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## TRANSLATIONAL PHYSIOLOGY |

# Urothelial expression of neuropilins and VEGF receptors in control and interstitial cystitis patients

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Departments of <sup>1</sup>Physiology and <sup>5</sup>Urology, University of Oklahoma Health Sciences Center, and <sup>2</sup>Oklahoma Medical Research Foundation (OMRF), Imaging Core Facility, Oklahoma City, Oklahoma; <sup>3</sup>VirtualScopics, Incorporated, Rochester; <sup>4</sup>Departments of Urology and Pathology, New York University Medical School, New York, New York; and <sup>6</sup>SibTech, Incorporated, Brookfield, Connecticut

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**Saban R, Saban MR, Maier J, Fowler B, Tengowski M, Davis CA, Wu X, Culkin DJ, Hauser P, Backer J, Hurst RE.** Urothelial expression of neuropilins and VEGF receptors in control and interstitial cystitis patients. *Am J Physiol Renal Physiol* 295: F1613–F1623, 2008. First published September 24, 2008; doi:10.1152/ajprenal.90344.2008.—Interstitial cystitis (IC) is a chronic and painful bladder syndrome of unknown cause with no reliable biological marker or effective therapy. Vascular endothelial growth factor (VEGF), which plays a key role in bladder inflammation, is closely associated with the vascular alterations observed in patients with IC. However, our recent findings of VEGF receptors (VEGF-Rs) and VEGF coreceptors on nonendothelial cells in human and mouse urothelium suggest that additional VEGF targets and functions are possible in IC bladders. We report here that VEGF-Rs and coreceptors (neuropilins; NRP) are strongly expressed in both the human bladder urothelium and in the human bladder cancer cell line (J82) and that the expression of NRP2 and VEGF-R1 is significantly downregulated in IC compared with control subjects. In addition, treatment of J82 cells with bacillus Calmette-Guérin (BCG), a novel treatment strategy for IC, upregulates the messages for NRPs and VEGF-Rs. Furthermore, intravesical instillation of an internalizable VEGF fluorescent tracer (scVEGF/Cy5.5) into mouse urinary bladders results in a marked ligand accumulation in the urothelium and bladder parenchyma, indicating that urothelial VEGF-Rs are functionally active and capable of ligand interaction and internalization. Our results suggest that the VEGF pathway is altered in IC, that urinary VEGF may gain access to the bladder wall via these receptors, and that BCG treatment may replenish the missing VEGF-Rs/NRP receptors. Together, these results suggest that levels of NRPs, VEGF-Rs, and VEGF are new putative markers for the diagnosis of IC and that modulating these receptors can be exploited as therapeutic strategies.

BCG (bacillus Calmette-Guérin); bladder cancer cell line (J82); molecular imaging; NIRF (near-infrared fluorescence); translational research; ChIP

INTERSTITIAL CYSTITIS (IC) is a chronic bladder syndrome characterized by urinary urgency, frequency, nocturia, pain, and sterile urine. Diagnosis of IC is primarily based on symptoms, as there is currently no available blood or urine tests due to lack of demonstrated biological markers. There are currently no

consistently effective treatments for IC, which remains an idiopathic heterogeneous disorder of unknown cause (31). However, intravesical administration of bacillus Calmette-Guérin (BCG) has been exploited as a promising option for the treatment of IC (37–39). Current evidence in IC supports multiple abnormalities in bladder urothelial physiology (18). Bladder epithelium permeability is thought to be increased in IC, and it may be related to abnormal expression of the proteins involved with epithelial adhesion and differentiation (21, 50). One theory for the cause of urothelial dysfunction in IC is that antiproliferative factor (APF), a frizzled 8-derived sialoglycopeptide produced by cells derived from IC patients, increases the permeability of the bladder (18).

Another putative candidate is vascular endothelial growth factor (VEGF). VEGF has been most intensively studied with respect to its actions on vascular cells, and in the bladder, increased staining of VEGF was reported in patients with glomerulations on hydrodistension, but not in patients who failed to show petechial bleeding or in controls (53). VEGF signaling is also part of the bladder's response to BCG (35, 43). Far less understood are the roles of VEGF and its receptors in epithelial cell biology, which were only reported recently (23, 24). We recently showed that VEGF signaling not only is part of the bladder's response to BCG but also appears to be active in urothelial cells, not just vascular endothelial cells, (35, 43) and represents a key response downstream of protease-activated receptor (PAR) activation (45). This new appreciation of VEGF's signaling role in bladder inflammation is supported by the emerging evidence that levels of various VEGFs are, in general, elevated at the site of inflammation (12, 19) and that infiltrating lymphocytes and other inflammatory cells represent an additional source of VEGF (17, 19).

Importantly, VEGF signaling in bladder urothelium is supported not only by expression of VEGF receptors (VEGF-Rs) but also by expression of VEGF coreceptors, neuropilins (43). Neuropilins (NRP), which were initially identified as a semaphorin receptor and mediator of axon guidance (10), function as coreceptors for VEGF and enhance its binding to VEGF-R2 (52). In this capacity, NRPs play a fundamental role in modulating the VEGF signaling involved in cancer development

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(20) and metastasis (7). The two related NRPs, NRP1 and NRP2, are expressed in both the nervous and vascular systems and are at the heart of the cross talk between these systems (15). However, recent evidence indicates that NRPs are expressed outside of the vascular system (36) and play a fundamental role in the activation of inflammatory cells, antigen-presenting cells (5), effector cells (6), and cancer cells (7). However, there is little information on the role of NRPs in bladder inflammation.

