Wilms tumor cells with WT1 mutations have characteristic features of mesenchymal stem cells and express molecular markers of paraxial mesoderm

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Wilms tumors (WTs) are genetically heterogeneous kidney tumors whose cells of origin are unknown. Tumors with WT1 mutations and concomitant loss of the wild-type allele represent a distinct subgroup, frequently associated with mutations in CTNNB1. Here, we describe the establishment and characterization of long-term cell cultures derived from five individual WTs with WT1 mutations. Three of these tumor cell lines also had CTNNB1 mutations and an activated canonical Wnt signaling pathway as measured by β-catenin/T cell-specific transcription factor (TCF) transcriptional activity. Four of the five Wilms cell lines had a stable normal karyotype for at least 25 passages, and four lines showed loss of heterozygosity of chromosome 11p due to mitotic recombination in 11p11. Gene expression profiling revealed that the WT cell lines are highly similar to human mesenchymal stem cells (MSCs) and FACS analysis demonstrated the expression of MSC-specific surface proteins CD105, CD90 and CD73. The stem cell like nature of the WT cells is further supported by their adipogenic, chondrogenic, osteogenic and myogenic differentiation potentials. By generating multipotent mesenchymal precursors from paraxial mesoderm (PAM) in tissue culture using embryonal stem cells, gene expression profiles of PAM and MSCs were described. Using these published gene sets, we found coexpression of a large number of genes in WT cell lines, PAM and MSCs. Lineage plasticity is indicated by the simultaneous expression of genes from the mesendodermal and neuroectodermal lineages. We conclude that WTs with WT1 mutations have specific traits of PAM, which is the source of kidney stromal cells.

INTRODUCTION

Wilms tumor (WT) or nephroblastoma is one of the most common childhood malignancies, affecting 1/10 000 children. The classic triphasic WT contains a mixture of undifferentiated blastemal, differentiated epithelial cells and stromal elements, whereas individual cell types may predominate in some tumors. These different components are thought to arise from a multipotential mesenchymal renal stem/progenitor cell, which fails to undergo a proper differentiation program (1). The epithelial and blastemal elements in triphasic WT mimic structures seen during normal nephrogenesis, whereas the stromal-predominant/stromal-type WT contains ectopic mesenchymal elements not usually seen in kidneys, mostly striated and smooth muscle cells (rhabdomyogenesis), as well as chondrocytes, osteocytes or adipocytes. Another term often used for these tumors is mesenchymal tumors with ectopic differentiation. These cell types are reminiscent of the differentiation potential of human mesenchymal stem cells (MSCs), which have the capacity to differentiate along a number of connective tissue lineages, such as cartilage, bone, adipocyte or muscle (2).

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Mutations in the WT1 gene, a zinc finger transcription factor, are found in 10–15% of WTs and are most frequent in stromal-type WT (3–8). We have reported that WT1 germ-line mutations are often associated with loss of the second wild-type allele, confirming Knudson’s two-hit hypothesis for the development of this specific subclass of WT (3,7). Intralobar nephrogenic rests (ILNR), which are thought to be precursor lesions are found in WTs derived from patients with germ-line WT1 mutations or WT1 deletions as in Wilms tumor, aniridia, genitourinary anomalies and mental retardation (WAGR) patients. It was shown that ILNR harbor homozygous WT1 mutations, whereas the associated tumors have additional CTNNB1 mutations, demonstrating the order of these genetic events (9).

A high level of WT1 expression is observed at the time of mesenchymal-to-epithelial transition during kidney development, pointing to its role in epithelial differentiation (10). Loss of a functional WT1 protein in mesenchymal cells of mutant tumors might provoke a faulty differentiation into myogenic, fat or cartilage lineages. WT1 is expressed in blastema and glomeruloid structures but is absent from stromal cells in WTs lacking mutations (8).

Mutations in exon 3 (destruction box) of CTNNB1 at amino acid positions that are targets for phosphorylation were identified in WTs (11–13). Owing to these mutations, β-catenin cannot be phosphorylated resulting in its stabilization and migration to the nucleus, where it activates Wnt target genes together with T cell-specific transcription factor/lymphoid enhancer-binding factor. The regulation of β-catenin phosphorylation is important for controlling Wnt signal transduction. CTNNB1 mutations are often associated with mutations in WT1 (8,12,13) and in our recent analyses, we found that all WT1 mutant tumors also had CTNNB1 mutations (14). These data suggest that there is a high selection pressure for WT1 mutant tumors to acquire additional mutations in CTNNB1. Nuclear accumulation of β-catenin, an indicator for activated Wnt signaling, was found in WTs with CTNNB1 mutations, although not all mutant tumors showed nuclear β-catenin staining (8,11). In CTNNB1 mutant tumors nuclear localization was observed in undifferentiated mesenchymal cells (8).

Tumors with wild-type WT1 are a distinct subgroup often with epigenetic alterations (loss of imprinting, LOI) in 11p15 targeting the H19 and IGF2 genes (15). Furthermore, most of these WT1 wild-type tumors express high levels of WT1 (C.D. and B.R.-P., unpublished data). Therefore, at least two genetically distinct molecular subgroups of WTs exist, underscoring the heterogeneity of this type of tumor.

WTs are thought to be derived from kidney progenitor cells, but the identification of human kidney stem cells remains elusive. Several reports have described the isolation of embryonic renal stem cells from mice and rats, which can differentiate into myofibroblasts, endothelial and epithelial cells as well as myogenic, osteogenic, adipogenic and neural lineages (16–19). In addition, kidney side population cells were isolated from embryonic and adult murine kidneys, capable of multilineage differentiation in vitro (20). Isolation of renal progenitors from human kidneys based on the expression of CD133 also revealed that these cells are capable of epithelial and endothelial differentiation (21). However, it has not been shown unequivocally that one type of kidney progenitor cell exists, giving rise to all components of the kidney. Pure progenitor cells to address these questions are not available.

The kidney is derived from the intermediate mesoderm, located between the lateral plate mesoderm and paraxial mesoderm (PAM). Nephrogenesis is a process of reciprocal inductive cell–cell interactions between the ureteric bud (UB), metanephric mesenchyme (MM) and stromal cells, ultimately resulting in mesenchymal-to-epithelial conversion and nephron formation. Most cells in the adult kidney are formed from either UB, giving rise to epithelial cells of the collecting duct or MM to the rest of the epithelial cells of the nephron. In addition, stromal cells are important for kidney morphogenesis as shown by targeted disruption of B2/Foxd1 (22). Foxd1 mutants displayed a reduced number of UB branches and nephrons, suggesting that Foxd1 is required for the formation of the renal capsule, establishing correct zones for induction and differentiation (23). Stromal cells in the kidney were believed to be derived from the intermediate mesoderm; however, fate mapping studies in the chick embryo showed that stromal cells of the developing kidney are derived from pax3 PAM cells (24). Furthermore, fate mapping studies of pax3 PAM progenitor cells in reporter mice (25) are consistent with those in the chicken and support the hypothesis that renal stromal cells derive from pax3 PAM progenitors. Pax3 is a transcription factor first expressed in early somitogenesis, specifically in limb muscle, PAM, neural crest and in the neural tube during embryonic development and is implicated in migration and differentiation, as well as formation of hypaxial muscle.

The cells of origin for the different forms of WTs are unknown. In order to better understand the biology of WTs, a cell culture model system is needed. Primary WT cells have a very limited lifespan in tissue culture and establishing cell lines from WTs has been notoriously difficult (26–30). So far, only a few WT-derived cell lines capable of long-term propagation have been established, some with highly complex karyotypes (31–33). In contrast, WT cells could be propagated to some extent after heterotransplantation into nude mice (34,35).

Here, we report a new method for the establishment of long-term homogenous WT1 mutant cell lines derived from WTs that have different genetic alterations in the WT1 gene. Characterization of these novel cell lines provides experimental evidence that the tumor initiating cells of WTs with WT1 mutations may reside in PAM.

