Interaction between metanephric blastema and ureteric bud: Novel view of origin of connecting tubulus and of Wilms's tumour

Doktoral (PhD) thesis

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1. Introduction

The development of kidney results from a well-coordinated reciprocal induction between metanephrogen mesenchyme (MM) and ureteric bud (UB). The branching UB induces the condensation, mesenchymal to epithelial transition of blastemal cells, proliferation of stromal cells and and finally results in functioning nephron. The molecular biological pathways regulating the differentiation of pluripotential cells is well-documented. However, two aspects the bilateral induction of MM and UB have not yet been cleared. The first one is the origin of the connecting tubulus (CNT) between the MM-derived tubuli and UB-derived collecting tubulus. The second question, which remains to be answered is the origin of Wilms's tumour (nephroblastoma) from the erronous interaction between the MM and UB.

1.1. Origin of connecting tubulus

Morphological studies suggested several decades ago the existence of a connecting piece (Verbindungsstück) between distal convoluted tubules (DCT) and cortical collecting ducts (CCD) and demonstrated biochemical and physiological differences between DCT and connecting tubules (CNT) in rabbit. CNT is an unique segment of the nephron which connects MM-derived distal convoluted tubule (DCT) and ureteric bud (UB)-derived CCD. Views on cellular origin of CNT are controversial. Most of our knowledge on development of CNT come from studies on mouse kidneys. Segment specific expression of Pou3f3 transcription factor has been detected in distal domain of renal vesicle (RV) and also in connecting segment in mouse, therefore it was suggested that the connecting segment arises from distal compartment of MM-derived RV. The lack of ureteric tip (UT) marker Calb1 in

tubular segment connected to RV further suggested that the connecting segment arises from the distal RV rather than the UT. The development of human kidney differs in several aspects from that of mouse kidney. In human kidney the branching of UB and development of the kidney is terminated in the 15. week and 36. week of gestation, respectively. Moreover, the distal part of nephron including the CNT, in contrast to other segments of the nephron, has a functional and morphological heterogeneity. Principal cells (PC) and intercalated cells (IC) occur exclusively in the collecting duct (CD) and CNT, supporting the common origin of the two segments. There are also different opinions on development of PC and IC cells as well.

1.2. Origin and definition of Wilms's tumor

Nearly 150 years ago Cohnheim has postulated that a developmental error and impaired differentiation within the embryonal Anlage may lead to tumor development. Wilms' tumor (WT), a malignant neoplasm in childhood is one of the best examples for arrested cellular differentiation. It is generally acknowledged that due to failure in bilateral induction between UB and MM, metanephric blastemal cells remain undifferentiated or undergo only partial differentiation. The arrested differentiation of MM results in WT of triphasic histology with nests of blastemal cells, epithelial tubules and stromal elements. WT recapitulates the morphology and molecular biology of nephrogenic zone of developing kidney.

Based on this presumption, WT was determined by the WHO in 2004 as follows: "Nephroblastoma is a malignant embryonal neoplasm derived from ***nephrogenic blastemal cells***. It mimics developing kidney and often shows a divergent pattern of differentiation". This definition was challenged in 2007 by a report ont he presence of UB-like tubular structures in triphasic WT. In spite of the

new finding, the latest WHO classification in 2016 retained the 2004 definition. Comparative gene expression analysis of developing human kidney and WT identified common expression fingerprint and presented UB-like epithelial structures in tumor tissue. Recently, ureteric bud derivatives were found not only in WT but also in nephrogenic rest (NR).

2. Objectives

The aim of our study was to establish the origin of CNT in human kidney. First we searched for possible connection between UB and SSB in H&E stained histological slides of foetal kidneys. Subsequently analysed the expression of markers defining different cell types of collecting duct system in foetal and adult human kidneys by immunohistochemistry. We showed that development of CNT in human differs from the model described in mouse and confirmed that CNT and CCD are composed of cells, occurring specifically in this segment of the nephron and which cannot be seen in other segment of the nephron, suggesting their common developmental origin.

Further aim of our study was to obtain new information on the involvement of UB in development and histology of WT. First, we have analysed the expression of RET, ROBO1 and SLIT2 genes, which are associated with the growth and branching of ureteric bud tip (UBT) in foetal kidney. Subsequently we searched for UB derivative tubular structures in nephrogenic rest and WT.

