The intermediate filament protein nestin is expressed in the developing kidney and heart and might be regulated by the Wilms’ tumor suppressor Wt1

Nicole Wagner\textsuperscript{1,2,*}, Kay-Dietrich Wagner\textsuperscript{1,2,*}, Holger Scholz\textsuperscript{3}, Karin M. Kirschner\textsuperscript{3}, and Andreas Schedl\textsuperscript{1,2}

\textsuperscript{1}INSERM U636 and \textsuperscript{2}Centre de Biochimie, Faculté des Sciences, Université de Nice, Nice, France
\textsuperscript{3}Institut für Physiologie, Charité-Universitätsmedizin Berlin, Berlin, Germany

*Authors contributed equally

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Contact information:

Kay-Dietrich Wagner, MD, PhD
INSERM U636
Centre de Biochimie
Faculté des Sciences
Université de Nice
Parc Valrose
06108 Nice
France
Phone: +33 492 07 69 49
Fax : +33 492 07 64 02
E-mail : kwagner@unice.fr
Abstract

Nestin is an intermediate filament protein originally described in neural stem cells and a variety of progenitor cells. More recently, nestin was detected in rat kidney podocytes. We show here that nestin is expressed in a developmentally regulated pattern in the kidney. Nestin was detected by immunohistochemistry in the condensing mesenchyme surrounding the ureter, in developing glomeruli, in podocytes of the adult kidney, and in a podocyte cell line. Nestin shared a strikingly overlap in expression with the Wilms’ tumor suppressor Wt1. Nestin was significantly up-regulated in a cell line with inducible Wt1 expression upon induction of Wt1. Co-transfection experiments in human embryonic kidney cells (HEK293) revealed stimulation of a nestin intron 2 enhancer element up to 6-fold by the Wt1(-KTS) splice variant. Nestin expression was significantly reduced in an inducible mouse model of glomerular disease. This model is based on podocyte-specific over-expression of Pax2 and associated with a loss of Wt1 expression [33]. Furthermore, also in the developing heart, nestin was found in an overlapping pattern with Wt1 in the epicardium and the forming coronary vessels. Strikingly, in the hearts of Wt1 knockout mice, nestin was barely detectable when compared to the hearts of wild-type embryos. Our results show that nestin is expressed at different stages of kidney and cardiac development and suggest that its expression in these organs might be regulated by the Wilms’ tumor suppressor Wt1.

Key words: Wilms’ tumor suppressor WT1, glomerular disease, podocyte, epicardium, coronary vessel, intronic enhancer
Introduction

Nestin is an intermediate filament protein, which was originally identified in neuroepithelial stem cells of rat, mouse, and human [20, 6, 40]. Nestin shows structural similarities to type III and type IV intermediate filament proteins, but due to the relatively low degree of protein sequence homology to the known 5 classes of intermediate filament proteins, nestin was defined as a new class VI intermediate filament [21]. Due to a very short N-terminus, nestin is unable to self-assemble and thus, requires interaction with other intermediate filament proteins like desmin, vimentin, or glial acidic protein (for review see [39]). Besides the unusual structural features, also regulation of nestin seems to be unique. Analyses of the rat promoter in transgenic mice indicate that nestin expression is regulated via enhancer elements in the first and second intron rather than the 5’-upstream region [41]. It has been suggested that enhancer elements in the first intron stimulate nestin expression in angiogenic endothelium [1], whereas enhancer elements in the second intron are required for expression in neuronal progenitor cells [41]. As transcriptional regulators of nestin Pou, Sox [14, 31], and the thyroid transcription factor-1 [22] have been identified until now.

Nestin has been considered as a marker for stem cells and progenitor cells in a variety of organs, i.e. it was detected in neuronal precursor cells, developing skeletal and cardiac muscle cells, mesonephric mesenchyme, pancreatic progenitor cells, and vascular endothelium among others (for review see [39]). It has been thought that nestin expression is transient in these progenitor cells and differentiation in specialized cells types results in down-regulation of nestin and expression of other cell-type specific intermediate filaments, e.g. neurofilaments in neurons [5]. Nestin expression in adult tissues was attributed to stem cell and progenitor cell populations and it was postulated that these progenitor cells are reactivated and proliferate and migrate in response to injury during tissue regeneration (for review see [39]). A recent study showing nestin expression in newly forming blood vessels and a high number of nestin-
positive endothelial cells after myocardial infarction might be in contrast to the concept of nestin expression exclusively in progenitor cell populations [24]. Another group reported nestin expression in rat kidney podocytes [42], which is again in conflict with nestin expression exclusively in proliferating progenitor cells, since podocytes are regarded as terminally differentiated post-mitotic cells.