RESULTS

Establishment of cell lines from five individual WTs with WT1 mutations

As WTs with WT1 mutations show mesenchymal differentiation, we hypothesized that the WT cells have features of MSCs. To test this hypothesis, we wished to establish long-term cell cultures from WTs with WT1 mutations. Initially, we cultivated explants from primary tumors in a tissue culture medium specifically developed for WTs (WT medium) (27). Cells established with this medium showed epitheloid morphology, but could not be cultivated for more
than 1 month and lost their epithelioid morphology after 2–3 passages (Fig. 1A). Next, we tested whether cells from WTs with WT1 mutations could be cultivated in a medium specifically developed for the propagation of MSCs (MSC growth medium, MSCGM). Therefore, tumor cell cultures were initiated in MSCGM and a parallel cell culture was set up in WT medium. The morphology of the WT cells in MSCGM was spindle shaped and resembled MSCs (Figs 1B and 4A, left panels). Analyzing cells grown in these two different media for the presence of WT1 mutations revealed that homozygous WT1 mutant cell lines could only be established in MSCGM, but not in WT medium. The presence of the mutation was analyzed by restriction enzyme digest using the enzyme Mse1, which cuts only the mutant DNA. DNA from the original tumor sample contains more mutant DNA as seen by the presence of two strong bands at 110 and 100 bp, whereas a faint band at 210 bp corresponding to the uncut wild-type DNA is seen (Fig. 1A, bottom, lane T). WT1 wild-type normal kidney (NK) cells (corresponding to the band at 210 bp) were selectively enriched when tumor explants from patient Wilms3 were cultivated in WT medium and only wild-type DNA was observed already at passage 2 (Fig. 1A, bottom, lanes P2 and P3). In contrast, at passage 1 in MSGM, the culture consisted of a mixture of cells with ~50% mutant and wild-type WT1 alleles (Fig. 1B, bottom, P1). A homogeneous population of homozygous WT1 mutant cells was observed after passage six (Fig. 1B, bottom P6 and P8). As this patient has a tumor-specific WT1 mutation, normal cells have only wild-type WT1 alleles (see below).

By using these culture conditions, we have now successfully established cell lines from five different WTs which are characterized by different homozygous WT1 mutations. The new WT-derived cell lines were cultivated for at least 60 population doublings. The doubling time varied between 1.8 and 3 days. The WT cell lines were termed Wilms1–5 in this work.

Identification of WT1 and CTNNB1 mutations in WT cell lines

A detailed description of the patient’s mutation status and the cell lines derived from their WTs is found in Table 1. Wilms1 with a homozygous WT1 p.S50X stop mutation and an additional p.S45F CTNNB1 mutation has been described before (36). The Wilms2 cell line has a homozygous p.R362X WT1 stop mutation due to a mitotic recombination in 11p11 and carries a p.S45Y CTNNB1 mutation. Wilms3 harbors a tumor-specific homozygous WT1 mutation, resulting in a C-terminal extension of the WT1 protein and a wild-type CTNNB1 gene. Wilms4, derived from a WAGR patient has a tumor-specific WT1 mutation in the remaining allele and a p.AS45 CTNNB1 mutation. Wilms5 is unusual, as the germ-line p.R390X WT1 mutation was lost in the tumor. This tumor is characterized by a novel homozygous WT1 mutation in exon 10 leading to an extended protein p.R433Ps84 and a wild-type CTNNB1 gene. The frameshift WT1 mutations in Wilms3, 4 and 5 in exon 10 lead to a stop codon in the 3′-UTR at the same position yielding an extended WT1 protein with 79 identical amino acids at the C terminus (Fig. 2A). The altered amino acid sequence does not follow the consensus sequence for a C2H2 type zinc finger (CX2–4C-X12-H-X3-H) and it is therefore unlikely that an intact Zinc finger 4 (ZF) is present in these mutant proteins.

Figure 1. Establishment of cell lines from primary WTs. (A) Top: Wilms3 cell culture initiated in WT medium. When the cells were kept in WT medium without subculturing, they retained their epithelial morphology and showed signs of epithelial differentiation. Bottom: analysis of DNA from the Wilms3 culture at passage 2 (P2) and 3 (P3) in comparison to the DNA extracted from the tumor (T) by restriction enzyme digest with Mse1 for the presence of the WT1 mutation. C: undigested wild-type control DNA. (B) Top: Wilms3 cell culture established in MSCGM, early passage cells at lower density. Bottom: disappearance of wild-type WT1 during passaging: in P1, the culture shows wild-type and mutant WT1 alleles, whereas in P6 all cells show only mutant WT1.
To test whether mutant WT1 transcripts are expressed, we performed quantitative real-time PCR (Q-RT-PCR). The level of WT1 mRNA varied between Wilms 1–5 and the Q-RT-PCR data were highly concordant with the microarray data (Fig. 2B). The presence of WT1 mutations in expressed mRNAs was verified by sequencing and reported for Wilms1 (36). The sequencing data for Wilms2, 3, 4 and 5 are not shown. All four major WT1 transcript isoforms were expressed, corresponding to transcripts lacking or containing exon 5 and lacking or containing the alternative splice site coding for three amino acids (KTS). The abbreviations for these four isoforms are WT1A (2/exon5/2KTS), WT1B (+exon5/2KTS), WT1C (2/exon5/+KTS) and WT1D (+exon5/+KTS). Next, we studied whether mutant WT1

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<td><strong>WT1</strong> mutation status of patient in blood DNA</td>
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<td>Wilms3 wild-type</td>
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<td>Wilms-cell line CLS1, unknown</td>
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Figure 2. WT cells with WT1 mutations express WT1 protein. (A) Amino acid sequence of normal and mutant WT1 proteins from Wilms3, Wilms4 and Wilms5 cells. The frameshift WT1 mutations lead to identical stop codons in the 3′-UTR yielding extended WT1 proteins with identical amino acids at the C terminus (yellow). The first altered amino acids are indicated (red). (B) WT1 mRNA expression levels in the five Wilms cell lines and MSCs. A highly concordant result is obtained by Q-RT-PCR and Agilent microarrays. The standard error was derived from three Q-RT-PCR experiments and from five individual microarrays. (C) Western blot analysis of WT1 expression in Wilms3 and M15 cells. n, nuclear extract; c, cytoplasm. (D) Fluorescence microscopy shows the subcellular localization of four transfected GFP-Wilms3 protein isoforms in live NIH-3T3 cells. The mutant WT1 isoforms are: A (2/2), B (+/2), D (+/+ ) and C (2/+). A more detailed description of mutant WT1 isoforms is given in the text.
proteins are expressed in the Wilms cell lines. Because the highest level of WT1 mRNA was observed in Wilms3, we initially analyzed whole cell lysates from this cell line using an antibody specific for the N-terminus of WT1. In this experiment, WT1 was not detectable and we decided to enrich the proteins by fractionation into nuclear and cytoplasmic proteins. With the help of a highly sensitive western blot kit, we detected the mutant WT1 protein in cytoplasmic extracts of Wilms3, with a slightly higher level of the +exon5 than the −exon5 isoform of WT1 (Fig. 2C). In comparison, in M15 cells, derived from mesonephric epithelial cells of the mouse embryo, WT1 is mostly found in the nucleus and these cells express more of the −exon5 isoform. After transfection of GFP–WT1 fusion proteins corresponding to the four mutant WT1 isoforms from Wilms3, a predominant cytoplasmic localization was observed for isoforms A (−/−), B (+/−) and D (+/+), however isoform C (−/+2) was also observed in the nucleus of NIH3T3 cells (Fig. 2D). Wilms1, 2, 4 and 5 express very low levels of WT1 mRNA and the WT1 protein was not detectable by immunoblotting presumably due to the detection limit.