We also asked, whether pluripotent UBT cells can further differentiate under tumorigenic condition. Therefore, we have analysed the expression of CA2, ATP6V1B1 and ATP6V0D2 genes marking progenitors of intercalated (IC) cells, along with SLC4A1 and SLC26A4, which are specific for mature α- and β- IC cells and also with AQP2 specific for principal cells (PC).

3. Material and methods

3.1. Sample collection

Formalin-fixed, paraffin-embedded foetal kidneys from 10 weeks (n=2), 12 weeks (n2), 15 weeks (n=2) and 17 weeks (n=5) gestational ages were obtained from the Department of Pathology, Medical School, University of Pecs, Hungary. Tissue samples were fixated in 4% buffered formaldehyde and embedded in paraffin for histological report. No known abnormalities were recorded for foetal kidneys. The use of tissue samples for this study was approved by the Ethics Committee of the University Pecs, Hungary (No. 5343/2014).

Histological samples of 12 selected WTs were obtained from the Wilms's Tumour Registry, Department of Pathology, University of Szeged, Hungary. All WT cases were pretreated and evaluated according to the UMBRELLA SIOP-RTSG 2016 protocol. In this study we included 8 mixed WT, two WT of regressive type, one each of blastemal type and with focal anaplasia. Moreover, we have analysed 6 PLNR, including a hyperplastic one of 6 mm in diameter and three ILNR. The use of Wilms's tumour and precursor tissues for this study was approved by the Ethics Commission of the Hungarian Medical Research Council (IV/8956-3/EKU). All procedures were in accordance with the ethical standards of the Institutional Research Committee and with the 1964 Helsinki Declaration.

3.2. Immunohistochemistry

After removing the paraffin and rehydration, the 4 μm thick sections were subjected to heat-induced epitope retrieval in citrate buffer, pH 6.0 or EnVision

FLEX Target Retrieval Solution, high pH (DAKO, Glostrup, Denmark) in 2100-Retriever (Pick-Cell Laboratories, Amsterdam, The Netherlands). Endogenous peroxidase activity was blocked with Envision FLEX Peroxydase Blocking Reagent (DAKO) for 10 minutes at room temperature. Slides were then incubated for one hour with the primary antibodies. We used antibodies marking distinct segments of the nephron and also marking PC, precursors of IC cells and α- and β IC cells: Anti-TMEM27, Anti-POU3F3, Anti-SCEL Anti-AQP2, Anti-CALB1, Anti-CA2, Anti-SLC26A4, Anti-SLC4A1, Anti-ATP6V1B1, Anti-ATP6V0D2, Anti-TMEM213, Anti-RET, Anti.-SLIT2, Anti-ROBO1, Anti-KRT17. EnVision FLEX horse-radish-peroxydase conjugated secondary antibody (DAKO) was applied for 30 minutes at room temperature. The signal was visualized with amino-ethyl-carbazol (AEC) or 3,3'-diaminobenzidin (DAB) (DAKO). Tissue sections were counterstained with Mayer's haematoxylin (Lillie's modification, DAKO) and after 10 seconds bluing in ammonium-hydroxide solution, were mounted by PERTEX (medite Ltd. Burgdorf, Germany) or Glycergel (DAKO). For negative control the primary antibody was omitted. The immune reaction was evaluated by both authors. Photographs were taken by a Leitz DMRBE microscope, equipped with HC PLAN APO 20x0.70 objective, and a ProgRes C14 camera (Leitz, Wetzlar, Germany).

4. Results and Discussion

4.1. Origin of CNT in mouse and human kidney

Expression of Pou3f3 in distal domain of RV and lack of UT-marker Calb1 in connecting segment in mouse kidney suggested that connecting segment arises from the RV. Therefore, we have analysed the expression of POU3F3 and CALB1 genes in human foetal kidney. We did not find POU3F3 expression in the RV stage in human

foetal kidney, it can be first seen in the distal domain of SSB. The UB tip (UBT) as well as the RV were consequently negative for CALB1 and the protein was expressed in UB trunk. In human kidney a clear segmentation can be seen first in SSB as distal, medial and proximal domains emerge. RV does not express POU3F3, positive immune reaction appears in distal compartment of SSB. Cells of UB tip (UBT) as well as the RV are consequently negative for CALB1, the protein is expressed only in UB trunk. These finding show the difference between development and segment specific differentiation of mouse and human kidney.

