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Expression of Pax2 in Human Renal Tumor-Derived Endothelial Cells Sustains Apoptosis Resistance and Angiogenesis

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The transcription factor Pax2 is known to play a key role during renal development and to act as an oncogene favoring renal tumor growth. We recently showed that endothelial cells derived from human renal carcinomas display abnormal characteristics of survival and angiogenic properties. In the present study we found that renal tumor-derived endothelial cells, but not normal endothelial cells, expressed Pax2 protein and mRNA. To down-regulate Pax2 expression, we transfected tumor-derived endothelial cells with an anti-sense PAX2 vector whereas we transfected normal human microvascular endothelial cells with a sense PAX2 vector to induce Pax2 expression. The inhibition of Pax2 expression in tumor-derived endothelial cells induced an increase in tumor suppressor PTEN expression and a decrease in Akt phosphorylation. In addition, decreased apoptosis resistance, adhesion, invasion, and *in vitro* and *in vivo* angiogenesis were observed. Conversely, Pax2 induction in normal endothelial cells conferred to these cells a proinvasive, proangiogenic phenotype similar to that of tumor-derived endothelial cells. These results indicate that Pax2 is involved in renal tumor angiogenesis and its expression may antagonize that of the PTEN tumor suppressor gene, affecting the Akt-survival pathway and promoting angiogenesis. (Am J Pathol 2006, 168:706–713; DOI: 10.2353/ajpath.2006.050776)

The paired-box 2 (PAX2) gene belongs to a family of nine homeobox genes^{1,2} that bind to DNA to initiate transcription of specific genes. In the embryo, Pax2 expression is restricted to the developing kidney, central nervous sys-

tem, eyes, and ears.^{3,4} In developing kidney, Pax2 is detectable in the caudal mesonephric duct, ureteric bud, and in the mesenchymal condensates induced in the ureteric bud.² Pax2 is down-regulated when the mesenchymal cells differentiate into tubular epithelial cells, and it is no longer expressed in the mature kidney.^{5–7} The relevance of Pax2 in the development of kidney is suggested by the severe kidney abnormalities, similar to congenital nephritic syndrome, observed in transgenic mice in which Pax2 expression was deregulated.⁸ Failure to repress Pax2 was found in hyperproliferative cystic tissue in a mouse model of polycystic kidney disease.⁹ In humans, Pax2 overexpression was associated with fetal and infantile multicystic dysplastic kidneys and autosomal dominant polycystic kidney disease.¹⁰ Moreover, Pax2 is up-regulated in adults during tubular epithelial cell regeneration after renal injury^{11,12} when the tubular epithelial cells dedifferentiate to an embryonic phenotype.¹³ Functional studies suggest that Pax2 is involved in cell survival and proliferation.¹⁴ In renal collecting duct cells, Pax2 was found to suppress caspase-2-induced apoptosis and to stimulate cell proliferation.¹⁵ Moreover, a pro-oncogenic potential has been ascribed to the PAX gene family^{16,17}; in fact, several chromosomal translocations involving members of the PAX gene family have been described in various human cancers, suggesting that altered regulation of PAX gene products can promote cellular transformation.^{18–20} In particular the inappropriate expression of Pax2 has been identified in Wilms tumor,¹⁹ renal cell carcinoma,²¹ breast cancer,²² and prostate cancer.²³ We recently detected Pax2 expression in an angiogenic tumor, Kaposi's sarcoma. In addition we

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demonstrated that the expression of Pax2 protein by Kaposi's sarcoma cells correlated with an enhanced resistance against apoptotic signals and with a proinvasive phenotype.²⁴