Previous results from our laboratories indicate that VEGF-Rs and NRPs play an important role in urothelial biology (43). Mouse bladder cells with accessible and functionally active VEGF-Rs were labeled in vivo via VEGF-R-mediated endocytosis with a new, internalizable fluorescent tracer, scVEGF/Cy5.5 (43). These and other studies with scVEGF/Cy5.5, presented evidence of a strong expression of VEGF-Rs and NRPs in the mouse bladder urothelium and pelvic ganglia that accumulates scVEGF/Cy5.5 following systemic administration (43). We also presented evidence that inflammation increases the number of cells with accessible VEGF-Rs and NRPs in the urinary bladder urothelium (43).

Three lines of evidence suggest that VEGF and neuropilin signaling could be aberrant in IC. First, the urothelium is "leaky" in IC and VEGF signaling modulates vascular permeability (42). Second, there is evidence of abnormal capillary growth (41). Third, there is the hypothesis for a connection between neural and epithelial function (3), an action that in other systems could be modulated by neuropilins. Here, we examined the expression of VEGF-Rs and neuropilins in human bladder urothelium in both control and IC patients. Our findings suggest a clear abnormality in the distribution of these key molecules that suggests they may play an important pathophysiological and diagnostic role in IC.

## MATERIALS AND METHODS

### Human Bladders

The research described here was approved by the University of Oklahoma Health Sciences Center. Animal experimentation was conducted according to IACUC protocol 05-0881 and the use of human tissues was approved by the Institutional Review Board (IRB protocol 08897) and adheres to the principles of the Declaration of Helsinki, as well as to Title 45, U.S. Code of Federal Regulations, part 46. Protection of Human Subjects, Revised November 13, 2001, and effective December 13, 2001. The same urothelial specimens that were collected for our previous study (50) were used in the present work. Specimens were collected from 6 controls and 27 patients (21 women and 6 men) meeting the current relaxed criteria for patient entrance into clinical studies of IC, as established by the National Institute of Arthritis, Diabetes, Digestive, and Kidney Diseases (25), including moderate to severe disease symptoms greater than 6 mo in

duration, an average age of 38.2 yr (range 23–63), and the undergoing of therapeutic cystoscopy and hydrodistension. Six female patients with an average age of 46.1 yr (range 21–66), who were known to be free of bladder mucosal disease and urinary tract infection and were undergoing a bladder suspension procedure for stress urinary incontinence, underwent bladder biopsy and served as controls.

**Specimen collection.** Patients with IC underwent cystoscopy and hydrodistension at 90 cmH<sub>2</sub>O for 5 min with urethral occlusion, followed immediately by biopsy with cold-cup rigid biopsy forceps of the posterior bladder wall through a 22Fr rigid cystoscope. Control samples were obtained in similar fashion from patients undergoing suspension for stress incontinence without hydrodistension. All samples were immediately fixed in formalin and subsequently mounted in paraffin.

### Immunofluorescence of Human Tissues, Human Bladder Cancer Cell Line (J82), and Mouse Tissues

Human bladders originally formalin fixed and paraffin embedded were deparaffinized and rehydrated in a series of xylene and ethanol washes and transferred to the Retriever (series 2100, Electron Microscopy Sciences; <http://www.emsdiasum.com/microscopy/products/histology/retriever.aspx>) in 1× R buffer A (pH 6.0 citrate-based retrieval solution). Slides were allowed to complete a heating cycle and given a minimum of 2 h to a maximum of overnight to cool (while still in the Retriever). Mouse bladders were frozen and post-fixed in 1% methanol-free formaldehyde (Polysciences), and the J82 cell line was fixed in 4% paraformaldehyde. All reagent incubations and washes were performed at room temperature. All tissues and cell lines were processed for immunofluorescence (IF) according to published methods (13). Briefly, slides were blocked for 45 min with 5% normal donkey serum (NDS; Jackson Immunolabs), then co-incubated with primary antibodies in 0.5% NDS for 1 h and 45 min in a humidified chamber. Following brief rinses with PBS, slides were incubated with appropriate secondary antibodies at the same time. All secondary antibodies were used at a 1:400 dilution and included donkey anti-rabbit IgG Alexa Fluor 488 conjugate (Molecular Probes; [probes.invitrogen.com](http://probes.invitrogen.com)), donkey anti-goat IgG Alexa Fluor 546, and donkey anti-rat IgG Alexa Fluor 488. Slides were washed, counterstained with 4',6-diamidino-2-phenylindole (DAPI), and coverslipped. Controls included single fluorophore-stained slides and slides stained with the omission of the primary antibody. Antibodies used in this work included anti-uropilin provided by Dr. X.-R. Wu (55), and commercially available antibodies are listed (see Table 2).

### Image Acquisition

All tissue cross sections were visualized with a Nikon Eclipse TE 2000-S inverted fluorescent microscope (Nikon; <http://www.nikoninstruments.com/>) and imaged at room temperature using a digital CCD camera (Roper Scientific; <http://www.roperscientific.com/>) driven by NIS-Elements AR2.3 Imaging software (Laboratory Imaging/Nikon; <http://www.nis-elements.com/>). DAPI staining was visualized using a DAPI filter set (340- to 380-nm excitation, 435- to 485-nm emission). Imaging of Alexa Fluor 488 utilized an excitation filter of

Table 1. Primers used for ChIP-Q-PCR

Gene	Alternative Name	Accession No.	Forward	Reverse
NRP1		NM_003873	tccaagttttctgcacattca	ttcctttgcaactggccttgta
NRP2		BC117413	ttcctttctcggaccactctc	ggcttttttcgcctttctttc
VEGF-R1	Flt1	DQ836395	cctttttcttttgccctcatt	tggacttccttcacaaaattca
VEGF-R2	Flk-1	AF063658	gaccagcaactgtccaagag	ctggcattgggtactgacttatg
VEGF-R3	Flt4	NM_182925.3	gggatacagcccaactttact	catcacacctcgtaacctgtc
Untr12			TGGACCTTTACCTGCTTTATCA	AGCAAGGACTAGGATGACAGAA

ChIP, chromatin immunoprecipitation; Q-PCR, quantitative PCR.



