, induced deficiency of Gfra1 in 4-week-old mice caused the depletion of germ cells, ending in the Sertoli cell-only phenotype [254].

We approached the identity of GDNF target cells by expression pattern of the receptors. Ret and Gfra1 were expressed by a small subset of spermatogonia (II, Fig. 4 B, C) and the expression levels of these receptors were downregulated during the first wave of spermatogenesis (II, Fig. 3 B, C). This was corroborated by Naughton et al. (2006), who found that the number of Ret+ spermatogonia decline with increasing age [251]. This may reflect the changing ratios of cell types or different usage of undifferentiated spermatogonia as stem cells during the first wave of spermatogenesis versus the steady state.

Recent studies have indicated that GDNF likely has a proliferative effect on a broader spectrum of undifferentiated spermatogonia than stem cells, and it may not regulate the decisions between self-renewal and differentiation per se [224]. Ebata et al. (2011) showed that GDNF with FGF2 increases the amount of dividing SSCs but not the total number of stem cells [363]. Thus, GDNF seems to promote the maintenance of SSCs by providing both new stem cells and offspring for differentiation. The germline stem cells overexpressing H-Ras overproliferated but underwent spermatogenesis in vivo, whereas the overexpression of cyclin D2, a Ras target gene, produced spermatogonial clusters [76]. Ras activation, downstream of GDNF, seems to drive nonspecific proliferation, but cyclins may be involved in the fate decisions of SSCs [76]. Accordingly, GDNF enhances the formation of short-chained spermatogonia in vitro [250, 256].

In juvenile GDNF-overexpressing mice, the proliferating spermatogonia formed large clusters protruding into the lumen of the seminiferous tubules (II, Fig. 2 B; FIG. 10 C). Clustering is normal behaviour of spermatogonia in vitro. In cultures supplemented with GDNF, SSCs proliferate in balls of cells with tight intercellular contacts [80, 247, 250]. Withdrawal of GDNF causes disintegration of the cluster and an increase in SSCs [80, 363]. Accordingly, SSC numbers reach a plateau when the clusters are formed and expansion of stem cells follows the passage of culture [363]. The cluster structure seems to have regulatory influence on the fate decisions of SSCs.

4.3.2 Spermatogonia in clusters, unable to differentiate, are removed by apoptosis (II)

In GDNF-overexpressing mice, human GDNF is expressed by spermatogonia, not only by Sertoli cells as in endogenous Gdnf (II, Fig. 4 A, D). The autocrine GDNF signalling loop is created in clustered spermatogonia that also express Ret and Gfra1 receptor (II, Fig. 4 E, F). Apoptosis analysis of GDNF-overexpressing mice showed TUNEL+ cells in both control and transgenic testes from the age of 2 weeks onwards (data not shown). Therefore, cell death during the first wave of spermatogenesis is also normally initiated in mutant mice (spermatogonia outside the clusters advance somewhat further along the differentiation pathway and express Kit receptor (II, data not shown). The normal density-dependent regulation of germ cell numbers does not function in transgenic mice, possibly because the
cells in the clusters, mainly undifferentiated spermatogonia of A$_{\text{singles}}$ and A$_{\text{aligned}}$ with short chains, do not reach the stage of apoptotic deletion (differentiating spermatogonia of A$_{2-4}$ [231]). The intensified elimination of surplus spermatogonia manifesting at 4 weeks of age by a nine-times higher level of cell death (II, Fig. 5 D, E) can be executed in the adluminal space by apoptotic pathways not available to spermatogonia in the basal compartment [274].

Our analysis of cell cycle kinetics showed that the overall proliferation of spermatogonia was not changed by the GDNF overexpression. The loss of the segmental distribution of mitoses (II, Fig. 5 A, B) and lower peak proliferation than in the wild type (II, Fig. 5 C) indicated that the spermatogonial differentiation is blocked. Accordingly, Fouchécourt et al. (2006) explained the decreased proliferation of spermatogonia in seminiferous tubules cultured with GDNF by the inhibitory effect of GDNF on differentiation, masking the mitogenic effect on SSCs [364].

In GDNF-overexpressing mice, the spermatogonia in clusters were not competent to enter meiosis during all-trans retinol treatment, but underwent apoptosis (II, Fig. 5 F, G). Either the adluminal space does not support meiosis or the high level of GDNF signalling is incompatible with the differentiation of spermatogonia [359]. The adult phenotype of GDNF-overexpressing mice (II) bears resemblance to that of Nanos2-overexpressing mice, in which a rim of Plzf+ spermatogonia is unable to differentiate [254]. Loss of GDNF signalling led to rapid downregulation of Nanos2 expression, suggesting that Nanos2 acts downstream to GDNF to maintain stemness [254].

A striking phenotype of GDNF-overexpressing mice was seen during the first round of spermatogenesis (II) or after transplantation of the transgenic germ cells that formed new clusters in recipient mice [360]. In adult GDNF-overexpressing mice, only a rim of spermatogonia remained (II, Fig. 2 E). Major defects during the first round of spermatogenesis can have a disruptive effect on steady-state germ cell differentiation [269, 273–275]. The mature blood-testis barrier may also restrict the expansion of the spermatogonial population [222, 365] or adult spermatogonia may be less responsive to GDNF. SSC activity can differ between juvenile and adult mice, according to environmental influences [224, 366]. The other regulatory factors in the mature stem cell niche may restrict the stem cell proliferation, unlike during the establishment phase in prepubertal mice.