It has been recently shown that endothelial cells derived from different tumors are genetically unstable.²⁵ In addition, we showed that endothelial cells derived from human renal carcinomas persistently display abnormal characteristics in terms of survival and angiogenic properties.²⁶ In the present study we evaluated whether renal tumor-derived endothelial cells (TECs) expressed the *PAX2* gene product and whether Pax2 was involved in their proinvasive and proangiogenic phenotype. For this purpose, TECs, which were found to express Pax2, were transfected with an anti-sense *PAX2* vector to inhibit the synthesis of Pax2 protein. In addition, normal human microvascular endothelial cells (HMECs), which were found not to express Pax2, were transfected with a sense *PAX2* vector to induce the synthesis of Pax2. Using these cell lines, we studied the effect of Pax2 expression on Akt-dependent pathway, cell survival, proliferation, adhesion, invasion, and *in vitro* and *in vivo* angiogenic properties.

Materials and Methods

Cell Lines

TEC lines used in this study were previously described and characterized.^{26,27} Briefly, renal TECs were isolated from six different renal clear-cell carcinomas using anti-CD105 antibody coupled to magnetic beads, by magnetic cell sorting (MACS System; Miltenyi Biotec, Auburn, CA).²⁶ Moreover, breast TECs were isolated from two ductal and a tubulo-lobular infiltrating breast carcinomas.²⁷ The expression of Pax2 in the tumors from which TECs were isolated was evaluated by immunohistochemistry using the anti-Pax2 antibody from Covance (Princeton, NJ) as previously described.²⁴ The cell lines were characterized as endothelial cells by morphology, positive staining for von Willebrand factor antigen, CD105, CD146, and vascular endothelial-cadherin; and negative staining for cytokeratin and desmin.²⁶ TEC lines were maintained in culture in endothelial basal medium (EBM) complete medium supplemented with epidermal growth factor (10 ng/ml), hydrocortisone (1 mg/ml), bovine brain extract (all from Cambrex Bioscience, Walkersville, MD), and 10% fetal calf serum (FCS).

HMECs were obtained from derma or from normal renal tissue using anti-CD31 antibody coupled to magnetic beads, by magnetic cell sorting (MACS System, Miltenyi Biotec). HMECs from derma were immortalized by infection of primary cultures with a replication-defective adeno-5/SV40 virus as previously described.²⁸ The previously characterized renal carcinoma cell line K1, obtained from a renal clear-cell carcinoma, was maintained in Dulbecco's modified Eagle's medium with 10% FCS.²⁹

Cloning of PAX2 Gene

Cloning of the *PAX2* gene was performed as previously described.²⁴ Briefly, 2 μ g of RNA were reverse-transcribed using oligo(dT) primers and 15 U of reverse transcriptase enzyme (Eppendorf, Hamburg, Germany). Five μ l of cDNA were amplified with forward and reverse primers (forward, 5'-ATGGATATGCACTGCAAAGCAGA-3'; reverse, 5'-CTAGTGGCGGTCATAGGCAG-3') covering the initial and terminal part of the coding sequence, respectively (NCBI GenBank no. GI409138), with *Taq*DNA polymerase (Invitrogen, San Diego, CA). Amplified DNA was ligated with the T/A cloning system into pTARGET mammalian expression vector (Promega, Madison, WI) for expression under the control of the cytomegalovirus promoter. We identified a clone with *PAX2* in correct orientation (S) and a clone with *PAX2* in inverse orientation (AS). DNA sequences were compared with the NCBI database using the BLAST program.

Transfection

HMECs or TECs were seeded in a T25 flask to reach confluence and transfected with 8 μ g of DNA and 20 μ l of LipofectAMINE 2000 reagent (Invitrogen) in Dulbecco's modified Eagle's medium plus 10% FCS without antibiotics, according to the protocol suggested by the manufacturer. As a control, cells were transfected with empty plasmid. Transfected cells were stably selected by culturing in the presence of 1 mg/ml geneticin (Sigma Chemical Co., St. Louis, MO). Successful transfection was evaluated by immunofluorescence and Western blotting. TECs and HMECs were stably transfected up to passage 10.

Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer's instructions; 2 μ g of RNA were reverse-transcribed using oligo(dT) primers and 15 U of reverse transcriptase enzyme (Eppendorf). Two μ l of cDNA were amplified with forward (5'-ATGGATATGCACTGCAAAGCAGA-3') and reverse (5'-CTAGTGGCGGTCATAGGCAG-3') primers and *Taq*DNA polymerase (Invitrogen). Reactions were performed for 30 cycles at a melting temperature of 52°C and analyzed with an ethidium bromide 1.5% agarose gel.

Western Blot Analysis

Western Blot analysis was performed for detection of Akt, P-Akt, PTEN, and Pax2, as previously described.^{24,30} Cells were lysed at 4°C for 1 hour in a lysis buffer (50 mmol/L Tris-HCl, pH 8.3, 1% Triton X-100, 10 μ mol/L phenylmethyl sulfonyl fluoride, 10 μ mol/L leupeptin, and 100 U/ml aprotinin) and centrifuged at 15,000 $\times g$. The protein contents of the supernatants were measured by the Bradford method. Aliquots containing 10 μ g of protein of the cell lysates were subjected to 10% sodium

dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and electroblotted onto nitrocellulose membrane filters. The blots were blocked with 5% nonfat milk in 20 mmol/L Tris-HCl, pH 7.5, 500 mmol/L NaCl plus 0.1% Tween (TBS-T). The membranes were subsequently immunoblotted overnight at 4°C with the relevant primary antibodies at the appropriate concentration. After extensive washings with TBS-T, the blots were incubated for 1 hour at room temperature with peroxidase-conjugated isotype-specific secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), washed with TBS-T, developed with ECL detection reagents (Amersham, Buckinghamshire, UK) for 1 minute, and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY). The following antibodies were used: anti-Pax2 polyclonal antibody (Covance), anti-human Akt1 and phospho-Akt1 (P-Akt) from Upstate Biotechnology (Lake Placid, NY), anti-PTEN antibody from Cell Signaling Technology (Beverly, MA), and anti- β -actin polyclonal antibody from Santa Cruz Biotechnology.

Apoptosis Assay

Apoptosis was evaluated using the terminal dUTP nick-end labeling assay (ApoTag; Oncor, Gaithersburg, MD). After serum withdrawal and/or treatment with 1 to 100 ng/ml of vincristine for 24 hours, cells were suspended in phosphate-buffered saline (PBS) and fixed in 1% paraformaldehyde in PBS, pH 7.4, for 15 minutes at 4°C followed by precooled ethanol/acetic acid (2:1) for 5 minutes at -20°C. Cells were treated with terminal deoxynucleotidyl transferase enzyme and incubated in a humidified chamber for 1 hour at 37°C and then treated with warmed fluorescein isothiocyanate-conjugated anti-digoxigenin for 30 minutes at room temperature. After washing, samples were mounted in medium containing 1 μ g/ml of propidium iodide and the cells were analyzed by immunofluorescence.

Cell Proliferation Assay

Cells were seeded into 96-well plates at a density of 8000 cells/well in EBM plus 10% FCS, and the proliferation was evaluated after 72 hours. DNA synthesis was detected as incorporation of 5-bromo-2'-deoxyuridine (BrdU) into the cellular DNA using an enzyme-linked immunosorbent assay kit (Chemicon, Temecula, CA) according to the manufacturer's instructions. Briefly, after washing, cells were incubated with 10 μ mol/L BrdU for 6 to 12 hours at 37°C, 5% CO₂, in a humidified atmosphere. Cells were then fixed with 0.5 mol/L ethanol/HCl and incubated with nuclease to digest DNA. BrdU incorporated into the DNA was detected using an anti-BrdU peroxidase-conjugated monoclonal antibody and visualized with a soluble chromogenic substrate. Optical density was measured with an enzyme-linked immunosorbent assay reader at 405 nm.