Table 2. Primary antibody characteristics

Antigen	Host Species	Against	Dilution	Code	Supplier	Address
AUM (urolakin polyclonal)	Rabbit	Bovine AUM	1:10,000		Dr. X.-R. Wu	
Neuropilin 1 (NRP1)	Goat (P)	Mouse	1:750	24-6800	eBioscience	www.ebioscience.com
Neuropilin-2 (NRP2)	Goat (P)	Rat	0.5625	AF567	R&D Systems	http://www.rndsystems.com
Neuropilin-2 (NRP2)	Rabbit	Human (NH <sub>2</sub> terminus)	1:100	36-1500	Zymed Laboratories, Inc.	South San Francisco, CA 94080
VEGF-R1 (Flt-1)	Rabbit	Human (COOH terminus)	1:50	RB-1527-P1	NeoMarkers	www.labvision.com
VEGF-R2 (Flk-1)	Rat	Mouse	1:50	550549	BD Pharmingen	

VEGF-R, VEGF receptor.

465–495 nm and an emission filter of 515–555 nm. Alexa Fluor 546 was imaged with an excitation of 528–553 nm and 590- to 650-nm emission range.

A secondary-only control slide was used to determine exposure time and to set minimum background fluorescence levels for each fluorophore imaged. Once set, exposure times were not changed during acquisition of each respective fluorophore in the staining series. Staining was considered positive only when the acquired signal exceeded the established background. An absence of signal bleed-through was determined using previously optimized multiaquisitions settings on single fluorophore-stained slides.

**Qualitative analysis of IF.** Qualitative analysis was performed using grayscale images. At least six randomly selected fields per cross section were visualized and photographed using a  $\times 40$  objective (0.75 NA) and used for quantitative analysis performed by two of the investigators (R. Saban and P. Hauser), who scored each section blindly for 1) polarization of stain in the apical urothelial cells (2, 29); 2) predominant stain in the nuclear region of apical urothelial cells; 3) predominant stain in the nuclear region of intermediate urothelial cells; and 4) presence of staining throughout urothelial layer. The following index was used: 1 (absent); 2 (reduced); or 3 (present). The statistical analysis was performed using Wilcoxon's rank sum test.

Results are expressed as means  $\pm$  SE. In all cases, a value of  $P < 0.05$  was considered indicative of significant difference.

**Quantitative analysis of IF.** Quantitative analysis was performed using grayscale images. At least six randomly selected fields per cross section were visualized and photographed using a  $\times 20$  objective (0.25 NA) and used for image analysis that was performed with Image Pro Analyzer software, version 6.0 (Media Cybernetics, Silver Spring, MD; <http://www.mediacy.com/index.aspx?page=IPP>). As the expression of NRPs and VEGF-Rs were predominantly observed in the urothelium, the free drawing tool was used to trace the limits of the urothelium (see Fig. 4) and to measure the total area. Then, the area occupied by cells stained by a particular antibody was calculated as the total area occupied by bright objects and presented as the percentage of the area covered by the urothelium (see Fig. 4) and were compared through a Welch *t*-test and adjusted for multiple comparisons using a Holm correction. An  $\alpha$  level of 0.05 was considered statistically significant.

#### Human Bladder Cancer Cell Culture

The human bladder cancer cell line J82 (HTB-1) was obtained from the American Tissue Culture Collection (ATCC). J82 cells were