**Karyotype and LOH analysis of Wilms cell lines**

Karyotype analysis revealed a stable normal karyotype in Wilms1, 2, 4 and 5. Wilms3 cells had a mosaic karyotype at passage 16: _4_7,XY,+18[19]/46,XY[4]. FISH using chromosome 11 centromere and telomere probes revealed that at passage two, trisomy 18 was present in 59% of the cells, increasing to 74% by passage 16. Cells established from the normal adjacent kidney of this patient had a normal karyotype, only present in tumor cells. As blood DNA was unavailable, we could not investigate whether this patient has a germ-line mosaicism for trisomy 18. Constitutional trisomy 18 has been described as a risk factor for WT development (37).

All Wilms cell lines had two copies of chromosome 11. Loss of heterozygosity (LOH) analyses showed that in four cell lines a mitotic recombination event had occurred between markers in 11p11 and 11p13, whereas the cell line from the WAGR patient (Wilms4) has retained both 11p parental alleles. Loss of the maternal allele could be identified in Wilms1 and 2, where parental DNA was available. Therefore, two copies of parental alleles (paternal uniparental disomy, UPD) from 11p11 → 11pter, containing the IGF2 gene, are present in these cells (Fig. 3). The IGF2 gene, a fetal growth factor, is normally only expressed from the paternal allele. LOI of IGF2 leading to expression from both parental alleles can be observed in WTs (15). High expression of IGF2 mRNA was observed by array analysis in all five Wilms cell lines and this was confirmed by Q-RT-PCR (Supplementary Material, Fig. S1).

**WT cell lines with WT1 mutations have a similar differentiation potential as human MSCs**

The morphological similarity of the WT cell lines with MSCs provoked the question, whether WT cells with WT1 mutations have a mesenchymal differentiation potential. To address this question, we performed in vitro differentiation experiments using established procedures and MSCs as positive controls. After induction of adipogenic differentiation, MSCs showed strong Oil Red O staining, which labels lipid vesicles. Wilms3 showed a few stained areas (Fig. 4A, panel Wilms3, Oil Red O), whereas the other four Wilms cell lines did not show any Oil Red O staining (see for example panel Wilms1, Oil Red O, Fig. 4A). A low level of FABP4 expression, a marker for fat differentiation was observed in uninduced Wilms3 and 5 and a slightly higher level in Wilms2. In all Wilms cells except Wilms4, FABP4 expression increased after induction of adipogenesis (Fig. 4B).

Terminal chondrocyte differentiation was analyzed by Safranin O staining after growth in pellet cultures for 2–3 weeks. Fibroblasts were used as negative controls (data not shown). Wilms1 and 2 could not be induced to terminal chondrogenic differentiation, whereas Wilms3 could be induced as demonstrated by positive Safranin O staining (Fig. 4A, panel Wilms1, Safranin O). Wilms4 and 5 were not analyzed for this differentiation pathway. Osteogenic differentiation was studied by determining the calcium concentration in Wilms cell culture supernatants in comparison to MSCs as positive and fibroblasts as negative controls. Wilms1, 2 and 3 showed some osteogenic differentiation with increased levels of calcium, whereas Wilms4 and 5 showed very low calcium levels (Fig. 4C).

Next, we investigated the potential of these cells for muscle differentiation. As positive control, we used human skeletal muscle myoblasts (HSMM) because we were not able to induce muscle differentiation in MSCs. Muscle differentiation of Wilms1–5 was initiated with reduced serum concentrations and the development of multinucleated cells was checked by phase contrast microscopy. HSMM cells showed an almost complete conversion to multinucleated cells with concomitant growth arrest, whereas all Wilms cell lines continued to grow in reduced serum. However, some multinucleated cells were detected in Wilms1 and Wilms2 and some cells appeared striated (data not shown). Using an antibody to titin, which recognizes a sarcomeric protein of differentiated striated...
muscle, in Wilms1 a few multinucleated cells were stained (Fig. 4A, right panels). Wilms2, 3, 4 and 5 did not show reactivity with this antibody, suggesting that a later stage of muscle differentiation cannot be achieved in these cells. We conclude from these results that the WT cell lines with WT1 mutations have a limited mesenchymal differentiation capacity, which varies between the tumors with different genetic alterations.
Gene expression profiling reveals a high degree of similarity between WT cells with WT1 mutations and human MSCs

To compare the WT cells and MSCs at the level of the transcriptome, we performed genome-wide expression analysis using oligonucleotide arrays (Agilent). The expression profiles of Wilms1–5, MSCs, NK cells and CLS1, a WT cell line without a WT1 mutation were compared by average linkage hierarchical clustering of the gene expression list. This analysis grouped MSCs with the Wilms cell lines (Wilms1–5) at a distance from NK and CLS1 (Fig. 5). Using correlation coefficient and Euclidean metric as distance measurements produced very similar trees. We conclude from these results that the transcriptomes of MSCs and WT cells with WT1 mutations have a high degree of similarity.

Next, we asked whether bona fide MSC genes are active in the Wilms cell lines. Human MSCs express specific surface markers which are commonly used for the identification and isolation of MSCs (2). We thus compared the expression levels of various characteristic MSC marker genes on microarrays. We found that CD49a, CD44, CD73, CD90, CD105 and CD102 genes are strongly expressed in all five Wilms cell lines (Fig. 6 and Supplementary Material, Fig. S2). In contrast, CLS1 with a wild-type WT1 gene did not express these MSC-specific genes, except for CD44 which is also expressed in CLS1 (Fig. 6 and Supplementary Material, Fig. S2). The expression of CD73, CD105 and CD90 was confirmed by Q-RT-PCR and a good overall correlation was observed between microarray and Q-RT-PCR (Fig. 6). In addition, FACS analysis further confirmed that CD73 and CD105 proteins were expressed on more than 99% of Wilms2, Wilms3, and MSC cells, whereas CD73 was observed on 15% of CLS1 only and CD105 was not expressed. The MSC-marker CD90 was positive on 80% of Wilms2, 72% of Wilms3, 45% of MSC and CLS1 was negative for this marker. These FACS results further confirmed the microarray data and the expression of MSC markers on the WT cell lines. CD34 is a marker for hematopoietic stem cells and CD31 a marker for endothelial cells both of which were not expressed in MSCs and Wilms1–5, however, CLS1 WT cells strongly expressed these genes (Supplementary Material, Fig. S2). In conclusion, WT1 mutant WT cells are highly related to MSCs.

To gain more specific information about the similarity of WT cells with MSCs, we analyzed their gene expression profiles and identified a shared gene signature. The Venn diagrams (Fig. 7A) show that 2774 genes are expressed at a higher level, whereas 2708 genes are expressed at a lower level in comparison to CLS1 cells (Fig. 7A). We then performed an ontological analysis on the up-regulated genes using EASE and identified a highly significant enrichment of specific GO terms (P < 0.001) in this gene set. The enriched GO terms are: anatomical structure development, multicellular organismal development, anatomical structure morphogenesis, developmental process, system development, organ development, organ morphogenesis and multicellular organismal process. Genes from the shared signature with an at least 500-fold difference to CLS1 are shown in a heat map (Fig. 7B). We also performed a gene set analysis of the shared gene signature using Globaltest and identified a significant enrichment of GO terms relating to diverse signaling pathways (Fig. 7C). The heat maps show the expression levels of representative pathway genes in NK (N), CLS1 (C) and the Wilms cell lines (Wilms1–5). A large number of genes from the TGFβ signaling pathway are expressed in the shared signature, e.g. TGFBR2, INHBA, DCN, THBS1, THBS2, FST, SMAD7, TGFBI, BMP4, ACVR1, RHOD and MAPK1 (Fig. 7C, panel TGFβ). The shared gene signature also contains significant numbers of genes from the Wnt, Hedgehog, MAPK, VEGF, mTOR and JAK-STAT signaling pathways (Fig. 7C). We expect that the expression of signaling pathway genes is correlated with pathway activity and future work will investigate this issue.