Adhesion Assay

Cells were cultured to confluence in gelatin-coated 24-well tissue culture plates. Before the assay, the monolayers were starved in EBM plus 0.1% bovine serum albumin. Renal carcinoma cells (K1) were detached with nonenzymatic solution (Sigma), labeled with the intravital red fluorescent cell linker PKH26 (Sigma) and added to the endothelial monolayers at the density of 5×10^5 cells. The adhesion assay was allowed to proceed for 30 minutes at 37°C, 5% CO₂, in a humidified atmosphere in static conditions. The plates were then washed three times with PBS to remove the unattached cells. Each experiment was done in triplicate. Labeled cells attached to the endothelial monolayer were counted in five fields (magnification, $\times 100$) by fluorescence microscopy and expressed as number cells/microscopic field.

Matrigel Invasion Assay

The invasion ability was evaluated using Transwell chambers (Costar, Cambridge, MA), in which the upper and the lower chambers were separated by 8- μ m pore-size polyvinylpyrrolidone-free polycarbonate filters. Briefly, before the invasion assay, filters were coated with 100 μ g/well of Matrigel (Becton Dickinson, Bedford, MA) diluted in culture medium. The lower compartment was loaded with medium plus 10% FCS. HMEC-c, HMEC-S, TEC-c, and TEC-AS cells (5×10^4 cells/well) were seeded onto the upper compartment and were incubated for 48 hours at 37°C, 5% CO₂, in a humidified atmosphere. Cells migrated to the underside of the filters were fixed with methanol, stained with Giemsa solution (Diff-Quik kit; Harleco, Gibbstown, NJ), counted in five fields (magnification, $\times 100$) in each well by light microscopy, and expressed as number of cells/microscopic field. Each experiment was done in triplicate.

In Vitro Angiogenesis Assay

The assay was performed as previously described.²⁶ Briefly, 24-well plates were coated with growth factor-reduced Matrigel (Becton Dickinson) at 4°C and incubated for 30 minutes at 37°C, 5% CO₂, in a humidified atmosphere. Cells (HMEC-c, HMEC-S, TEC-c, and TEC-AS) were seeded on the Matrigel-coated wells in RPMI plus 5% FCS at the density of 5×10^4 cells/well. After a 6-hour or 24-hour incubation, cells were observed with a Nikon inverted microscope (Nikon Corporation, Tokyo, Japan), and the experimental results were recorded. The extent of capillary-like structures was measured with the MicroImage analysis system (Cast Imaging srl, Venice, Italy) and expressed in arbitrary units.

In Vivo Angiogenesis Assay

For the *in vivo* studies, an *in vivo* Matrigel angiogenesis assay was performed, in which cells were subcutaneously injected into SCID mice (Charles River, Jackson Laboratories, Bar Harbor, ME; $n = 5$ for each experimen-

tal condition). Briefly, cells were harvested using trypsin solution (Sigma), washed with PBS, resuspended in 250 μ l of Dulbecco's modified Eagle's medium, and added to 250 μ l of growth factor-reduced Matrigel at 4°C. Cells were injected subcutaneously into the mid-abdominal region of SCID mice via a 26-gauge needle and a 1-ml syringe. At day 6, mice were killed, and the plugs were recovered and processed for histology. Typically, gels were cut out by retaining peritoneal lining for support, fixed in 10% buffered formalin, and embedded in paraffin. Sections (3 μ m) were cut, stained with hematoxylin and eosin or with a Masson trichrome reaction, and examined under a light microscopy system. Morphometric analysis was performed to count vessels that were expressed as percent area per field. Vessel structures were counted only if showing a patented lumen with red globuli and/or leukocytes. The vessels area was planimetrically assessed using the MicrolImage analysis system (Casti Imaging srl). The human nature of endothelial cells was assessed by immunofluorescence with an anti-HLA class I polyclonal antibody (Santa Cruz Biotechnology).