Thus, we analyzed whether nestin might be expressed in cell types independent of progenitor populations and describe nestin expression in the developing kidney and heart. Furthermore, we investigated molecular mechanisms, which might contribute to nestin expression in these tissues and establish a role for the Wilms’ tumor suppressor Wt1 in the regulation of nestin expression.
Material and Methods

Animals

All animal experiments were performed in accordance to the French law. A heterozygous (Wt1<sup>+/−</sup>) breeding pair [C57BL/6 strain] was obtained from the Jackson Laboratory (Bar Harbor, ME) and genotyped by PCR according to the protocol provided. Inducible double transgenic Z/Pax2;Cre<sup>Tx</sup> mice were generated by crossing Z/Pax2 transgenic animals with tamoxifen-inducible Cre-deleter mice [4, 33]. Double transgenic offspring was identified by PCR using the following primers: YAC-F 5’-ACTTCACCTCGGCCCTTGATAG-3’ (forward primer); YAC-B 5’-GTGGAGAGTCAGACTTGAAAG-3’ (reverse primer); Cre-F 5’-CGCAGAAACCTGAAGATGTTCGCGA-3’ (forward primer); Cre-B 5’-GGATCATCAGCTACACCAGACAGACG-3’ (reverse primer). Tamoxifen induction was performed as described [4, 33].

Immunohistochemistry and immunocytochemistry

Staged embryos (the morning of vaginal plug was considered E0.5) and isolated organs from adult mice were fixed overnight at 4°C in 3% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Three µm paraffin sections were cut and transferred onto gelatin-coated glass slides. Tissue sections were permeabilized with 0.1% Triton X-100 in PBS and blocked by incubation for 1 hour in 10% normal serum (in PBS, 0.1% Triton X-100, 3% BSA), which was obtained from the same species as the secondary antibody. Sections were incubated (16 hours, 4°C) with primary antibodies diluted 1:100 in PBS, 0.1% Triton X-100, 3% BSA: anti-Nestin antibody from mouse (MAB353, Chemicon), anti-Wt1 antibody from rabbit (C-19, sc-846, Santa Cruz Biotechnology). Tissue sections were stained with a peroxidase technique. For the mouse monoclonal antibody, the antigen detection was performed with Vector M.O.M. immunodetection Kit (Vector Laboratories, PK-2200,
Burlingame, CA), for the polyclonal antibody with a biotinylated antibody against rabbit
(Vector Laboratories), followed by incubation with peroxidase coupled Streptavidin (Sigma).
Visualization was achieved with DAB substrate (Vector Laboratories, cat. # SK-4100); in the
case of the double-labeling of Wt1 and Nestin, the latter was visualized using VIP substrate
(Vector Laboratories, cat. # SK-4600). An indirect immunofluorescence double labeling
technique was used to mark Wt1 and nestin expressing cells [32]. In case of cultured
podocytes, these were after washing with PBS fixed with 3% PFA. The podocytes or sections
were incubated (16 hours, 4°C) with primary antibodies each diluted 1:100 in PBS, 0.1%
Triton X-100, 3% BSA: polyclonal anti-Wt1 from rabbit (C-19, sc-846, Santa Cruz
Biotechnology) and monoclonal anti-Nestin from mouse (MAB353, Chemicon). The reaction
products were visualized by incubation (1.5 hours, room temperature) with Cy2– and Cy3-
conjugated secondary antibodies. Slides were viewed under an epifluorescence microscope
(DMLB, Leica) connected to a digital camera (Spot RT Slider, Diagnostic Instruments) with
the Spot software (Universal Imaging Corp.) or alternatively with a Zeiss NhO 2photon
confocal microscope.