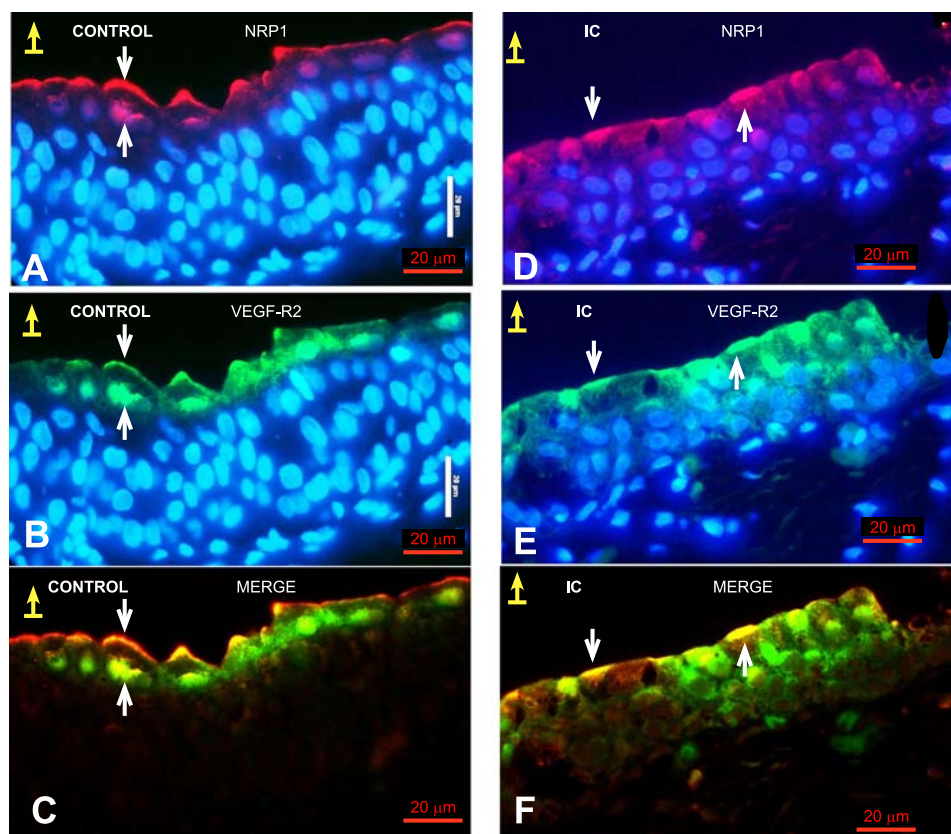


Fig. 1. Expression of neuropilin (NRP) 1 and VEGF receptor (VEGF-R2) in control and interstitial cystitis (IC) bladder urothelium. Representative photomicrographs indicate that control human urothelial cells express NRP1 (A) and VEGF-R2 (B) and that the two receptors are localized in the plasma membrane and nuclear regions (C). Although the bladder urothelium from IC patients also expresses NRP1 (D) and VEGF-R2 (E), a distinct pattern of distribution is suggested by a predominant expression of NRP1 and VEGF-R2 in the cytoplasm (F). White arrows indicate cells expressing both receptors being studied, and yellow arrows point to the bladder lumen.

seeded and cultured in MEM supplemented with 10% FBS, 100  $\mu$ M nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 2 $\times$  MEM Vitamin Solution. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> until 90% confluence was reached. In addition, HeLa cells (ATCC, Manassas, VA) were cultured in DMEM with 10% FCS, glutamine, and 100 U penicillin-streptomycin, and used for IF.

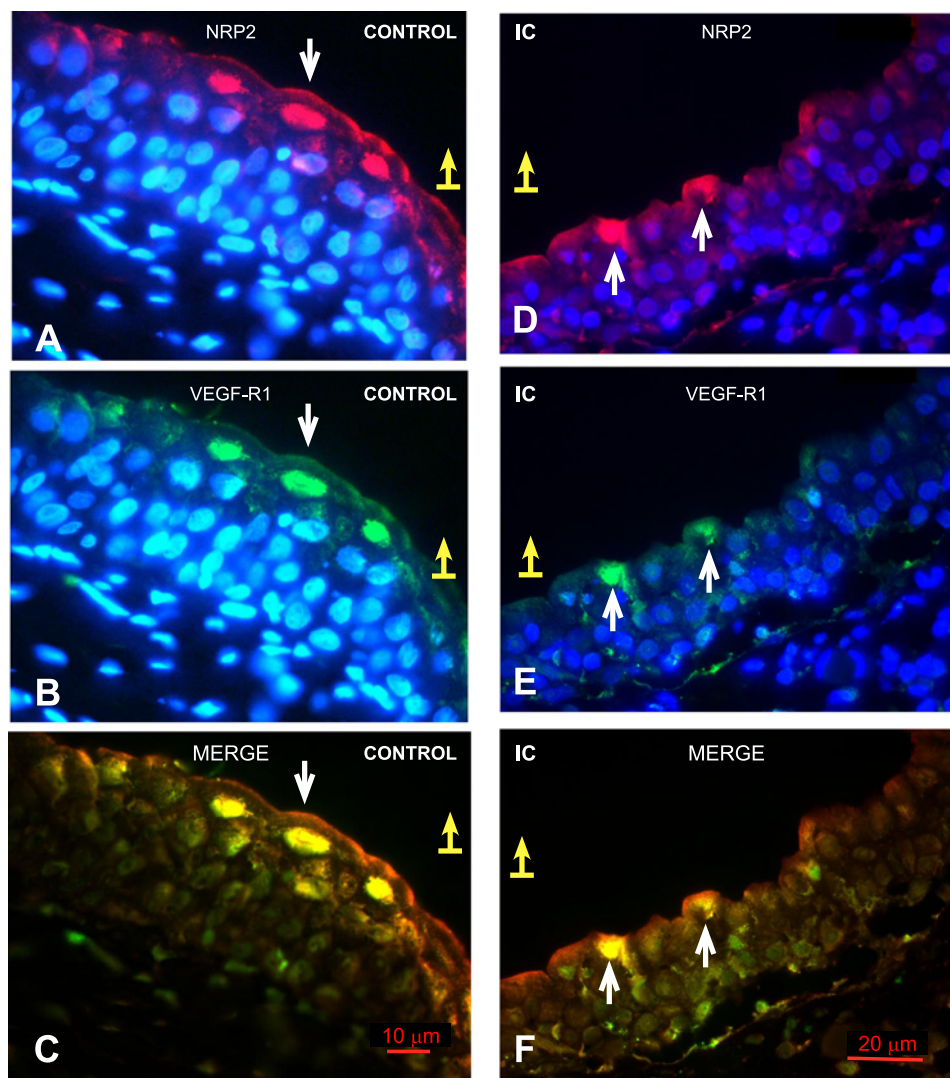
**Challenge of J82 with BCG.** The J82 cell line was used to determine the effects of BCG on the expression of VEGF-R1, VEGF-R2, NRP1, and NRP2. For IF, cells were cultured on coverslips (500  $\mu$ l media  $\cdot$  62.5  $\mu$ l cells<sup>-1</sup>  $\cdot$  well<sup>-1</sup>), whereas for chromatin immunoprecipitation (ChIP)/quantitative real-time PCR (Q-PCR) analysis, the cells were grown in 75-mm<sup>2</sup> flask (10 ml media  $\cdot$  1 ml cells<sup>-1</sup>  $\cdot$  flask<sup>-1</sup>). All cells were serum starved overnight and challenged for 24 h with BCG [8.1  $\times$  10<sup>6</sup> colony-forming units (CFU)/ml]. Following 24 h of challenge, cells were isolated and frozen for ChIP-Q-PCR, as described below or fixed in 4% paraformaldehyde for IF.