Results

Pax2 Expression by TECs

The expression of Pax2 was evaluated in different lines of TECs derived from six renal and three breast carcinomas. All TEC lines derived from renal carcinomas expressed Pax2 as demonstrated by both Western blot (Figure 1A) and reverse transcriptase-polymerase chain reaction (data not shown). In contrast, TECs derived from breast carcinomas did not express Pax2 (Figure 1A). This correlated with Pax2 expression in the renal carcinomas but not in the breast carcinomas used for the extraction of TECs, as detected by immunohistochemistry (Figure 1, B and C). Normal HMECs obtained from derma or kidney did not express Pax2 (Figure 1A). To evaluate the relevance of Pax2 expression by renal TECs, a renal TEC line was stably transfected with a *PAX2* anti-sense vector (TEC-AS), to obtain the inhibition of Pax2 expression, or with a control vector (TEC-c). Moreover, HMECs were stably transfected with a *PAX2* sense vector (HMEC-S), to obtain the expression of Pax2, or with a control vector (HMEC-c). TEC-AS no longer expressed Pax2 protein or mRNA (Figure 1, E, J, and K) in respect to control TEC-c transfected with the empty vector (Figure 1, D, J, and K). HMEC-S transfected with *PAX2* sense vector expressed both Pax2 protein and Pax2 mRNA (Figure 1, G, J, and K) at variance with HMEC-c transfected with empty vector (Figure 1, F, J, and K).

Effect of Pax2 on PTEN Expression and Akt Phosphorylation

We previously demonstrated in TECs a decreased expression of PTEN, a tumor suppressor gene that regulates Akt phosphorylation.²⁶ To test whether Pax2 expression influences PTEN and Akt-dependent pathway, we

Figure 1. Pax2 expression in TECs and HMECs. **A:** Western blot analysis of Pax2 protein expression by TEC lines derived from six different renal carcinomas (renal TEC) and from three different breast carcinomas (breast TEC) and by normal HMECs derived from kidney (HMEC). **B** and **C:** Representative micrographs of immunohistochemical staining for Pax2 in a renal carcinoma (**B**) and in a breast carcinoma (**C**). In the renal carcinoma several cells showed nuclear peripheral staining for Pax2, whereas no positive cells were detected in the breast carcinoma. The immunoperoxidase staining was performed in the absence of a nuclear counterstaining because Pax2 is a nuclear antigen. **D–G:** Representative micrographs showing Pax2 expression by immunofluorescence staining of TEC-c (**D**), TEC-AS (**E**), HMEC-c (**F**), and HMEC-S (**G**). Transfection of TECs with *PAX2* anti-sense vector (TEC-AS), but not with vector alone (TEC-c), markedly reduced the staining for Pax2. Conversely, the minimal staining for Pax2 of HMECs transfected with vector alone (HMEC-c) was increased after transfection with *PAX2* sense vector (HMEC-S). **J:** Representative Western blot analysis of Pax2 protein expression by TEC-c, TEC-AS, HMEC-c, and HMEC-S, showing a band corresponding to 46 kD. **K:** Representative expression of Pax2 mRNA by reverse transcriptase-polymerase chain reaction analysis on TEC-c, TEC-AS, HMEC-c, and HMEC-S. *PAX2* cDNA was used as control. Results obtained with the TEC-c cell line were identical to those obtained with all of the wild-type TEC lines studied. All micrographs are representative of three individual experiments. Original magnifications: $\times 250$ (**B**, **C**), $\times 400$ (**D–G**).

compared the PTEN levels and Akt phosphorylation, after serum starvation, in TECs and HMECs expressing or not Pax2. The results indicate that the expression of Pax2, as it occurred in TEC-c, correlated with a decreased expression of PTEN and an increased phosphorylation of Akt (Figure 2). When the expression of Pax2 was down-regulated by anti-sense transfection, the levels of PTEN were increased and the ratio P-Akt/Akt markedly reduced. Conversely, transfection of HMECs with *PAX2* sense markedly depressed PTEN expression and enhanced P-Akt/Akt ratio in respect to HMEC-c (Figure 2). These results suggest a regulatory role of Pax2 on a tumor suppressor gene such as PTEN and on the Akt-dependent survival pathway.