**Nestin reporter constructs**

Sequences of the human *nestin* gene (NCBI accession no. AF004335) were amplified from
blood cell-derived DNA using the Expand Long Template PCR System (Roche Diagnostics).
Sequence information of the primers that were used for PCR amplification of intron 2
sequence of the *nestin* gene is given in Table 1. The PCR products were ligated into the *KpnI*
and *HindIII* sites of the pGL2basic reporter plasmid (Promega) and verified by automated
dideoxy sequencing.
Cell culture

Human embryonic kidney (HEK) 293 cells (accession no. ACC 305) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (PAA Laboratories, Pasching, Austria) supplemented with 10% FCS (Biochrom KG, Berlin, Germany), 100 IU/ml penicillin (Invitrogen, Karlsruhe, Germany), and 100 µg/ml streptomycin (Invitrogen) and routinely split at a 1:10 ratio twice per week. Mouse podocytes were a gift of K. Endlich [29]. The podocytes carrying a temperature-sensitive mutant of the immortalizing SV40 large T antigen under control of the interferon-γ-inducible H-2Kb promoter [13, 26] were kept in RPMI 1640 nutrient (Invitrogen), supplemented with 10% FCS (Invitrogen), 100 IU/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and were cultivated at 32 °C in the presence of 10 IU/ml mouse recombinant interferon-γ (Invitrogen, permissive conditions) or at 38 °C without interferon-γ for a minimum of two weeks to induce differentiation [29]. Stable U2OS cells (clone UB27) with the tetracycline repressible Wt1(-KTS) form were grown as described [8].

Cell transfection experiments and reporter gene assays

For transfection experiments, the HEK293 cells were grown to approximately 60% confluence in 24-well tissue culture plates. One hundred ng of the reporter constructs together with 25 ng of a cytomegalovirus (CMV)-driven β-galactosidase plasmid and 125 ng of expression constructs encoding different Wt1 forms were transiently co-transfected with the use of the Fugene6® reagent (1µl per well) according to the supplier’s protocol (Roche Diagnostics, Mannheim, Germany). The pGl2basic vector (Promega, Mannheim, Germany) and the pCB6+ plasmid were transfected for control purpose. Luciferase activities were
measured in the cell lysates after 48h using the firefly luciferase system (Promega) on a Microlite TLX1 luminometer (Dynatech Lab. Inc., Alexandria, Virginia, USA). A Beckman DU604 spectrophotometer (Beckman Coulter, Krefeld, Germany) was used for determination of β-galactosidase activities in each sample [34, 35, 37]. Values are presented as relative light units normalized to β-galactosidase activities for internal control of transfection efficiencies. The results shown are averages of at least 3 transfection experiments each performed in duplicate.

**Real-time RT-PCR**

RT-PCR was performed with 2 µg of total RNA as described elsewhere [37, 38]. Real time PCR was performed on the Light Cycler Instrument® (Roche) using the Platinum® SYBR® Green kit (Invitrogen). The following primers were used for PCR amplification: mouse nestin (NCBI accession no. NM_016701), 5’-CTGCAGGCCACTGAAAAGTT-3’ (forward primer), 5’-GACCCTGCTTCTCTGCTC-3’ (reverse primer); mouse Wt1 (NCBI accession no. NM_144783), 5’-AGAGCAGCCAGCTACCATCGCAA-3’ (forward primer), 5’-GGCTGCCTGTGCAACTGTCA-3’ (reverse primer), mouse GAPDH (NCBI accession no. M32599), 5’-ATTCAACGGGCACAGTCAAGG-3’ (forward primer), 5’-TGGATGCAGGGATGATGTT-3’ (reverse primer); human nestin (NCBI accession no. NM_006617.1), 5’-GCCAGGTTGGAACAGAGGT-3’ (forward primer), 5’-CATCTTGAGGCTGCGACAGCT-3’ (reverse primer); human GAPDH (NCBI accession no. NM_002046.3), 5’-GAAGGTTAAGGTCGGAGTCA-3’ (forward primer), 5’-CAGGAGGCATTGCTGATG-3’ (reverse primer).
Statistics