#### ChIP- and Q-PCR-Based Assays of J82 Cell Line

To confirm whether BCG treatment would alter gene expression, we used ChIP combined with Q-PCR, as described previously (46). ChIP/Q-PCR was used because it is quantitative and has the added advantage of using the DNA transcriptome and, therefore, measures genes that are actively being transcribed. This represented an advance

over Q-PCR alone that is based on RNA and, therefore, more susceptible to RNA degradation and instability. Briefly, the J82 cell line (10<sup>6</sup> cells/treatment) was treated as described above, frozen, and shipped to Genpathway (<http://www.genpathway.com>) for querying the chromatin for gene transcription by ChIP/Q-PCR (Genpathway's TranscriptionPath Query assay) (26). Cells were exposed briefly to formaldehyde for cross-linking of the proteins and DNA together, followed by sonication to fragment the DNA into pieces of  $\sim$ 300–500 base pairs. An antibody against RNA polymerase II (Genpathway proprietary information) was then used to precipitate the DNA transcriptome (44). The Ab-protein-DNA complexes were purified using beads coupled to protein A. The DNA was isolated from the complexes using a combination of heat to reverse cross-linking, RNase, and proteases, and then purified using phenol extraction and EtOH precipitation. The final ChIP DNAs were then used as templates in Q-PCR using primer pairs specific for each gene of interest and designed using primer 3 ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)). Details of the primer sequences are given in Table 1, and primary antibody characteristics are shown in Table 2. Q-PCR was carried out using *Taq* polymerase (iQ SYBR Green Supermix, Bio-Rad). The designed primers shared 100% homology with the target sequence, but no significant homology with other sequences. Q-PCRs were run in triplicate, and the values were transferred into copy numbers of DNA using a standard curve of genomic DNA with known copy numbers. The

Fig. 2. Expression of NRP2 and VEGFR-1 in normal and IC bladder urothelium. Representative photomicrographs indicate that normal human urothelial cells express NRP2 (A) and VEGF-R1 (B) and that the two receptors are localized in the plasma membrane and nuclear regions (C). The bladder urothelium from IC patients also expresses NRP2 (D) and VEGF-R1 (E), and these receptors are expressed in the same cells (F). White arrows indicate cells expressing both receptors being studied, and yellow arrows point to the bladder lumen.





resulting transcription values for each gene were also normalized for primer pair amplification efficiency using the Q-PCR values obtained with Input DNA (unprecipitated genomic DNA). Results are presented as transcription events detected per 1,000 cells for each gene tested.

**Statistical analysis of ChIP/Q-PCR.** The difference between two mean values was analyzed with an unpaired Student's *t*-test (GraphPad Prism software, version 4.0; GraphPad Software, San Diego, CA). A nominal *P* value <0.05 was considered statistically significant.

#### Near-Infrared Fluorescent Imaging

Female mice were fed a low-chlorophyll diet for 2 wk to reduce autofluorescence in the intestinal region (54), and the abdominal hair was removed. Mice were anesthetized with isoflurane, transurethrally catheterized as previously described (44), and instilled with 150  $\mu$ l of one of the following substances: pyrogen-free saline (*n* = 20) or scVEGF/Cy5.5 (scVEGF/Cy is the trade name for scVEGF/Cy5.5), an engineered single-chain VEGF labeled with Cy5.5 dye, which identifies cells with accessible and functionally active VEGF-Rs (43). Twenty-four hours after instillation, anesthetized mice were immediately placed on a heating pad inside a FluorChem HD2 cabinet (Alpha Innotech, San Leandro, CA) equipped with a Chromalight multiwavelength illuminator with a Cy5.5 excitation filter ( $620 \pm 60$  nm), and images were captured with 4-megapixel cooled camera (F2.8, 28- to 70-mm zoom lens) with a Qdot 705 emission filter ( $705 \pm 10$  nm). The FluorChem HD2 was coupled to a dedicated computer, and

images were first acquired and stored with AlphaEase FC 32-bit software (Alpha Innotech). Following near-infrared fluorescent (NIRF) imaging, mice were euthanized with pentobarbital sodium (100 mg/kg ip), the abdomen was opened and scanned for NIRF, and tissues were removed rapidly and frozen for IF.

#### Reagents

scVEGF/Cy is the trade name for scVEGF/Cy5.5. scVEGF/Cy and inactive tracer (inVEGF/Cy) were from SibTech (Brookfield, CT), and BCG (TheraCys) was purchased from Aventis-Pasteur.

#### RESULTS

##### Expression of VEGF-Rs and NRPs by Human Urothelium

Our previous study showed that both NRPs and VEGF-Rs are expressed with high density in nonendothelial cells in human bladders (43). Here, we compared human bladder biopsies obtained from control (stress urinary incontinence patients) and IC patients. In bladder biopsies of control subjects, intense immunostaining for NRP1 (Fig. 1A), VEGF-R2 (Fig. 1B), NRP2 (Fig. 2A), and VEGF-R1 (Fig. 2B) was noted, and all receptors were localized in the urothelial layer. The staining was the strongest in the superficial umbrella cells with