Figure 2. Effect of Pax2 expression on PTEN levels and Akt phosphorylation. **A:** Representative Western blot analysis of PTEN expression from cell lysates of TEC-c, TEC-AS, HMEC-c, and HMEC-S. Equal protein loading was verified by β -actin detection. The densitometric analysis of PTEN was performed on three individual experiments and data are expressed as mean $\pm$ SD. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c. **B:** Representative Western blot analysis of P-Akt and Akt expression from cell lysates of TEC-c, TEC-AS, HMEC-c, and HMEC-S. The densitometric analysis of P-Akt/Akt ratio was performed on three individual experiments and data are expressed as mean $\pm$ SD. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c.

Pax2 Expression in TECs and HMEC-S Protects against Apoptosis and Stimulates Proliferation.

One of the most peculiar characteristics of normal endothelial cells is the occurrence of apoptosis in the absence of serum. To verify the effect of Pax2 expression on endothelial cell apoptosis, TEC-c, TEC-AS, HMEC-c, and HMEC-S were incubated for 24 hours in the absence of serum or with increasing doses of vincristine (1 to 100 ng/ml). As shown in Figure 3A, cells that expressed high levels of Pax2 protein (TEC-c and HMEC-S) were protected against apoptosis induced by serum deprivation or by vincristine. In contrast, TEC-AS and HMEC-c exhibited a higher sensitivity to apoptosis. In addition, the transfection with *PAX2* anti-sense vector reduced the growth of TEC-AS in respect to TEC-c, whereas the transfection with *PAX2* sense vector enhanced the growth of HMEC-S in respect to HMEC-c (Figure 3B). These results suggest that Pax2 promotes the resistance of tumor endothelial cells to apoptosis and stimulates cell proliferation.

Effect of Pax2 on Tumor Cell Adhesion to TECs and on Matrigel Invasion

TECs exhibited enhanced adhesive properties to carcinoma cells in respect to normal endothelial cells and increased ability to invade Matrigel (Figure 4). The transfection with *PAX2* anti-sense vector markedly reduced the adhesion of carcinoma cell line K1 on TEC-AS in respect to TEC-c (Figure 4A). Conversely, the expression of Pax2 induced a significant increase of K1 adhesion to HMEC-S in comparison with HMEC-c. Similar results were obtained in the invasion assay, in which loss of Pax2 protein expression in TEC-AS significantly inhibited the ability of TECs to invade Matrigel. In contrast, the ac-

Figure 3. Pax2 expression is associated with apoptosis resistance and enhanced cell proliferation. **A:** Apoptosis was evaluated by the terminal dUTP nick-end labeling assay as a percentage of apoptotic cells after 24 hours of incubation. Control cells were incubated in the presence of 20% FCS. Apoptosis was induced by serum withdrawal (0% FCS) or by treatment with increasing doses of vincristine. The graphic shows that TEC-c and HMECs transfected with the *PAX2* sense vector (HMEC-S) were resistant to apoptosis in respect to TECs transfected with the *PAX2* anti-sense vector (TEC-AS) and HMEC-c. Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c. **B:** Cell proliferation was evaluated by BrdU incorporation. Cells were seeded in 96-well plates at the density of 8×10^3 cells/well, and the proliferation was evaluated after 72 hours of incubation. The graphic shows a significant reduction of proliferation in TEC-AS in respect to TEC-c and, conversely, a significant increase of proliferation in HMEC-S in respect to HMEC-c. Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c.

quirement of Pax2 expression by HMEC-S induced the ability to invade Matrigel in normal endothelial cells (Figure 4B).