In conclusion, the accumulation of undifferentiated spermatogonia when GDNF was overexpressed and the depletion of SSCs in the case of low Gdnf dosage showed that GDNF is essential to spermatogonial self-renewal. Our original findings and the subsequent results from other labs, indicate that GDNF, secreted by Sertoli cells, regulates the proliferation of a subset of undifferentiated spermatogonia expressing Ret and GFRα1. This makes GDNF a crucial niche factor behind the continuing maintenance of stem cells in the testis.
5. CONCLUDING REMARKS

Programmed cell death during kidney development has been recognized since the 1990s, but little is known about its regulation. Modern cell-fate mapping and high-resolution visualization techniques, combined with embryonic organ culture, may help to elucidate the ontogeny of dying cells and the roles played by PCD, especially during the early stages of kidney development.

In the need for viable transgenic animal models, some central questions concerning the physiological roles of BMP4 have only partially been resolved. BMP4 is an essential para- or autocrine factor in many mesenchymal cell populations, only recently acknowledged as effectors in kidney morphogenesis. The periductal mesenchyme surrounding the WD regulates the anterior-posterior patterning of the caudal nephric mesenchyme by directing the site of UB outgrowth. The mesenchyme around the ureter, in addition to forming the ureteric smooth muscle, has a modulating effect on ureteric growth and differentiation.

BMP4 acts as a survival and differentiation factor on stromal cells. The stromal compartment of the embryonic kidney includes various cell types whose ontogeny and lineage relationships are elusive, not to mention their differentiation cues and survival demands that are almost completely unknown. The adverse effect of BMP4 on the early CC emphasizes the unique nature of the condensation process of this primary cap and subsequent establishment phase of the progenitor cell population for the nephric epithelium.

Our original finding of GDNF as a crucial growth factor for the self-renewal of undifferentiated spermatogonia has contributed to the development of culture protocols for rodent SSCs. Nevertheless, the appropriate in vitro conditions for long-term maintenance of SSCs from most species, including humans, are still lacking. Culture and transplantation techniques have made it possible to study the proliferation of SSCs, but the current protocols do not support the differentiating germ cells. This has complicated the study of SSC fate decisions. Currently, the molecular mechanisms behind the choice between self-renewal and commitment to the differentiation pathway are only tentatively known. Fate decision of a single spermatogonium can be accidental. The maintenance of the stem cell pool is likely dependent on inputs from several soluble factors and physical contacts within the testis stem cell niche, interacting with cell-intrinsic regulators and favouring self-renewal.
Acknowledgements

This work was carried out between years 1995 – 2000 at the Institute of Biotechnology, University of Helsinki. I want to thank Professor Mart Saarma, former Head of the Institute, for providing excellent environment for scientific work.

I am grateful to my supervisors, Professor Hannu Sariola, who gave me opportunity to work in his group and allowed me scientific freedom and Dr. Tiina Immonen, who provided sound advice and reason. Encouragement of my supervisors and Professor Juha Voipio made this thesis project possible.

I want to thank Professor Jamie Davies and Professor Olli Jänne for reading of my manuscript carefully and giving valuable advice to improve my dissertation. I would like to acknowledge Professor Heikki Rauvala and Graduate School for Molecular Biology and Biotechnology, Helsinki for funding and excellent post-graduate education.

Special thanks go to Professor Irma Thesleff for creating a radiant Developmental Biology Group to Viikki during the last years of the past millennium. I will always remember coworkers from the Tooth Group with great warmth, especially Johanna Pispa, Soile Keränen, Carin Sahlberg, Tuija Mustonen, Päivi Kettunen, Marja Mikkola, Pekka Nieminen and Thomas Åberg.

Marja-Leena Peltonen, Virpi Syvälahti, Elina Korhonen, Birgitta Tjäder and Anna Hanninen are acknowledged for their technical assistance and friendship. Patience and good advice of Riikka Santalahti and Merja Mäkinen were also invaluable.

Late colleague Dr. Xiaojuan Meng is remembered with deepest gratitude. I am also indebted to all my cowriters: e.g. Maria Lindahl, Mervi Hytönen, Marjo Hyvönen, Kirsi Sainio, Tiina, Auri Tenhunen and Petri Rossi. Members of the Kidney Group, the ‘old’ ones, Kirsi, Kirmo Wartiovaara, Juan and Marjo and new companions at the BI, e.g. Tiina and Mervi, are all thanked for their help and support. In our ensemble life might have been sometimes rough but never boring. Also the personalities of the Molecular Neuroscience group enhanced the exiting working environment.

The miracle in my life: Love of my children, Aurelia, Daniel, Letitia and Rhea. My husband Pekka, my parents Osmo and Liisa, my favourite cousin Jaana and my godmother Saara have supported me through these years. My sincere affection belongs to my late grandfather, Mauri, who gave me his time when I was a child and shared his passion for nature, history and reading. I also thank all those people, from the mothers at the children's playground to various officials, who aided me to tie the loose ends of my studies.

Helsinki, April 2014
References


59

References


[34] M. J. Donovan, T. A. Natoli, K. Sainio, A. Amstutz, R. Jaenisch, H. Sariola and J. A. Kreidberg, "Initial differentiation of the metanephric mesenchyme is independent of


alpha8beta1 is critically important for epithelial-mesenchymal interactions during kidney morphogenesis," *Cell*, vol. 88, pp. 603-613, 1997.


A. Dziarmaga, M. Eccles and P. Goodyer, "Suppression of ureteric bud apoptosis rescues


O. Michos, A. Goncalves, J. Lopez-Rios, E. Tiecke, F. Naillat, K. Beier, A. Galli, S. Vainio and