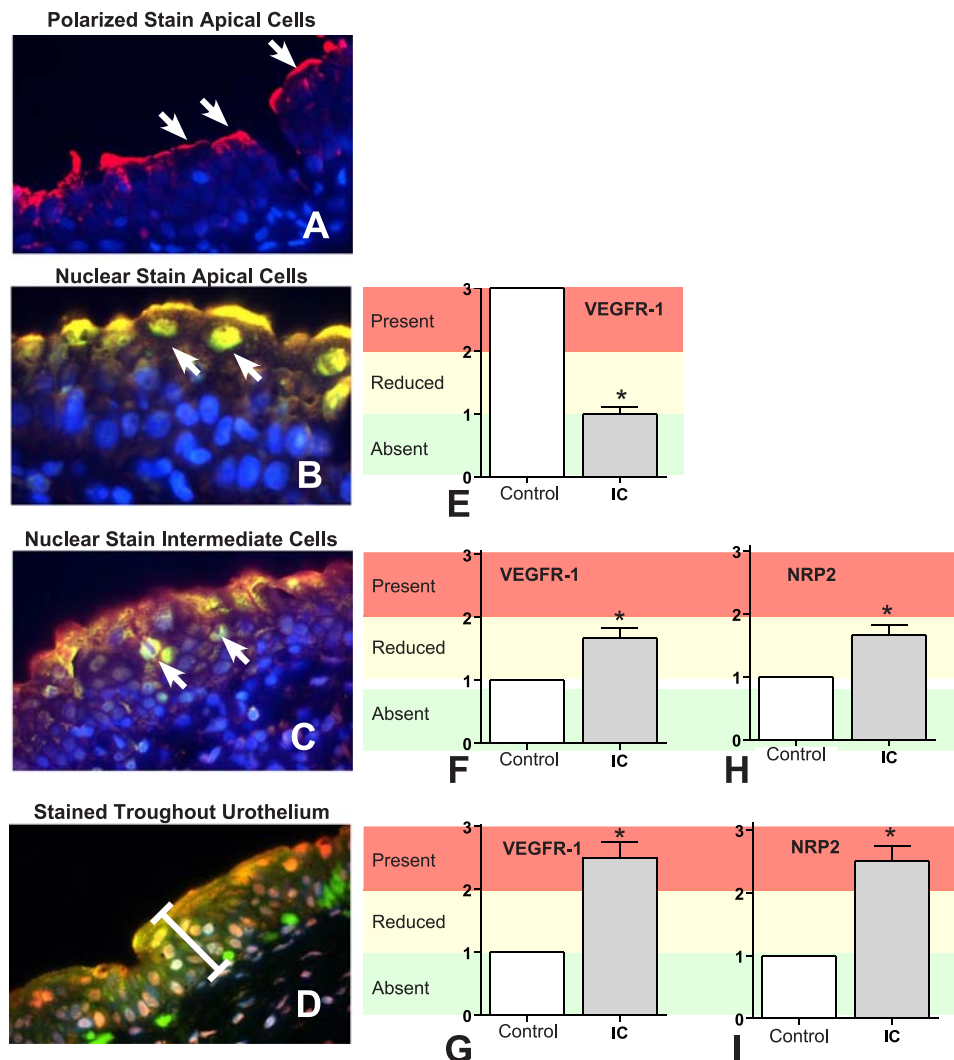


Fig. 3. Qualitative analysis of the immunofluorescence (IF) staining. Distribution of IF was focused on 4 different patterns, and their representative photomicrographs illustrate polarization of the staining toward the luminal surface (A), predominant staining in the nuclear region of the apical cells (B), predominant staining in the nuclear region of the intermediate urothelial cells (C), and whether the IF was observed throughout the urothelial cell layer (D). The following index was used: 1 (absent), 2 (reduced), or 3 (present). Values are means  $\pm$  SE. Only the groups that presented a statistically significant difference in the distribution of the particular receptor staining between control and IC are being presented (E–I). \*Statistically significant difference (*P* < 0.05).

only sparse staining in the lamina propria and detrusor layers, most probably in blood vessels and inflammatory cells (data not shown). Within the urothelium, NRP1 and VEGF-R2 (Fig. 1C) and NRP2 and VEGF-R1 (Fig. 2C) were stained particularly strongly in the luminal surface area and in the proximity of the nuclei of the cells.

Although bladder biopsies obtained from IC patients also showed strong expression of NRP1 (Fig. 1D), VEGF-R2 (Fig. 1E), NRP2 (Fig. 2D), and VEGF-R1 (Fig. 2E), the pattern of their staining was noticeably distinct from those observed in control bladders. Therefore, qualitative and quantitative analyses were performed to further characterize the receptor distribution.

#### Qualitative Analysis of IF Staining

Distribution of IF was characterized using four different patterns, and their representative photomicrographs are illustrated in Fig. 3, A–D. These included 1) polarization of the staining toward the luminal surface (Fig. 3A); 2) predominant staining in the nuclear region of the apical cells (Fig. 3B); 3) predominant staining in the nuclear region of the intermediate urothelial cells (Fig. 3C); and 4) whether the IF was observed throughout the urothelial cell layer (Fig. 3D). In control individuals, the expression of all receptors seemed to be polarized in the apical cells, whereas IC patients presented a nonsignificant trend for a decrease in polarization of the

staining. IC patients presented a significant reduction of staining of VEGF-R1 in the nuclear region of the apical cells (Fig. 3E) and an increased staining in the nuclear region of intermediate cells for VEGF-R1 and NRP2 (Fig. 3, F and H, respectively). In addition, control bladders presented VEGF-R1 and NRP2 predominantly in the apical cells, whereas in IC patients both VEGF-R1 and NRP2 were expressed throughout the urothelium (Fig. 3, G and I, respectively).

#### Quantification of IF Results

The total area occupied by the urothelium was measured by tracing each microphotograph, as illustrated in Fig. 4A. The area delimited with the tracing tool was then automatically computed (in  $\mu\text{m}^2$ ) by the Image Pro software. Next, the area occupied by each bright object in each channel was calculated by using the count function, and the sum of the areas of all bright spots was automatically calculated and resulted in the total area occupied by the Alexa 488 and Alexa 546 stains, as represented in Fig. 4, B and C, respectively. Subsequently, the percentage of the total urothelial area occupied by Alexa 488- and Alexa 546-positive stains was calculated for each field, and the results of all fields were averaged and are presented as means  $\pm$  SE in Fig. 4D. These results indicate that NRP2 and VEGF-R1 were the highest expressed receptors in control urothelium and were significantly reduced in IC patients.