4.1.2. In human kidney ureteric bud tip (UBT) is connected to distal compartment of S-shaped body (SSB)

We searched for a possible connection between of RV and UB in H&E stained histological slides of foetal kidneys of different gestational ages. We did not detect any sign of fusion between RV and UB. In human kidney UB derived cells, progenitors for emerging CNT, grow out from the UBT towards the distal compartment of MM-derived SSB. This developmental step can easily be followed in serial section of haematoxylin & eosin stained foetal kidneys. We observed several UBT with outgrowth of epithelial tubular structure towards to distal compartment of SSB. The cuboid cells of emerging CNT and SSB were flattened in area of future fusion. In some of the slides a complete fusion between the UBT outgrowth and distal domain of SSB has been seen with continuous lumen between the UBT and SSB.

4.1.3. PC and IC cells and their progenitors in foetal kidney

PC and IC cells occur exclusively in CNT and CD. To establish the development of PC and IC cells in foetal kidney, we have applied immunohistochemistry for AQP2 marking PC and also for CA2 marking precursors of IC cells. We found a strong CA2 positive staining in developmentally younger cortical

domain of UB trunk. In medullary CD only single cells displayed CA2 positive staining. In contrary to CA2 expression, cortical UB trunk showed only weak AQP2 staining, and the staining intensity was gradually increased towards its medullary domain. Neither CA2 nor AQP2 expressed in other tubular cells of foetal kidney.

We have also analysed the occurrence of SLC4A1, ATPase H+ Transporting V0 Subunit D2 (ATP6V0D2), ATP6V1D1 and SLC26A4 marking α-and *β*-IC cells. We have detected IC cell specific ATP6V1D1 expression in cortical UB trunk in 10 weeks old kidneys. In 10-15 weeks old kidneys in addition to the fine line of immune reactivity on the surface of tubules some cells expressed ATP6V0D2 at apical plasma membrane forming a "cap" like structure. Several tubular cells were positive for ATP6V1B1 in 17 weeks old kidney and also for the ultimate *β*-IC marker SLC26A4. This finding indicates that many of the emerging IC cells in the early gestational age have an immature phenotype. The expression of SLC26A4 and ATP6V1B1 indicates that these cells may correspond to nonα-non*β* IC cells.

4.1.4. Expression of segment specific genes in adult kidney

Each segment of the human kidney, with exception of the CNT and CD displays homogeneously cells of segment specific morphology and function. AQP2, a PC marker expressed only in cells of CNT and CD, preferentially in medullary CD, which corresponds to the specific function of PC. The CA2 antibody, which marks the precursor cells for PC and IC cells showed positive reaction in CNT and also in CCD and OMCD cells. The distribution of cells in the CNT and CD shows a mosaic structure corresponding to the function of PC and IC cells, which express genes regulating the electrolit and water balance. Expression of AQP2 occurs preferenctially in the last segments of the distal nephron, whereas α-IC cell marker SLC4A1 and *β*-IC cell marker SLC26A4 are expressed in CNT and CCD (Table 1)

Table 1. Expression of segment and cell specific genes in human adult kidn

Gene	PCT	LH	MD	DCT	CNT	CCD	OM
TMEM27	++	-	-	-	-	-	-
POU3F3	-	++	+++	+	-	-	-
SCEL	-	-	-	++	-	-	-
AQP2	-	-	-	-	+	++	++
CA2	-	-	-	-	+	+++	+
SLC4A1	-	-	-	-	++	++	+
ATP6V1B1	-	-	-	-	++	++	+
ATP6V0D2	-	-	-	-	++	++	+
TMEM213	-	-	-	-	++	++	+
SLC26A4	-	-	-	-	+	++	-
CALB1	-	-	-	-	+	+++	-

PCT – proximal convoluted tubules; LH – Loop of Henle; MD – macula
distal convoluted tubules; CNT – connecting tubules; CCD – cortical c
OMCD – outer medullary collecting duct; IMCD inner medullary coll

4.2. Wilms's tumour

4.2.1. Ureteric bud markers in foetal kidney.

First, we have analysed the cellular localisation of proteins encoded by genes listed in Table 2 in foetal kidney. We observed ROBO1 and RET expression not only in UBT but also in the UB trunk. No ROBO1 or RET expression has been seen in metanephric mesenchyme (MM)-derived structures such as condensed blastemal cells, renal vesicles (RV) or S-shaped body (SSB). The ROBO1 ligand SLIT2 showed a weak expression in cells of UBT and UB trunk and also in MM-derived blastemal cells and emerging epithelial structures including RV and SSB.