It is not possible to definitely identify the cells from which WTIs originate in the tumor tissue of WT patients, as these tumors are heterogeneous and show diverse differentiation patterns. Here, we searched for alternative approaches to clarify this issue. A recent study presented the induction of PAM with the generation of multipotent mesenchymal progenitors using ES cells that were cultivated on murine OP9 stromal cells (38). In this work, the mesenchymal precursors were isolated by flow sorting using a CD73-specific antibody and subsequently analyzed by genome-wide expression profiling. By comparing MSCs derived from embryonal stem cells with primary adult MSCs a specific gene signature consisting of 412 genes was identified. We compared the gene expression profiles of Wilms1–5 with this MSC-specific gene list and found that the majority of MSC-specific genes were also expressed in the five Wilms cell lines (Supplementary Material, Table S1). These data further support the similarity
of the WT cell lines to MSCs, suggesting that WTs with \textit{WT1} mutations originate from cells of the mesenchymal lineage.

\textbf{WT cells with \textit{WT1} mutations express typical molecular markers of PAM}

Murine embryonal stem cells have been used for guided differentiation into three germ layers (39). This enabled the generation of mesoendoderm, neuroectoderm, lateral plate mesoderm and PAM in tissue culture. The different cell types were sorted by flow cytometry using specific surface markers and analyzed by genome-wide expression profiling. We used data from the published gene lists for mesoendoderm (CDH1+/PDGFRα+), neuroectoderm (SSEA1+/KDR−/PDGFRα−), lateral plate mesoderm (CDH1−/KDR+/PDGFRα+) and PAM (CDH1−/KDR−/PDGFRα−) (39) and compared these with our gene expression data from the five Wilms cell lines and MSCs. This analysis revealed that Wilms1–5 cells are negative for the mesendoderm marker CDH1 and the lateral plate mesoderm marker KDR/FLK1 and positive for the paraxial marker PDGFRα. The immunofluorescence analysis of Wilms3 with PDGFRα showed that all cells express this surface receptor (Supplementary Material, Fig. S3A). Although there is some overlap in the gene expression profile between these lineages, the marker profile expressed in the WT cells with \textit{WT1} mutations allocates these to PAM. The gene list of specific PAM cells includes PDGFRα, PDGFRβ, MSX1, RUNX2, TWIST2, TBX4, TBX20, HAND1, DOK4 and several collagen genes as well as the anterior–posterior markers \textit{HOXA10, HOXA11, HOXB6, HOXC10} and \textit{HOXD1}. With the exception of TBX4, TBX20 and HAND1, these PAM-specific genes are also expressed in the Wilms1–5 cells and a heat map of the expression of paraxial marker genes is shown in Supplementary Material, Figure S3B.

As it was recently shown that kidney stromal cells are derived from PAM (24), we studied the expression of kidney stromal markers. It is known that cortical and medullary interstitial cells in E15.5 mouse kidney express \textit{Meis1} (40). Using the information available through the comprehensive gene expression analysis of individual compartments of day E15.5 mouse kidneys (40), we found that the \textit{Meis1} positive cells also express FoxD1 as well as \textit{Wt1} (Supplementary Material, Table S2). \textit{MEIS1} and \textit{FOXD1} are expressed in all Wilms cell lines as well as in MSCs. The expression of \textit{FOXD1} was confirmed by Q-RT-PCR and a good correlation

Figure 6. Expression of characteristic mesenchymal stem cell markers. On the left, the RNA expression analysis of specific mesenchymal stem cell markers \textit{CD73}, \textit{CD90} and \textit{CD105} in WT cell lines by microarray and validation by Q-RT-PCR is shown. The surface expression of \textit{CD73}, \textit{CD105} and \textit{CD90} of CLS1, MSC, Wilms2 and Wilms3 was analyzed by flow cytometry (FACS). Staining with isotype controls is indicated by gray histograms and the percentage of positive cells is indicated.
between the microarray data and Q-RT-PCR was observed (Supplementary Material, Fig. S1). The expression of stromal marker genes and molecular markers of PAM suggest that the tumor initiating cells for WTs with \textit{WT1} mutations reside in PAM.

Identification of a WT-specific gene signature

Next, we wished to identify genes whose expression is specific for the WT cells. We thus compared the gene expression profiles of Wilms1–5 cells with that of MSCs and identified a WT-specific gene signature (WT signature). Microarray analysis revealed a significant differential expression of 721 genes between MSCs and Wilms1–5 cells. Of these differentially expressed genes, 322 had an at least 1.8-fold higher and 399 an at least 1.8-fold lower expression level in Wilms1–5. A GO term enrichment analysis using EASE showed that genes from the tube development pathway were significantly up-regulated including \textit{EDNRA}, \textit{BMP2}, \textit{BMP4}, \textit{HHEX}, \textit{SIM2}, \textit{RHOJ}, \textit{FOXF1}, \textit{TBX3}, \textit{PAX3} and \textit{LAMA5}. Other enriched GO terms not reaching statistical significance relate to organ morphogenesis (\textit{P}-value 0.06), regulation of astrocyte differentiation (\textit{P}-value 0.085) and mesenchymal development (\textit{P}-value 0.088). GO terms relating to keratin filament and...
multicellular organisinal development were significantly enriched in the group of 399 genes with strongly reduced expression levels in WT cells.

In order to get a deeper insight into the function of the WT signature, we used a knowledge driven approach and extracted biological information about individual genes by screening the literature. On the basis of this comprehensive analysis, a large number of genes could be assigned to specific functional groups and these data are depicted in Table 2. We noted that the WT signature consists of a large group of genes encoding transcription factors that play a role in embryonic development or pattern formation and another large group of genes with a function in neurogenesis, axon development and axon guidance. Other functional groups relate to the cell cycle, diverse signaling pathways, blood vessel development, muscle differentiation and other biological processes (Table 2).

The most highly expressed genes from the WT signature include PAX3, a gene expressed in the presomatic and PAM, developing somites, limb muscle, neural crest and neural tube. The expression of this gene was confirmed by Q-RT-PCR (Supplementary Material, Fig. S1). PAX3 is also a master regulator of myogenesis. Other highly expressed genes are HOXD10, which controls skeletal development and HOXD11 which is expressed in metanephric blastema of the kidney. Additional genes from the WT signature are also regulators of kidney development, for example the transcription factors HOXA11, HOXC11, SALL1, TCF21 and the bone morphogenic proteins, BMP2 and BMP4. The role of HOXD11/HOXA11 in kidney development was demonstrated by double-knockout mice which display variable kidney hypoplasias (41).

Other highly expressed genes have a function in malignant tumors. For example ARHGDIB, a Rho guanosindophosphate dissociation inhibitor which affects tumor growth and malignant progression of gastric carcinomas, the DNA methyltransferases DNMT1 and DNMT3B which are up-regulated in the colorectal adenoma–carcinoma sequence, and DKK3 (11p15) which is expressed in pancreatic cancer and osteosarcomas. PIK3C2B is a component of the PI3K signaling pathway, which is expressed in metanephric blastema of the kidney. Additional genes from the WT signature are also regulators of kidney development, for example the transcription factors HOXA11, HOXC11, SALL1, TCF21 and the bone morphogenic proteins, BMP2 and BMP4. The role of HOXD11/HOXA11 in kidney development was demonstrated by double-knockout mice which display variable kidney hypoplasias (41).