ANOVA with Bonferroni-test as post-hoc test and Students’ t-test were performed as indicated. A $P$-value of less than 0.05 was considered statistically significant.
Results and Discussion

Nestin is expressed in the developing and adult kidney

Although nestin expression has been described in a variety of developing organs and cell types e.g. neuronal precursor cells [10], developing cardiomyocytes [15], vascular endothelial cells [24], or Sertoli cells [11], until recently, beside one report on nestin expression in rat podocytes [42], little was known about nestin expression during normal embryonic kidney development. We could show here that nestin is expressed at embryonic day 12.5 (E12.5), the earliest time point studied, in the condensing mesenchyme surrounding the ureter (Fig.1A) although we can not exclude that nestin is expressed already earlier. At later stages of development, nestin expression is prominent in the more mature glomeruli in the deep cortex of the kidney, but also detectable in more immature glomerular progenitors i.e. comma- and s-shaped bodies, and in the most outer kidney cortex in mesenchymal progenitor cells surrounding the ureteric bud branches (Fig.1B). In the adult kidney, nestin expression is restricted to the glomeruli (Fig.1C), which is in agreement to a previous report on nestin expression in rat podocytes [42].

Nestin and Wt1 are up-regulated in podocytes upon induced differentiation

To analyze the expression and subcellular localization of nestin in more detail, we made use of a recently established mouse podocyte cell line, which carries a temperature-sensitive mutant of the SV40 large T antigen and can be differentiated into a podocyte cell type by culturing at 38 °C [29]. It has been shown that these differentiated cells express the podocyte proteins nephrin, podocalyxin, podocin, CD2AP, synaptopodin, and Wt1 and form foot-process-like structures [29]. Interestingly, in the great majority of differentiated podocytes, we could also detect nestin expression (Fig.2). Nestin showed the typical filamentous staining in differentiated cells, which are characterized by nuclear Wt1 Expression.
Furthermore, nestin protein was enriched in the developing processes of the cells, which might resemble foot-process-like structures (Fig. 2b). To analyze whether nestin might be differentially expressed in differentiated versus undifferentiated podocytes, we performed quantitative RT-PCR as described [33, 37] and detected a significant increase in nestin expression upon induced differentiation of the podocytes (Fig. 2c). Interestingly, also expression of the major podocyte transcription factor Wt1 became up-regulated in differentiated versus undifferentiated cells (Fig. 2d).

**Nestin and Wt1 share an overlapping expression pattern in the kidney in vivo**

Since the nestin expression pattern during kidney development closely resembles the pattern described for Wt1 [2, 30] and the expression of both, Wt1 and nestin, is overlapping in the podocyte cell line and changed in a comparable manner during induced differentiation of these cells, we investigated by immunohistochemical double-staining whether both proteins show an overlapping expression in the kidney in vivo. At embryonic day 12.5, the earliest time point studied, high Wt1 expression was detected in the condensing mesenchyme surrounding the branches of the ureteric bud, whereas Wt1 signal was weak in the loose mesenchyme as reported [2, 30]. The Wt1-positive cells in the condensing mesenchyme showed co-expression of nestin (Fig. 3a). Interestingly, nestin was enriched mostly in the basal parts of the mesenchymal cells, which face the ureter. At embryonic day 15.5, nestin and Wt1 shared an overlapping expression in glomerular precursors (Fig. 3b), where again, nestin showed localization in the basal parts of Wt1-positive cells. Confocal microscopy revealed nuclear Wt1 expression and nestin protein localization in the cytoplasm and processes of podocytes in glomeruli of the adult mouse kidney (Fig. 3c). This specialized localization of nestin during kidney development and in podocytes in culture might suggest a
role in cell polarity or cell process formation, although future studies are required to support this speculation.