CA2 identify progenitor cells committed to differentiate into PC and IC cells in adult kidney. The CA2-positive cells were located in developmentally younger cortical domain of the UB in close vicinity to but not in the UBT. Immature IC cells in cortical UB trunk showed positive staining with IC cell markers ATP6V1B1 and ATP6V0D2. Most of the positive tubules displayed a thin line of ATPase positivity on the luminal surface of cells and only a few showed a cap-like positivity as seen in adult kidneys. Strong AQP2 protein expression was observed in the medullary UB trunk corresponding to the medullary collecting duct (CD), but the cortical UB trunk and UBT were negative. The expression of the genes the in distinct type of cells and areas in foetal kidneys is summarized in Table 2. None of the genes, with exception of the SLIT2, has been detected in MM-derived structures of the foetal kidney.

Table 2. Expression of ureteric bud and cell specific gene products in human foetal kidney

	RET	ROBO1	SLIT2	CA2	ATPase	AQP2
UBT	+	+	+	-	-	-
C-TRUNK	+	+	+	+	+	-
M-TRUNK	+	-	-	-	-	+
MM-DER	-	-	+	-	-	-

UBT – ureteric bud tip, C-TRUNK – cortical UB-trunk, M-TRUNK – medullary UB-trunk, MM-DER – metanephric-mesenchyme-derivative. – negative, + positive.

4.2.2. Ureteric bud-derivatives in nephrogenic rest

During our analysis, we have identified cellular components in Wilms' tumour derived from UBT. It is generally accepted, that Wilms tumor is associated with and develops from NR. Therefore, we have analysed 9 NR including a hyperplastic one of 6 mm in diameter by applying antibodies used for analysis of foetal kidney and WT.

We found a positive reaction with RET and ROBO1 in three of the perilobular nephrogenic rests (PLNR) including the hyperplastic ones. Two of the three intralobular nephrogenic rests (ILNR) also showed positive immunoreaction with RET and ROBO1 antibody. Positive immunreaction was also seen with CA2 antibody in solid growing epithelial cells of the hyperplastic rest and tubular structures of two ILNR. Moreoever, two ILNR contained scattered tubular cells displaying positive staining with ATP6V0A4 és ATP6V1B1 antibodies.

4.2.3. Ureteric bud-derivatives in Wilms' tumour

ROBO1, which is expressed in UBT in foetal kidneys showed a strong expression in UB-like tubules embedded in nodular/serpentine growing blastemal cells or in mesenchymal stroma. No ROBO1 expression was seen in tubular structures resembling renal vesicles. The expression of SLIT2, corresponding to its expression in normal foetal kidney, was detected not only in the UB-like structures, but also in the surrounding blastemal cells as well. SLIT2 immunoreaction was seen in tubular structures embedded in circular growing proliferative stroma and also in renal vesicle-like tubules.

RET is expressed in UBT cells and also in UB-like tubules surrounded by blastemal cells and UB-like tubules in fibrotic stroma. None of the RV-like tubular structures displayed RET positive staining. ROBO1, SLIT2 and RET, which are expressed in UBT cells in normal foetal kidney, showed a positive immunoreaction in UBT-like tubules in WT. UBT cells were consequently negative for CA2. Positive CA2 staining was detected in cells of UB trunk in foetal kidney and in elongated tubular structures resembling cortical UB trunk in WT. We detected immature IC cells displaying ATP6V1B1 and ATP6V0D2 immunoreaction in some of the tubular structures in WT. However, none of the WT showed positive staining with SLC4A1