Coexpression of genes from the WT1 and/or CTNNB1 mutant WT signature in WT cell lines

WTs with WT1 mutations show diverse differentiation patterns and for this reason the tumor tissue consists of heterogeneous

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<td>Transcriptional regulation, transcription factor activity</td>
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Continued
Regulation of muscle differentiation with a high expression difference between Wilms cell lines and MSC are MSC. The same genes may appear in more than one functional category, genes Manually curated and selected list of up-regulated genes, fold change versus TGF Hh signaling/target gene Extracellular matrix Cell–cell signaling/adheren junctions Hh signaling/target gene Cell–cell signaling/adheren junctions TGFβ signaling RA signaling Adipogenesis Chondrogenesis/osteogenesis Regulation of muscle differentiation

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Manually curated and selected list of up-regulated genes, fold change versus MSC. The same genes may appear in more than one functional category, genes with a high expression difference between Wilms cell lines and MSC are highlighted in bold.

cell populations. In contrast, WT-derived cell lines represent a homogeneous cell population. It is evident that microarray data derived from WTs correspond to the average gene expression pattern of the different cell types present in the tumor and the differentiation states of these cells. In order to investigate whether the WT1 mutant cell lines express similar genes as WTs in vivo, we compiled a list of genes characteristic for WTs with WT1 and/or CTNNB1 mutations from existing microarray data (13,43–45). It is surprising that comparing the gene lists from these four publications not a single gene is present in all four tumor signatures (Supplementary Material, Table S3). This could be explained by the various array formats that were used or by different treatment regimens of the analyzed WTs (13,43–45). We found that 194 named genes of this merged gene list were also present on our array format. Importantly 140 (72%) of these WT signature genes are either expressed in all five or at least in one of the Wilms cell lines (Supplementary Material, Table S3). Of note seven of these genes that are coexpressed in tumors in vivo and the Wilms cell lines are PAM marker genes (Supplementary Material, Table S3, highlighted in blue). We also observed that WTs in vivo express a number of genes related to muscle differentiation, osteogenesis and endothelial cell function, which are not expressed in the Wilms cell lines (Supplementary Material, Table S3, highlighted in green and brown). This could be explained by the fact that the Wilms cell lines have features of multipotent mesenchymal precursors and thus do not express differentiation specific genes. In summary, these results demonstrate the overall similarity of the characteristic gene expression profile of the WT1/CTNNB1 mutant WTs in vivo with the established WT1 mutant cell lines. Several genes were selected from this list and were analyzed by Q-RT-PCR using RNA from all cell lines and for comparison RNA isolated from tumor material of Wilms1, Wilms2 and Wilms4. Overall, a good correlation was found between microarray data and Q-RT-PCR for AXIN2, DKK2, MET, SIM2 as well as PDGFRA, a marker for PAM, which is also highly expressed in MSCs (Supplementary Material, Fig. S4).

### β-Catenin/TCF-dependent Wnt signaling in WT cell lines

As Wilms1, 2 and 4 cells have a mutant CTNNB1 gene, we analyzed whether β-catenin/TCF-dependent transcriptional activity is affected by these mutations. To address this issue, we used the TOPflash reporter assay, which measures β-catenin-dependent Wnt signaling in living cells. As negative control, we used the FOPflash reporter construct with mutant TCF4 consensus binding sites. After transfection of TOPflash and FOPflash constructs into the Wilms cell lines, the TOP/FOP ratio was determined. Wilms1, 2 and 4 showed a TOP/FOP ratio between 4 and 6.5, whereas Wilms3 and 5 with a wild-type CTNNB1 gene had a ratio of 1 and 0.5, respectively. These data demonstrate that the β-catenin-dependent Wnt signaling pathway is activated in Wilms cell lines with CTNNB1 mutations (Fig. 8A).

Activation of the Wnt signaling pathway causes nuclear accumulation of β-catenin protein. We therefore analyzed the subcellular localization of β-catenin by immunofluorescence assays and immunoblotting. All five Wilms cell lines showed cytoplasmic β-catenin and small amounts of β-catenin in the nucleus as well as in cell–cell contacts (Fig. 8B). Very strong nuclear staining was observed in SW80 control cells (data not shown). The immunofluorescence results were confirmed by western blot analysis which detected β-catenin in the nucleus and cytoplasm in all Wilms cell lines (Fig. 8C).

To get an insight into the consequences of mutant β-catenin for Wnt signaling-dependent gene regulation, we compared the gene expression profiles of the Wilms cells with mutant CTNNB1 mutations to those with a wild-type gene. Surprisingly, only 15 named genes were differentially expressed (Table 3). We identified AXIN2 in the group of genes which are up-regulated in CTNNB1 mutant cells and confirmed the differential expression by Q-RT-PCR (Supplementary Material, Fig. S4, higher expression in Wilms1, 2 and 4). AXIN2, is a Wnt target gene with a role in negative-feedback...
regulation of Wnt signaling. In contrast, PAX8 expression is up-regulated in Wilms cells with a wild-type CTNNB1 gene. PAX8 participates in the regulation of mesenchymal–epithelial transition in the kidney. Interestingly, two of the unknown transcripts expressed at a lower level also map to the PAX8 gene locus but in an antisense orientation. The Wnt-signaling pathway is known to be involved in WTs with WT1 and CTNNB1 mutations. We therefore asked whether WTs with WT1/CTNNB1 mutations express the same Wnt target genes as our Wilms cell lines. To investigate this issue, we used a list of β-catenin/Wnt target genes from the Wnt homepage (www.stanford.edu/~rnusse/wntwindow.html) as well as new Wnt target genes described in the literature and checked their expression levels on microarrays. We then compared our expression data with existing data of high-density microarrays of WTs (43,44) and found that most Wnt target genes that are expressed in WTs in vivo are also expressed in Wilms1–5 cells (Supplementary Material, Table S4). These results show that the WT cell lines faithfully reflect the in vivo situation regarding activity of the Wnt signaling pathway. The establishment of WT cell culture models is an important prerequisite for further investigations of WTs with WT1 mutations.

DISCUSSION

We report here for the first time the successful establishment of long-term homozygous WT1 mutant cell cultures derived from WTs with WT1 mutations. This was achieved by using a tissue culture medium specifically developed for the growth of MSCs. This new method is of more general importance as we were able to establish cell lines from five individual WTs with diverse WT1 mutations. It is interesting to note that long-term cell lines could not be established from WTs with a wild-type WT1 gene using MSCGM, demonstrating that these tumors are biologically different (unpublished data).

Table 3. Differentially down- and up-regulated genes in CTNNB1 mutant and wild-type WT cell lines

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<td>(liprin beta 2)</td>
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Figure 8. Activation of the Wnt signaling pathway in WT cells with mutant CTNNB1 genes. (A) Transient transfection assay of the five Wilms cell lines with TOP- and FOPflash reporter plasmids and analysis of luciferase activity. The TOP to FOP ratio is displayed relative to the internal renilla control plasmid. Standard deviations were derived from three independent experiments. (B) Immunofluorescence analysis of β-catenin expression MSCs, Wilms2 and Wilms3 cells. β-catenin was visualized after incubation with an antibody specific for β-catenin followed by a fluorochrome-conjugated anti-rabbit secondary antibody. The cell lines and CTNNB1 mutation status are indicated on top of each panel. Magnification ×40. (C) Western blot analysis of β-catenin expression in the five WT cell lines. After incubation with an antibody specific for β-catenin, the same blot was used to detect alpha-tubulin which is a cytoplasmic protein. c, cytoplasm; n, nuclear extract.
Remarkably in four of our Wilms cell lines the homozygous mutant WT1 alleles are due to a similar mitotic recombination event between 11p11 and 11p13 leading to UPD from 11p11 → 11pter. This resulted in paternal UPD in two cases analyzed and consequently expression of the imprinted IGF2 gene in 11p15 from both alleles. Only one cell line derived from the WAGR patient had retained both parental copies of the short arm of chromosome 11.