**Wt1 stimulates nestin directly**

The co-localization of nestin and Wt1 throughout kidney development raised the interesting possibility that Wt1, which acts as a major transcriptional regulator (for review see [28], might directly stimulate nestin expression. More than 20 different Wt1 proteins are generated by alternative mRNA splicing [12], the use of alternative translation start sites [3, 27], and RNA editing [30]. The major isoforms are generated by alternative splicing of exon 5, which encodes 17 amino acids, and the use of two alternative splice donor sites at the end of exon 9, which results in the insertion/omission of a tripeptide (lysine-threonine-serine, KTS) in the zinc finger domain of the Wt1 protein [12]. The Wt1(+KTS) forms with the tripeptide insertion have a presumed role in mRNA processing [7, 9, 16, 18], whereas Wt1(-KTS) proteins, which lack the KTS peptide, function mainly as transcription factors (reviewed in [19, 28]).

To analyze a potential direct stimulation of nestin by Wt1, we made use of a stable osteosarcoma-derived cell line (U2OS cells), which expresses the Wt1(-KTS) splice variant under control of a tetracycline repressed promoter [8]. Removal of tetracycline from the culture medium resulted in a nearly 6-fold increase in the expression of nestin as determined by quantitative RT-PCR (Fig. 4A) indicating that Wt1 could stimulate expression of nestin. Since it is known that nestin is mainly regulated by stimulation of intronic enhancer elements [1, 14, 22, 23, 31], we addressed the question whether Wt1 would activate an intronic enhancer of the nestin gene directly. For this purpose, a 1.7 kb sequence containing intron 2 from the human nestin gene (NCBI accession no. AF004335) as well as several deletion mutants were cloned and ligated into the pGL2 basic reporter plasmid (Fig.4B). The
luciferase reporter was transiently transfected into human embryonic kidney cells (HEK293) along with Wt1 expression constructs and a CMV-β-gal plasmid for normalization of transfection efficiencies. Co-transfection of Wt1(-KTS) stimulated the activity of the nestin intron 2 enhancer significantly, whereas Wt1(+KTS) had no effect (Fig.4C). To narrow-down the region containing elements for activation of the nestin enhancer by Wt1, we co-transfected several reporter constructs with different 3’- or 5’-truncations in HEK293 cells. The transfection experiments yielded a ≈300 bp sequence that was required for stimulation of the nestin enhancer by Wt1 (Fig.4B). Constructs, which contained nestin 5’- or 3’-intron 2 sequences of the identified 300 bp regulatory sequence were not stimulated by Wt1 (Fig.4C,e) indicating specificity of the 300 bp element for activation by Wt1. Also a reporter construct containing 2.9 kb of 5’ upstream sequence of the nestin gene was stimulated neither by Wt1(-KTS) nor by Wt1(+KTS) (0.99±0.09 and 0.95±0.1, respectively). Sequence analysis of the identified 300 bp intron sequence that was required for stimulation by Wt1 revealed a high GC content of the sequence, which is typical for Wt1 binding sites [8]. We could detect a potential binding element (5’- GGGAGGACGTGGAGGAGAGGG-3’, which showed high similarity to the Wt1 binding site in the nephrin promoter [38], although we did not confirm binding to Wt1 protein experimentally.

Besides the activation of nestin by Wt1, which we showed here, a variety of transcription factors i.e. Sox1/2/3, Sox11 [31], Brn-1, Brn-2, Brn-4 [14], and TTF-1 [22] have been reported to stimulate nestin. These factors have been shown to be required for nestin expression in the central nervous system, but might be of minor relevance for nestin expression in podocytes because they were not reported to be expressed in the developing kidney.
Nestin is down-regulated in an inducible mouse model of glomerular disease

To gain further insights into the relevance of nestin expression in the kidney, we made use of our recently established inducible mouse model of glomerular disease [33]. The animals express the Pax2 transcription factor ectopically in podocytes, which results in podocyte de-differentiation, glomerular disease with severe proteinuria and high lethality and on the molecular level in reduced expression of Wt1 and its downstream target genes. Immunohistochemical analysis of kidney sections from Pax2 over-expressing mice revealed a dramatic reduction in glomerular nestin expression compared to healthy control animals (Fig.5A). This reduction in nestin expression was confirmed by quantitative RT-PCR, which showed a decrease in nestin expression to approx. 30% of the control levels (Fig.5B). Whether this reduced nestin expression simply reflects the de-differentiated phenotype of the podocytes or whether it is a direct effect of the reduced Wt1 expression remains to be clarified, although our in vitro data on Wt1-dependent stimulation of nestin expression support the latter possibility. Our observation of reduced nestin expression in glomerular disease is in apparent contrast to a recent report, which described up-regulation of nestin in a rat model of puromycin aminonucleoside-induced nephrosis [42]. It is possible that the different models reflect distinct kidney diseases involving various molecular mechanisms. Unfortunately, the authors did not measure Wt1 expression in their model. However, a definite description of the role of nestin in kidney disease can be expected from nestin studies in patients and animal models with defined inducible up-/down-regulation of nestin.