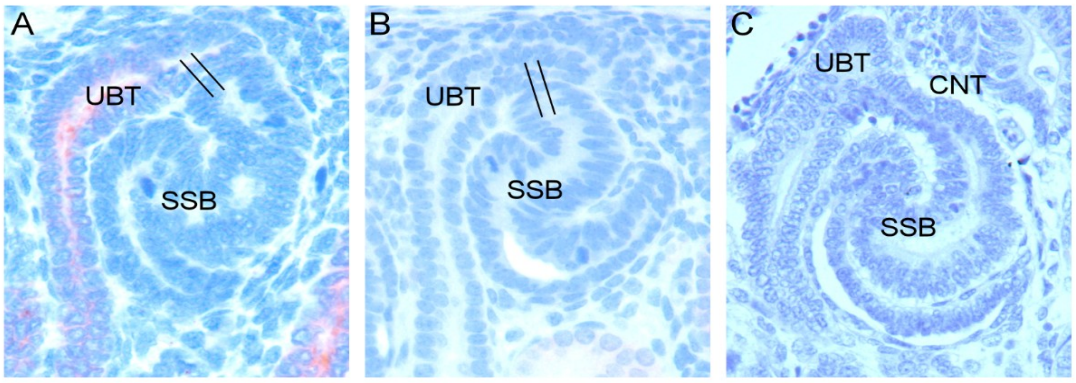
and SLC26A4, which are marker of mature α- and β-IC cells. No any structures or cells of WT displayed positive staining with AQP2.

4.2.4. Differentiation of UB-derived cells in nephrogenic rest and Wilms's tumour

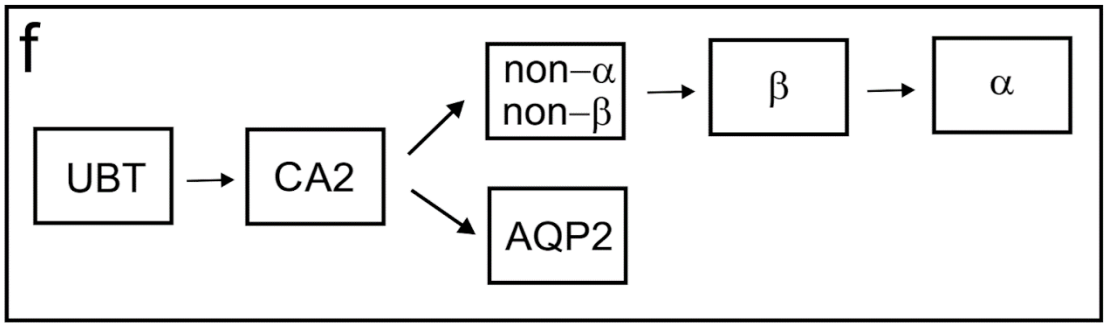
In this study we have analysed not only the occurrance of UBT derivative structures in WT and nephrogenic rests, but also the potential of UBT to differentiate into PC and IC cells under tumorigenic conditions. We have detected the expression of CA2 gene in NR and triphasic WT. CA2 marks the progenitors of both PC and α- and β-IC cells. We found positive staining with ATP6V1B1 and ATP6V0D2 antibodies in WT and NR. Our findings indicate that cells of UBT can differentiate into CA2-expressing cortical UB trunk and immature IC cells, not only in normal foetal kidneys but also in tumorigenic conditions in NR and WT. However, UBT derivatives cannot be converted into functional β- and α-IC cells in NR and WT as they do it during development of normal kidney..