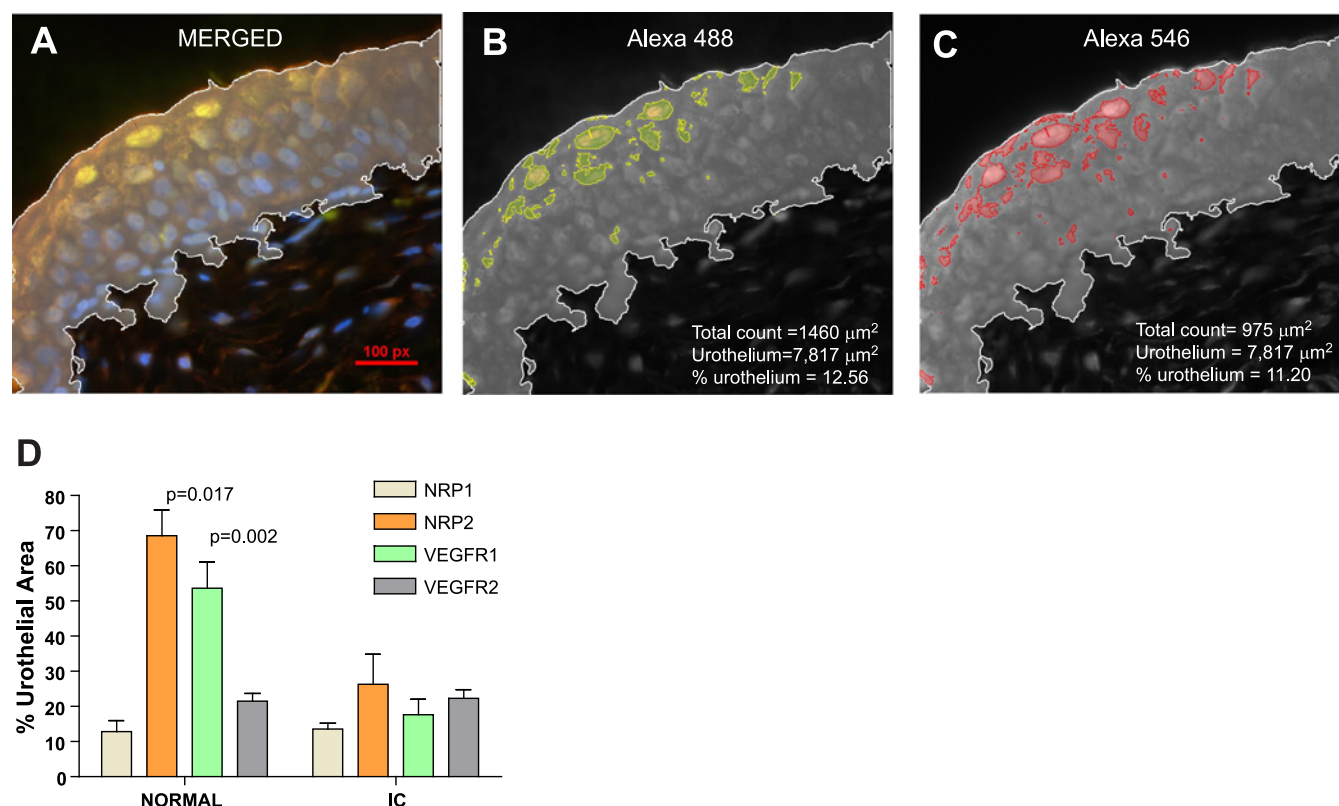


Fig. 4. Quantification of IF results. A: representative photomicrograph illustration of the total area occupied by the urothelium. B: representative photomicrograph sections stained for VEGF-R1. C: representative photomicrograph sections stained for NRP2. The area occupied by the bright objects in each channel (Alexa 488 and 546) was calculated by using the count function and presented as total count or percentage of the area occupied by the urothelium. D: image analysis of the bladder urothelium. At least 6 random fields/cross section obtained from 5 normal and 6 IC patients were visualized and used for image analysis that was performed with the Image Pro Analyzer and expressed as the percentage of the total area of the urothelium positive for each antibody. Values are means  $\pm$  SE. \*Statistically significant difference ( $P < 0.05$ ) between normal and IC.

### scVEGF/Cy5.5 Tags Urothelial Cells Following Intravesical Instillation

Since upper layers of urothelium express VEGF-Rs and VEGF coreceptors, and VEGF is found in human urine (14, 30, 34), we tested whether urinary VEGF could be absorbed through the urothelial apical surface. Anesthetized mice were instilled with scVEGF/Cy5.5, a fluorescent tracer that binds to and is internalized by VEGF-Rs (4, 43). Twenty-four hours after instillation, the mice were subjected to NIRF imaging followed by fluorescent microscopy of bladder histological sections. We found that instillation of the scVEGF/Cy5.5 tracer led to strong fluorescence in the abdominal region (area inside the red circle in Fig. 5A), and when the abdomen was opened (Fig. 5B) fluorescence was detected in the urinary bladder (yellow dotted circle), as well as in collecting lymphatics (red arrows) and the kidneys (green arrows). Microscopy of cross sections of the urinary bladders isolated from instilled mice indicated a predominant accumulation of scVEGF/Cy5.5 fluorescence in the bladder urothelium (Fig. 5C, white dotted

line). However, scVEGF/Cy5.5 fluorescence was also detected in suburothelial regions with distinct accumulation of fluorescence in individual cells in these structures (white arrows, Fig. 5C). Anti-uroplakin antibody clearly labeled the bladder (Fig. 5D) and ureteral epithelium (Fig. 5E) and further illustrates that both urothelial and suburothelial cells internalize scVEGF/Cy5.5 tracer following intravesical instillation. These results suggest that the apically localized VEGF-Rs in the urothelium might be a major route of interaction with urinary VEGF.