Nestin is expressed in the developing epicardium and coronary vessels

To investigate whether the overlapping expression of nestin and Wt1 is specific for the kidney, we performed double-immunolabeling for both proteins in the developing heart, another known site of Wt1 expression [17, 25, 32, 37]. At embryonic day 12.5, nestin and
Wt1 shared an overlapping expression pattern mainly in the epicardium. At E15.5 both proteins were co-expressed in the developing coronary vessels (Fig. 6A). The nestin expression in the developing coronary vessels of mouse embryos is in agreement with a previous study showing nestin expression in a variety of developing vessels [24]. Interestingly, these authors reported also nestin immunoreactivity in coronary vessels after myocardial infarction, which we had identified as a site of Wt1 expression earlier [32]. To further characterize a regulation of nestin by Wt1, we analyzed nestin expression in Wt1 wild-type and knockout hearts at different time points of embryonic development. E15.5 represented the latest possible time point to obtain viable Wt1 knockout embryos [37]. Immunohistochemistry revealed a loss of nestin expression in the hearts of Wt1 knockout mice compared to wild-type littermates suggesting that nestin might be activated by Wt1 also in vivo (Fig. 6B).

In summary, we detected nestin expression in a developmental specific pattern in the kidney and in adult podocytes. As an additional site of nestin immunoreactivity, we identified the epicardium and developing coronary vessels of the heart. In both organs, nestin shared an overlapping expression with the Wilms’ tumor suppressor Wt1. Up-regulation of nestin in a cell line with inducible Wt1 expression, stimulation of the activity of a nestin enhancer element by co-transfection with Wt1(-KTS), and the loss of nestin in the hearts of Wt1 knockout mice suggests that nestin is regulated by the Wilms’ tumor suppressor Wt1.

Note added in proof:
A similar study by Chen et al. entitled “Differential expression of the intermediate filament protein nestin during renal development and its localization in adult podocytes” is scheduled
Acknowledgements

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References


Figure Legends

Figure 1: Immunohistochemical analysis of nestin expression in the developing kidney. A: At embryonic day 12.5 (E12.5), the earliest time point studied, nestin expression is detectable in the condensing mesenchyme surrounding the ureter. B: At E15.5, nestin is highly expressed in the developing glomeruli and also detectable in the cortical mesenchyme surrounding ureteric branches (arrow in B,a). C: In the adult kidney, nestin is specifically expressed in glomeruli. Immunohistochemical analysis was performed as described recently (Wagner et al., 2005). Sections were counterstained with hematoxylin (Sigma). Scale bars indicate 100 µm.

Figure 2: Immunocytochemical detection and quantitative RT-PCR analysis for nestin and Wt1 in a podocyte cell line with inducible differentiation. After growth for two weeks under differentiating conditions at 38 °C, the majority of podocytes express nestin (Cy3, red fluorescence). Note the filamentous staining for nestin with strong expression in foot-process-like structures. Nestin (red) and the Wilms’ tumor suppressor Wt1 (Cy2, green speckles in the nucleus) are co-expressed in individual cells (b). Counterstaining of the nuclei was performed with Dapi. Scale bars indicate 10 µm. Quantitative RT-PCR reveals an increased expression of nestin (c) and Wt1 (d) in differentiated podocytes (white bars) when compared to undifferentiated cells grown at 32 °C in the presence of interferon-γ (black bars). Nestin and Wt1 mRNA was normalized for GAPDH transcripts. Data are presented as means±S.E.M. (n=6 each, **P<0.01, ***P<0.001).