The five Wilms cell lines can be grown for prolonged time in culture, irrespective of the presence or absence of an activated mutant β-catenin, suggesting that the mutant WT1 protein enables long-term survival. It is evident that the mutant WT1 proteins cannot bind to their normal target sequence in DNA, because they lack intact ZF domains. However, they have an intact N-terminal region which is required for protein–protein interactions. We showed here that Wilms3 cells express a mutant WT1 protein which is mislocated in the cytoplasm. It is a likely possibility that mutant WT1 proteins fulfill novel functions which are not related to their DNA binding activity. It has been shown that complete inactivation of Wilms in knockout mice was associated with apoptosis of mesenchymal cells (46). In contrast, the Wilms cell lines with mutant WT1 are viable and do not show any signs of apoptosis. It can be concluded from these results that mutant WT1 is biologically active and therefore these mutations can be classified as gain-of-function mutations. In this context, it is interesting to note that siRNA-dependent inhibition of Wilms in kidney organ cultures isolated at day 11 and cultured for 72 h resulted in abnormal proliferation (47). It is evident from these results that WT1 has a role in apoptosis and cell cycle regulation. We consider it possible that gain-of-function mutations of WT1 may have an oncogenic function independent of β-catenin.

Origin and nature of WTs with WT1 mutations

WTs with WT1 mutations have stromal-type histology, but it is unclear where these WTs originate. A characteristic feature of WTs with WT1 mutations is the presence of ectopic mesenchymal differentiation, predominantly skeletal muscle but also fat, and rarely cartilage and bone, reminiscent of human MSCs and known derivatives of PAM. Here, we used a combination of approaches for an experimental analysis of the origin and nature of WTs with WT1 mutations. A comparison of genome-wide expression profiles showed that the WT cell lines cluster with MSCs at a distance from NK cells or CLS1, the WT cells without a WT1 mutation. This analysis provided the first evidence that WT cells are related to MSCs. We then analyzed a gene signature specific for multipotent mesenchymal precursors which were derived from human embryonic stem cells after the induction of PAM in tissue culture (38) and showed that the majority of these genes were also expressed in the five Wilms cell lines. Moreover, the Wilms cell lines express RNA and protein of the highly specific MSC marker genes CD73, CD90 and CD105. To further substantiate the MSC-like nature of WT cells with WT1 mutations, we induced adipogenic, chondrogenic and osteogenic differentiation as well as muscle differentiation. Our results demonstrate that the Wilms cell lines have a limited capacity to differentiate into some of these mesenchymal lineages. This reflects the in vivo situation quite well, as WTs with WT1 mutations show only incomplete mesenchymal differentiation patterns. Collectively, these data suggest that WTs with WT1 mutations could originate from multipotent mesenchymal precursor cells of the PAM lineage. Similar evidence came from a comparison of gene expression data from Wilms cell lines with existing gene expression data from kidney development. Although the Wilms cell lines expressed only a limited number of kidney specific genes, they all expressed the stromal marker genes FOXD1 and MEIS1.

The origin of stromal cells in the kidney has only recently been resolved. It was reported that stromal cells in the kidney are derived from a pax3+/ domain of PAM and not from the intermediate mesoderm as all other cells of the adult kidney (24). However, it is important to note that in Pax3Cre/R26R mice, β-galactosidase is also expressed in cells that are derivatives of the metanephrogenic mesenchyme and therefore Pax3 expression is not exclusively found in stromal cells (25). It is impossible to obtain PAM from humans; however, human embryonal stem cells can be differentiated into diverse germ layers including PAM (39). A PAM-specific gene set was derived after guided differentiation of ES cells and flow sorting of PAM cells with specific surface markers (39). We analyzed the PAM cell-specific signature in the five Wilms cell lines and detected a highly significant overlap. By using this approach, we could show expression of paraxial mesodermal genes, demonstrating their close relationship to this embryonic tissue. A low level of Wilms is expressed in PAM derived from ES cells (S. Kume, personal communication). Of note, we found that Wilms is expressed in cortical (nephrogenic) and medullary interstitium, in the same compartment that expresses Meis1 and FoxD1, corresponding to stromal cells of the kidney (40 and Supplementary Material, Table S2). These data show that stromal-type WTs are related to kidney stromal progenitor cells, which originate in PAM. Our results, however, do not definitely prove that WTs originate in PAM. It is also a possibility that WTs initiate in immature MM and loss of a functional WT1 by mutation may change the fate of these cells towards the stromal lineage. Future work is required to resolve this issue.

The predominant differentiation type in stromal-type WTs is a rhabdomyogenic pattern. Pax3 is necessary and sufficient to induce myogenesis in MSCs (48). In addition, Pax3+/− mice have multiple skeletal defects, a delay in muscle differentiation and a decrease in muscle mass. Pax3 is essential for the expression of Six1 and Eya2 during skeletal myogenesis (49), all three genes being expressed in the Wilms tumor cell lines. The expression of these genes in the WT cell lines with WT1 mutations might explain their predominant rhabdomyogenic differentiation pattern in vivo and their limited muscle differentiation potential in vitro. Expression of Pax3 in WT samples with myogenic differentiation as well as in MM and in the stromal compartment of the developing mouse kidney was recently demonstrated by immunohistochemistry (50). As Pax3 promotes survival in embryonic and malignant cells and decreased Pax3 leads to cell death in embryonal rhabdomyosarcoma, melanoma and myoblast cells, this gene may have an important contribution for (tumor) cell survival.
WT gene signature

The WT signature contains 322 up-regulated genes and the largest group of genes belongs to the class of transcription factors including six HOX genes. HOXD10 is expressed 350-fold higher in WTs than MSCs and PAX3 102-fold higher. This demonstrates that, despite their similarity with PAM and MSCs, the tumor cells also show significant differences regarding the expression of transcription factors. It is evident that a tumor-specific transcriptional program is activated in the Wilms cell lines and future work is needed to investigate the impact of these transcription factors for WTs with WT1 mutations. Another large group of genes has a role in neurogenesis, axon development or axon guidance, followed by genes involved in G-protein signaling. Ontological analysis of the WT signature revealed tube development as a significantly up-regulated GO term. Of note, the WT signature contains a number of genes which have been found to be highly expressed in tumors or are known members of oncogene families. It is interesting that among these are several genes that are associated with poor prognosis or metastatic potential; both properties are not usually an attribute of genes that are associated with poor prognosis or metastatic potential; both properties are not usually an attribute of WT1 mutant tumors.

β-Catenin/TCF-dependent Wnt signaling in CTNNB1 mutant WTs

The role of β-catenin/TCF-dependent (canonical) Wnt signaling for nephron development and branching nephrogenesis is well established (51,52). Canonical Wnt signaling was observed in epithelia of the branching UB and in mesenchyme during transition into renal tubules and is rapidly down-regulated in maturing nephrons and becomes undetectable in postnatal kidney. In transgenic animals with a Ctnnb1 gain-of-function mutation, mesenchymal-to-epithelial transition of induced renal progenitors is blocked by the continued β-catenin activity (53). Therefore, a regulated activation with ensuing inactivation of Wnt signaling is critical for normal nephrogenesis. Furthermore, Wnt signaling is implicated in vertebrate development. During morphogenesis, massive cell movements take place which establish and shape the germ layers (54). The Wnt/planar cell polarity pathway (PCP) regulates specific cell behaviors and the migration of paraxial and lateral plate mesoderm cells (55). Wnt5a, a ligand acting in PCP and the downstream target prickle are involved in cell migration from the posterior streak; in the anterior streak this is antagonized by Wnt3a to generate the non-migratory medial mesoderm (56). WNT5a as well as PRICKLE2 are highly expressed in the Wilms cell lines and therefore these cells may have a defect in the cross talk with other cells. Recent data showed that Wnt signaling is a major target of WT1 by competition of WT1 with TCF4 for binding to CBP (58). As in all the developmental processes Wnt signaling is only temporarly active and if WT1 might have a role in its down-regulation, this would suggest that tumor development is caused by an inappropriately active Wnt signaling due to lack of repression by inactive WT1.