In Vitro and in Vivo Angiogenesis

We compared TEC-AS and HMEC-S with TEC-c and HMEC-c for the ability to organize when plated on Matrigel in capillary-like structures. Within a few hours TECs formed an extensive network of ring-like structures (Figure 5, A and B) whereas TEC-AS markedly lost this ability (Figure 5, A and C). Moreover, HMEC-S significantly increased the ability of normal endothelial cells to organize in capillary-like structures (Figure 5, A, D, and E).

To evaluate the involvement of Pax2 in *in vivo* angiogenesis, TEC-c and TEC-AS were injected subcutaneously within diluted Matrigel in SCID mice. After 6 days,

Figure 4. Pax2 expression is associated with enhanced adhesion and invasion properties of endothelial cells. **A:** The adhesion of the renal carcinoma cell line K1 to the endothelium was evaluated. Labeled K1 cells were added to a monolayer of TEC-c, TEC-AS, HMEC-c, and HMEC-S, and incubated for 30 minutes at 37°C, 5% CO₂, in a humidified atmosphere in static conditions. After washing, adherent cells were counted in each well. Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c. **B:** Invasion of TEC-c, TEC-AS, HMEC-c, and HMEC-S toward Matrigel-coated filters was evaluated. Cells were seeded onto Matrigel precoated upper well (100 μ g/well) and incubated for 48 hours at 37°C, 5% CO₂, in a humidified atmosphere. The lower well was loaded with medium plus 10% FCS. Cells that migrated to the underside of the filters were fixed with methanol, stained with Giemsa solution, and counted in five microscopic fields in each well. Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c. Original magnifications, $\times 100$.

plugs were recovered and processed for histological analysis. We previously demonstrated that TECs, at variance with normal endothelial cells, spontaneously organized in human microvessels containing blood cells.²⁶ The loss of Pax2 in TEC-AS was associated with a significant reduction in this ability (Figure 6). These data suggest that Pax2 expression contributes to the angiogenic properties of TECs.

Discussion

In the present study, we demonstrated that human endothelial cells derived from renal carcinomas expressed Pax2 and that its expression contributed to the apoptosis resistance and the proangiogenic properties character-

Figure 5. *In vitro* angiogenesis. **A:** The ability of endothelial cells to form capillary-like structure within Matrigel was evaluated. TEC-c, TEC-AS, HMEC-c, and HMEC-S (5×10^4 cells/well) were plated on growth factor-reduced Matrigel in RPMI plus 5% FCS, and the extent of capillary-like structure formation was observed after 6 and 24 hours. **B** and **C:** Representative micrographs showing the network of ring-like structures formed by TEC-c (**B**) and by TEC-AS (**C**) after 6 hours of incubation. **D** and **E:** Representative micrographs showing the network of ring-like structures formed by HMEC-c (**D**) and by HMEC-S (**E**) after 6 hours. Data are expressed as mean $\pm$ SD of three independent experiments performed in triplicate. Analysis of variance with Newmann-Keuls multicomparison test was performed: * $P < 0.005$ TEC-AS versus TEC-c; § $P < 0.005$ HMEC-S versus HMEC-c.

istic of these cells. Renal cell carcinoma is characterized by resistance to radiation and chemotherapy³¹ and by an abundant angiogenesis with development of abnormal blood vessels.^{32–34} We recently demonstrated that tumor endothelial cells derived from renal carcinomas differ from normal microvascular endothelial cells because they do not undergo senescence and maintain a constant activated phenotype with the ability to grow and organize in the absence of serum in capillary-like structures.²⁶ *In vivo*, when implanted subcutaneously in SCID mice, these cells are able to form vascular structures connected with the mouse vasculature.²⁶ These observations are consistent with the finding that TECs derived from different tumors are phenotypically and genetically different from normal endothelium^{25,35} and may be resistant to chemotherapeutic drugs.³⁶