Figure 3: Immunohistochemical co-localization of nestin (purple, VIP substrate) and Wt1 (brown, DAB substrate) in the developing embryonic kidney and in glomeruli of the adult kidney (nestin – Cy2, green; Wt1 – Cy3, red, confocal image). At E12.5, Wt1 is expressed in
the condensing mesenchyme surrounding the ureteric bud and nestin expression is strongly enriched in the basal parts of Wt1-positive cells (a). At later stages (E15.5), nestin and Wt1 expression becomes restricted to comma-shaped bodies (arrow) and developing glomeruli (arrowhead) in the developing kidney (b). In the adult kidney, Wt1 is detectable in the nuclei and nestin in the cytoplasm and processes of podocytes (c). Scale bars indicate 30 µm.

Figure 4: A: Quantitative RT-PCR demonstrating nestin expression in a stable osteosarcoma cell line (clone UB27) with inducible expression of Wt1 [8]. The cells were grown in the presence of tetracycline (+tet) to suppress Wt1. Note that removal of tetracycline from the culture medium (-tet) enhanced the expression of nestin significantly. Nestin mRNA was normalized for GAPDH transcripts. Data are presented as means±S.E.M. (n=5 independent induction experiments, *P<0.05). B: Schematic illustration of the nestin intron 2 enhancer element constructs, which were used for co-transfection experiments with Wt1 expression constructs. Sequences of the human nestin gene (NCBI accession no. AF004335 were amplified by PCR, cloned into the pGL2basic reporter plasmid (Promega), and verified by automated dideoxy sequencing. C: Normalized luciferase activities for the different nestin intron 2 enhancer element constructs co-transfected with Wt1 expression constructs in HEK293 cells. Normalized luciferase activities were increased by co-transfection of Wt1(-KTS). The Wt1(+KTS) protein, which has been implicated in mRNA processing rather than transcriptional regulation [7, 9, 18], had no statistically significant effect. Note that an intron 2 construct, which contained sequences 3’ of the identified enhancer element was not stimulated by co-transfection with Wt1 expression constructs (e). Shown are means±S.E.M. from 5 transfection experiments each (*P<0.05, ***P<0.001, ANOVA with Bonferroni-test as post-hoc test).
**Figure 5:** Reduced nestin expression in an inducible mouse model of glomerular disease [33].

A: Immunohistochemical detection of nestin (brown, DAB substrate) in the glomeruli of vehicle-injected control animals (a) and severe reduction of nestin expression in mice with glomerular disease (b, arrows point to the glomeruli). Sections were counterstained with hematoxylin (Sigma). Scale bars indicate 100 µm. B: Quantitative RT-PCR confirms a significant reduction in nestin expression in animals with glomerular disease (white bar) compared to vehicle-injected controls (black bar). Nestin expression is normalized to GAPDH transcript levels. Data are presented as means±S.E.M. (n=6 each, ***P<0.001).

Note that this inducible mouse model of glomerular disease is associated with a severe reduction of Wt1 expression [33].

**Figure 6:** Cardiac expression of nestin during mouse embryonic development. A: Immunohistochemical co-localization of nestin (purple, VIP substrate) and Wt1 (brown, DAB substrate) in the epicardium at E12.5 and in the epicardium and developing coronary vessels (arrows) at E15.5. B: Immunodetection of nestin (brown, DAB substrate) in the hearts of wild-type (Wt1+/+) and Wt1-deficient mouse embryos (Wt1−/−) at E12.5 and E15.5. In contrast to nestin expression in the epicardium and developing coronary vessels (arrows) in wild-type embryos, nestin was barely detectable in the Wt1−/− mutant hearts. Sections were counterstained with hematoxylin (Sigma). Scale bars indicate 30 µm.
Table 1: DNA oligonucleotides used for PCR amplification of intron 2 of the human *nestin* gene

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Fig. 1
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**Fig. 4**

A. Nestin/GAPDH mRNA levels as measured by qRT-PCR. The relative levels of Nestin mRNA are normalized to GAPDH mRNA expression.

B. Schematic representation of the luciferase reporter constructs used in the experiments.

C. Bar graphs showing relative luciferase activity. The graphs compare the activity of various constructs in the presence or absence of specific proteins.
Fig. 5
Fig. 6