Another novel observation from our studies is that only 15 of the 20 000 expressed genes show a significantly different level in the CTNNB1 mutant and wild-type tumor cells. One of the genes expressed at a higher level in the mutant cells is a well-known TCF/β-catenin target gene, AXIN2, a negative regulator of Wnt signaling. In contrast, PAX8, with a known function in kidney development is up-regulated in CTNNB1 wild-type WT cell lines. This small number of differentially expressed genes in CTNNB1 mutant versus wild-type tumor cell lines suggests that the activation of other signaling pathways is also important. Indeed, our comparative pathway analysis demonstrated that in the shared transcription profile of the five WT cell lines with MSCs, many members of the transforming growth factor-β, mitogen-activated protein kinase, calcium, Hedgehog, JAK-STAT, p53, mTOR and vascular endothelial growth factor signaling pathways are expressed. If this gene expression pattern reflects pathway activity, WT cells and MSCs are regulated by the same signaling pathways.

Outlook

The establishment of long-term cell cultures derived from WTs with WT1 mutations is essential for experimental approaches to unravel the impact of WT1 and CTNNB1 for the development of WTs. The Wilms cell lines are a novel tool to study the function of genes over-expressed in the WT signature and they allow to investigate the role of wild-type and mutant WT1 for differentiation and Wnt signaling. A detailed understanding of the biology of WTs with WT1 mutations is an essential prerequisite for developing efficient therapies.

MATERIALS AND METHODS

Patient and tumor characteristics

Patient numbering is identical to the cell line numbering. All cell cultures were initiated from fresh WT samples obtained from 1998 to date and only those with a germ line or tumor-specific WT1 mutation were included in this study. Patient 1 was described previously (36). Briefly, the girl presented with bilateral tumors at 1 year of age with fetal rhabdomyomatous histology. The tumors did not respond to the SIOP-2001 pre-surgery chemotherapy (4 weeks of Act D and VCR) and were removed by kidney sparing surgery. After 1 year without further cytotoxic treatment, she developed again bilateral tumors. The right tumor showed triphasic histology, whereas the left tumor showed fetal rhabdomyomatous histology and the cell culture was established from the left tumor. Patient 2 presented at 1 year of age with unilateral, classical triphasic WT which was completely resected upfront. In addition, this male patient had bilateral cryptorchidism.

Patient 3 developed a unilateral WT at 11.5 months. He was preoperatively treated according to SIOP-2001/POH protocol (4 weeks of Act D and VCR). The stromal type tumor showed fetal rhabdomyomatous histology. Patient 4 was born with bilateral cryptorchidism and bilateral corneal opacities. A retarded psychomotor development was noticed, and at age of 11 months he presented with a renal mass in one kidney. The diagnosis of WAGR syndrome was suspected based on mental retardation, sporadic aniridia associated
with glaucoma and cryptorchidism. Cytogenetic analysis on peripheral blood lymphocytes demonstrated a 46, XY, del(11)(p13) karyotype, a deletion encompassing the WT1 and PAX6 genes. Upon diagnosis, a partial nephrectomy of the lower pole of the left kidney was performed. Pathology showed classic nephroblastoma and multiple foci of intralobar nephroblastomatosis in the surrounding tissue. The biopsy of the right kidney showed abnormal tubular and vascular structures with an interstitial inflammatory reaction which overall was compatible with dysplastic features; there was no evidence of nephrogenic rests or tumor.

Patient 5 had hypospadias and developed unilateral WT at the age of 2 years. He was preoperatively treated according to SIOP-2001/GPOH protocol (4 weeks of Act D and VCR). Pathology reported a tumor-like lesion of the kidney corresponding to a massive intralobar nephroblastomatosis with a rhabdomyoblastic stroma component.

Cell culture

In Duesseldorf, the tumor cell culture was initiated by manual dissection of the tumor with scissors into 1 mm cubicles, which were placed into the respective growth media, without disturbing for 7 days. Half of the medium was replaced with fresh growth medium every 3 days until outgrowth was observed. After that the medium was changed every 3 days and subculturing was started after cells became almost confluent. In Barcelona, the tumors were enzymatically digested and the culture was initiated in DMEM. After the first passage, the cells were shipped to Germany. Thereafter, cells were kept at low density with a medium change every 3 days. Growth medium was either WT medium as described (27) or Human MSCGM (Lonza). The following cell lines were purchased: human MSCs (Lonza), HSMM (Lonza) and WT-CLS1 (Cell Lines Services). The study was approved by the local Ethics Committee (Nr. 2617) and parents gave written consent that leftover tumor material can be used for research.

Mutation and LOH analysis

All WT1 exons were analyzed for mutations after amplification using the Expand High fidelity PCR system (Roche) and denaturing high performance liquid chromatography (WAVE, Transgenomic) as a pre-screening method (primers and conditions in Supplementary Material, Table S5). To get an optimal amplification of exon 1, 0.5 M Betain (Sigma) was added. Exons showing aberrant patterns were sequenced on an ABI 3100 automated capillary DNA sequencer (Applied Biosystems) using cycle sequencing procedure and the BigDye terminator kit. Exon 3 of CTNNB1 was analyzed as described before (11). LOH analysis was performed on blood and tumor DNA from the same patient. Four CA-repeat markers from chromosome 11p and q were used (primer sequences and PCR conditions are described in Supplementary Material, Table S6).

Protein extraction and western blot analysis

For preparation of nuclear and cytoplasmic extracts, 1 x 10^7 cells were washed once in PBS containing 1 mM MgCl2, resuspended in hypotonic buffer (10 mM HEPES pH 7.9, 300 mM sucrose, 50 mM NaCl, 1 mM EDTA pH 8.0, 1.5 mM MgCl2, 0.6% NP40, 0.5 mM DTT, 0.5 mM PMSF, 1 mM sodium metabisulfite, 1 mM benzamidine–HCl) and immediately spun at 1700 g for 5 min. The supernatant was removed and saved as first wash fraction. The cells were resuspended in the same buffer and incubated on ice for 30 min, followed by disruption of the cells in a homogenizer with a B pestle (at least 20 strokes). Nuclei were pelleted in a refrigerated microcentrifuge at 5700 g for 12 min and the supernatant saved as cytoplasmic fraction. Nuclei were resuspended in buffer B (50 mM Tris–HCl pH 7.5, 20% glycerol, 10% sucrose, 0.42 M KCl, 5 mM MgCl2, 0.1 mM EDTA, 2 mM DTT, 1 mM PMSF, 1 mM sodium metabisulfite, 1 mM benzamidine–HCl) and placed on a rotating wheel at 4°C for 30 min. The nuclear extract was spun at 18400 g for 30 min at 4°C. The supernatant was removed as nuclear fraction. Protein concentration was determined with a Bradford assay (BioRad). 100 μg of the cytoplasmic and nuclear fractions were separated on 10% SDS–PAGE and transferred to polyvinylidene fluoride membrane (BioRad). β-Catenin (1:500, E5, Santa Cruz Biotechnology) and α-tubulin (1:1000, T9026, Sigma) were detected using an ECL kit (GE Healthcare). For detection of the mutant WT1 protein, blocking was done in Super Block Blotting Buffer in PBS (Thermo Scientific) and the first antibody was an N-terminal specific antibody (1:220; clone 6F-H2, DAKO) and the second antibody was a stabilized peroxidase conjugated goat anti-mouse antibody (H+L, Thermo Scientific). Antibody-bound proteins were detected with the Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific).