5. Conclusion, Novel Results

5.1. Origin of connecting tubulus in human kidney



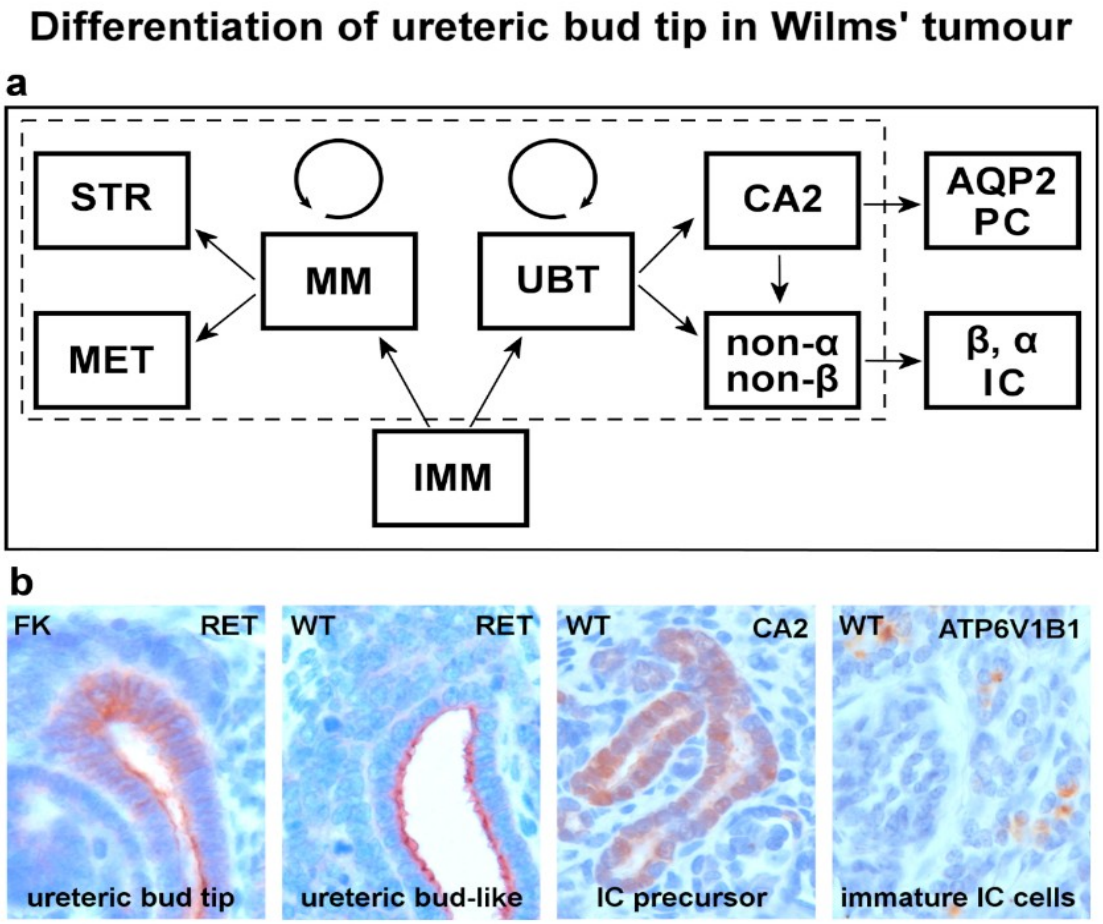
Based on detailed histological examination of a series of human fetal kidneys we concluded that proliferating cells growing out from the ureteric bud tip are connected to the distal domain of S-shape body. After fenestration a continous lumen appears between ureteric bud tip and distal domain of S-shape body.



Immunhistology confirmed that CNT originates from cells of UBT and therefore it is an integral part of distal nephron. Cells of UBT differentiate to CA2 positive precursor cells and during the UB branching in developmentaaly older medullar CD differentiate to AQP2 positive PC cells and in junger cortical CD cells into non-α non-β-IC, and finally β-IC és α-IC cells.

(Sárkány és Kovács, Histochem Cell Biol, etc 2021)

5.2. Novel model for development of Wilms' tumour



(Sarkany et al. Scientific Reports, közlésre elküldve).

a, Pathway of normal kidney development and tumorigenesis of WT. Both metanephric mesenchyma (MM) and ureteric bud (UB) derives from the intermediary mesenchyme (IMM) and both retain self-renewal capacity to generate appropriate number of nephrons. MM differentiates into proximal tubular system through mesenchymal to epithelial transition (MET) and into kidney stroma (STR). Cells of ureteric bud tip (UBT) have bipotential characteristics, give rise to daughter cells with self-renewal capacity, which remains in UBT domain and to cells left behind in the UB trunk. The UBT differentiate into CA2 positive precursor cells, which convert into AQP2 positive principal cells (PC), and also give rise to immature nonα-nonβ intercalated (IC) cells, which in normal kidney differentiate to functional β- and α-IC

cells. Differentiation phases of embryonal kidney which are recapitulated during develoment of WT are bracketed. Fully differentiated PC and IC cells cannot be seen in WT. **b,** Immunhistochemistry of distinct phases of UBT differentiation in WT. UBT-derivative cells can differentiate into CA2 positive IC cell precursors and immature nonα-nonβ intercalated cells in WT. FK- foetal kidney; WT – Wilms' tumor.

We have demonstrated unequivocally that not only MM-derived cells but also bi-potential cells of UBT are involved in development of NR and WT. Therefore, the WHO definition of Wilms's tumour that „nephroblastoma is a malignant embryonal neoplasm derived from ***nephrogenic blastemal cells"*** is obsolete. We suggest the following definition: ***"Wilms' tumor is a malignant embryonal neoplasm derived from pluripotential cells of nephrogenic blastema and of ureteric bud".***

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