In the present study, we found that renal endothelial cells derived from renal carcinomas expressed Pax2, a transcription factor that has been involved in tumor growth.^{16,20} Indeed, it has been demonstrated that inhibition of Pax2 expression in renal carcinoma cells by anti-sense nucleotides inhibits tumor cell growth.²¹ Our observation that the endothelial compartment in Pax2-positive renal carcinomas also expressed Pax2 may sug-

Figure 6. Effect of Pax2 down-regulation on *in vivo* angiogenesis by TECs. **A:** TEC-c and TEC-AS were implanted subcutaneously in SCID mice within growth factor-reduced Matrigel, and the formation of the organized vascular structures was quantitatively evaluated after 6 days. **B** and **C:** Representative micrographs showing the formation of canalized vessels in mice implanted with TEC-c (**B**) and in mice implanted with TEC-AS (**C**). Only the vascular structures containing red blood cells were counted as vessels. The human nature of implanted endothelial cells was assessed by immunofluorescence staining for human HLA class I (not shown). Data are expressed as mean $\pm$ SD of five individual experiments. Mann-Whitney nonparametric test was performed (* $P < 0.005$).

gest that tumor endothelial cells in renal carcinoma derive from dedifferentiated tumor cells, as suggested for leukemia,³⁷ and therefore express Pax2. Alternatively, TECs may represent a dedifferentiated endothelial population displaying an embryonic renal phenotype that results in Pax2 expression. Indeed we previously showed that TECs express the embryonic marker HLA-G, a non-classical HLA antigen expressed on fetal and maternal placental endothelial cells.²⁶ Our finding that TECs derived from the three Pax2-negative breast carcinomas did not express Pax2 suggests that the endothelial expression of this transcription factor is limited to tumors that overexpress Pax2. However, previous studies have suggested that *PAX2* gene function is required only at certain stages of tumor cell development because it may be transiently expressed by several nonrenal tumors.²⁰

Herein we investigated the functional implication of Pax2 expression by endothelial cells. We found that down-regulation of Pax2 expression by TECs transfected with *PAX2* anti-sense resulted in an enhanced expression of the tumor suppressor gene PTEN, which is down-regulated in TECs in respect to normal endothelial cells. These results suggest that the expression of Pax2 may control the transcription of PTEN. In addition, it has been previously demonstrated that Pax2 possesses an oncogenic potential due to down-regulation of other tumor suppressor genes, such as p53.³⁸ PTEN is a phosphatase that regulates the PI3K-dependent activation of Akt.³⁹ Indeed, Akt phosphorylation was significantly enhanced in TECs and correlated with an increased resistance to apoptotic signals.²⁶ In the present study we demonstrated that down-regulation of Pax2 expression correlated with a decreased Akt phosphorylation and an enhanced sensitivity to apoptosis induced by serum deprivation or by chemotherapeutic drugs such as vincristine. These results are consistent with a previously reported anti-apoptotic activity of *PAX2* gene expression in tubular epithelial cells.¹⁵ Several studies have indicated that TECs are resistant to chemotherapeutic and anti-angiogenic drugs.^{36,40} Because of the involvement of PI3K/Akt survival pathway in such resistance, the combined use of anti-angiogenic and anti-PI3K drugs has been suggested.^{41,42} Our results may provide a rational for the described effects of activin A, which is known to

down-regulate Pax2 expression¹¹ and to repress tumor growth through inhibition of angiogenesis.^{43,44}

In the present study we found that the expression of Pax2 not only correlated with an enhanced survival of endothelial cells but also with an enhanced *in vitro* and *in vivo* angiogenic activity. In fact, suppression of Pax2 production induced the loss of the proinvasive and proangiogenic phenotype in TECs. Conversely, the acquisition of Pax2 in normal endothelial cells induced a phenotype similar to that of TECs.

In conclusion, these results indicate that Pax2 is involved in renal tumor angiogenesis because its expression may down-regulate PTEN, a tumor suppressor gene that modulates the Akt-survival pathway, and may control steps involved in angiogenesis, such as survival, proliferation, invasion, and cell organization.

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