Differentiation analysis

Adipogenic, chondrogenic and osteogenic differentiation was done using reagents from Lonza and as described by the manufacturer. Non-induced control cells were kept in the respective maintenance media. After the complete cycles of induction and maintenance, cells were processed for adipogenic differentiation by staining with Oil Red O and cells from a parallel flask were used for RNA isolation (RNasy, Qiagen). For osteogenic differentiation, cells were harvested by scraping them in the presence of 0.5 M HCl and a calcium Liquicolor assay was performed according to the manufacturers instructions (Stanbio Laboratory, USA). For chondrogenic differentiation, the pellet cultures were embedded in freezing medium, cryo-sections were prepared and stained for glycosaminoglycans with Safranin O. For the analysis of myogenic differentiation, HSMM cells (Lonza) were used as controls. Differentiation was induced with MSCGM (Lonza) supplemented with 2% horse serum for 1 week. Multinucleated cells were documented with phase contrast microscopy. For immunofluorescence analysis, cells were seeded in four chamber slides (BD Biosciences) and grown in reduced serum. After 1 week, cells were fixed with 2% paraformaldehyde and 4% sucrose in PBS for 15 min at RT. Blocking was carried out with PBS containing 4% FCS and 0.1% Tween 20 for 30 min, followed by incubation with a titin-specific antibody at a 1:100-fold dilution (H300, sc-28536; Santa Cruz Biotechnology) in blocking solution for 1 h. Cells were
washed with PBS and incubated with goat-anti-rabbit Alexa
fluor 488 antibody (Molecular Probes) for 1 h in the dark.
Finally, cells were mounted with ProLong Antifade (Molecu-
lar Probes) supplemented with DAPI and analyzed by fluo-
rescence microscopy.

For the semi-quantitative RT-PCR analysis of the FABP4
gene for adipogenic differentiation, the primers (for: 5’-TA
TGAAGAAGTAGGATGGGGC-3’; rev: 5’-CCACCCACC
AGTGTATCATCCTC-3’) were used for amplification and ali-
quots of the reaction were removed after 25, 30 and 35 cycles.

Luciferase reporter assays in WT cells

Wilms cells were plated in six-well plates at a concentration of
1.5 × 10^5 cells/well in MSCGM without antibiotics. At a con-
fluence of 50–80%, the cells were cotransfected with TOP- or
FOPflash-plasmids (Upstate Biotechnology) and either
pRL-TK (Wilms1, 2, 4 and 5) or pRL-CMV (Wilms3) (Renilla
luciferase) plasmid (Promega) for internal control of transfection
efficiency, using Lipofectamine LTX Reagent according to the pro-
colto of the supplier (Invitrogen). All assays were done at least in duplicates and the Firefly and
Renilla luciferase activities were measured using the Dual-
Luciferase Reporter Assay System (Promega) 24 h following
transfection. The Renilla luciferase activity was used to nor-
malize the transfection efficiency and the relative luciferase
activity was used to calculate the TOP to FOP ratio.

Immunfluorescence and FACScanalysis

For staining of β-catenin, Wilms cell lines and MSC were
seeded in four chamber slides (BD Biosciences) at 0.5–2 × 10^5
cells/well. At a confluence of 80%, cells were washed
with PBS and fixed with 3% paraformaldehyde for 15 min at
room temperature, followed by a PBS wash. Cells were
blocked and permeabilized in Blocking Solution consisting of
5% normal rabbit serum in PBS and 0.3% Triton X-100
(Sigma) for 60 min at room temperature. Cells were incubated
overnight at 4°C with the β-catenin antibody (1:150, # 9587,
Cell Signaling). After washing with PBS, the slides were incu-
bated 120 min in the dark with fluorochrome-conjugated anti-
rabbit secondary antibody (1:500, Alexa Fluor 546; Invitro-
gen). After mounting with Antifade (Invitrogen), they were
viewed and documented with a Zeiss Axiosplan microscope.
For the analysis of PDGFRA expression, the cells were
blocked with 50% goat serum, followed by incubation with
the antibody for 1 h at 4°C. The cells were washed with PBS
and fixed with 3% formaldehyde, followed by incubation with
an FITC labeled anti-mouse antibody.

Flow cytometric analysis was performed on a FACS Canto
Flow cytometer (BD Biosciences) and the results were ana-
yzed using DIVA software. Fluorescein isothiocyanate-
conjugated monoclonal antibody to CD90 (clone F15-42-1-5;
IgG1) and isotype controls were obtained from Beckman-
Coulter (Krefeld, Germany); phycoerythrin-conjugated
monoclonal antibody to CD73 (clone AD2; IgG1) from BD
Biosciences (Heidelberg, Germany); phycoerythrin-
conjugated monoclonal antibody to CD105 (clone 166707;
IgG1) from R&D Systems (Wiesbaden, Germany).

RNA isolation, gene expression analysis and Q-RT-PCR

Total RNA was isolated from the cell lines using the RNeasy
Mini Kit (Qiagen). NK RNA was isolated from the cell culture
established from normal adjacent kidney from Wilms3 and
grown in WT medium. Labeling of the RNA in the One-Color
format was performed as described by the manufacturer
(Agilent Technologies). The cRNA was hybridized to 4 ×
44 K ‘Whole Human Genome Oligo Microarrays’ (Agilent)
and ‘Spike-In positive controls’ (Agilent) were added as
internal controls. Microarrays were scanned with an Agilent
Technologies Scanner G2505B and scans were quantified
using Agilent Feature Extraction V10.1.1.1 Software. The raw
data were deposited in GEO, accession Nr. GSE18058.

The gene expression analysis of each cell line was repeated
with the same RNA (technical reproduction) and twice
using RNA from another passage (biological reproduction).
In addition, after establishing two new cell lines, the gene
expression analysis was repeated with new RNA isolated
from all five cell lines in parallel. For the Q-RT-PCR,
cDNA was synthesized using TaqMan Reverse Transcription
Reagents (Applied Biosystems) using total RNA. The
Q-RT-PCR was performed in triplicates using TaqMan gene
expression assay probes from Applied Biosystems for
WT1 (Hs00240913_m1), PAX3 (Hs00240950_m1), IGF2 (Hs0100
5963_m1), AXIN2 (Hs01063168_m1), FOXD1 (Hs002701
17_s1), PDGFRA (Hs00183486_m1), SIM2 (Hs0023192
7_m1), DKK2 (Hs00205294_m1), MET (Hs01565580_m1),
NTSE (CD73) (Hs00159686_m1), THY1 (CD90) (Hs002642
35_s1), ENG (CD105) (Hs03986114_s1) and TaqMan Universal
PCR Master Mix (no AmpErase UNG). The analysis was
performed on an ABI PRISM 7900HT Sequence Detection
System. The expression level of these genes in each sample
was normalized to an endogenous control gene, GAPDH
(4323764T).

Statistical analysis

Four software packages were used for statistical analysis: LIIMMA (R-library, Bioconductor, http://www.R-project.org)
(57) for normalization and search for differentially expressed
genes, pvclust (R-library, http://www.is.titech.ac.jp/~shimo/prog/pvclust/) for cluster analysis, and globaltest (R-library, Bio-
conductor) (58,59) and MEV (Multi-Experiment Viewer, TIGR)
(60) for meta-analysis of KEGG pathways and GO terms, respect-
ively. Before performing statistical tests, all array data were nor-
malized using quantile normalization and filtered for genes
considered to be unexpressed everywhere by removing those
genes not exceeding a mean intensity of 200 in any cell line.

To determine relationships between cell lines, hierarchical
cluster analysis was applied to the filtered expression data
using pvclust. In addition to arranging the cell lines in a
cluster tree, pvclust allows to assess the significance of the
observed branches by producing bootstrap probabilities as
well as approximately unbiased P-values. Differential gene
expression between CLS1, MSC and WT was tested pair
wise for each WT cell line separately, and only genes reported
by LIIMMA as being differentially expressed in all tests for the
contrasts of interest were considered for further analysis by
dividing them into sets of up- and down-regulated genes.
Biological meaning of these gene sets of differentially expressed genes was investigated using MEV’s EASE module (Expression Analysis Systematic Explorer), testing for the presence of over-represented GO terms. To test for association of pathways with the cell lines, a globaltest with KEGG pathways was performed on the filtered arrays, comparing WT with MSC and the pooled WT and MSC data with CLS1. For all statistical tests, a standard P-value of 0.05 was used as significance level. Bootstrap P-values (pvclust) and adjusted resampling P-values (EASE) are based on sample sizes of 10,000 and 1,000, respectively.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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