### Expression of VEGF-Rs and VEGF Coreceptors in Human Bladder Cancer Cell Line

To develop an in vitro model for urothelial VEGF-Rs and VEGF coreceptors, we investigated whether a human bladder cancer cell line (J82) expresses VEGF-Rs and NRPs. Judging by ChIP-Q-PCR analysis with corresponding primers (Table 1), genes for VEGF-R1, VEGF-R2, VEGF-R3, NRP1, and NRP2 were actively transcribed in J82 (Fig. 6). Interestingly, 24-h treatment of J82 with BCG further enhanced transcription

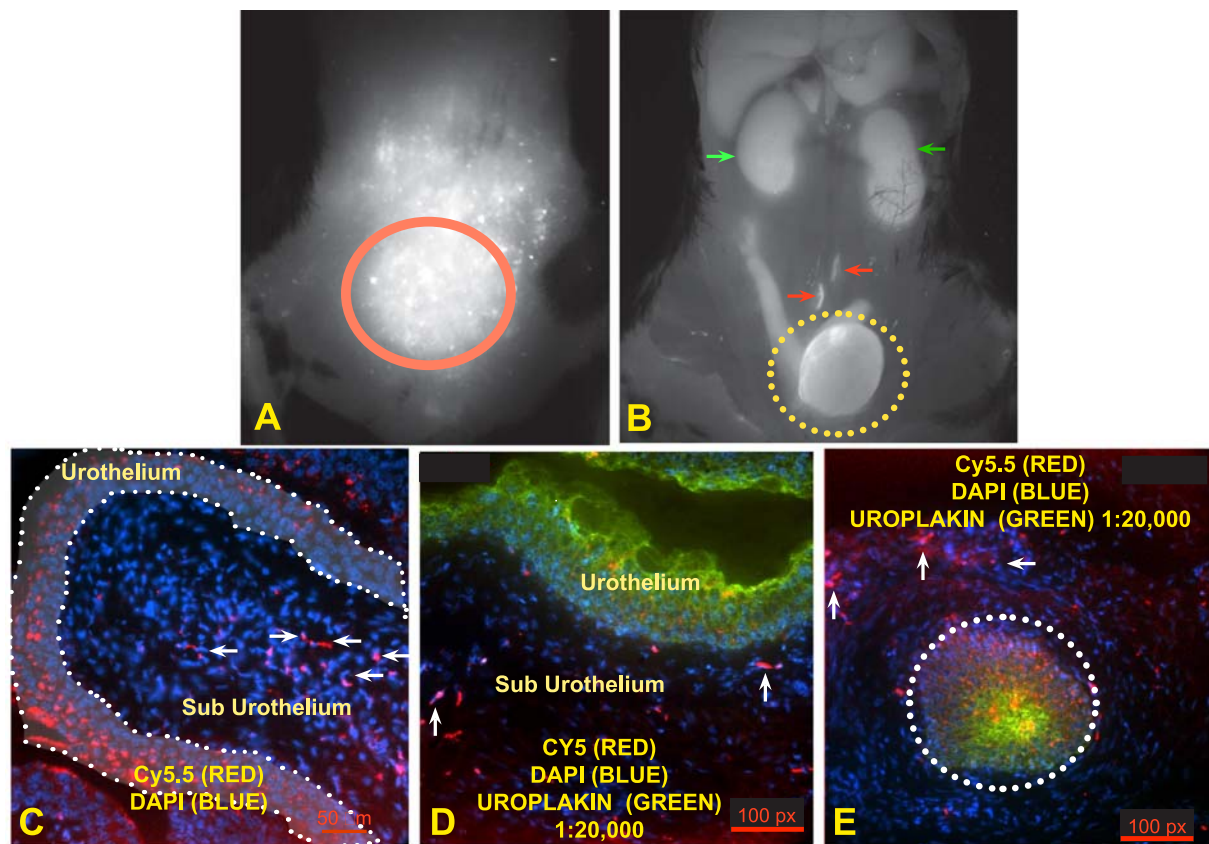


Fig. 5. Accumulation of scVEGF/Cy5.5 in the urinary bladder following intravesical instillation. Female C57BL/6 mice ( $n = 4$ ) were fed a low-chlorophyll diet for 2 wk to reduce autofluorescence in the intestinal region, and the abdominal hair was removed. Mice were anesthetized and instilled with 150  $\mu$ l of scVEGF/Cy5.5 containing  $\sim 0.5$  nmol of the tracer and placed on a heating pad inside a FluorChem HD2 (Alpha Innotech, San Leandro, CA) equipped with a Chromalight multiwavelength illuminator with a Cy5.5 excitation filter ( $620 \pm 60$  nm), and images were captured with 4-megapixel Cooled camera (F2.8, 28- to 70-mm zoom lens) with a Qdot 705 emission filter ( $705 \pm 10$  nm). The FluorChem HD2 was coupled to a dedicated computer, and images were first acquired and stored with AlphaEase FC 32-bit software (Alpha Innotech). A: representative photograph of the fluorescence in the abdominal region that reached a peak at 2 h after instillation. B: 24 h later, the skin was opened and the gastrointestinal tract was removed to permit a better visualization of the pelvic floor. Interestingly, not only the urinary bladder (yellow dotted line) but also collecting lymphatics (red arrows) and the kidneys (green arrows) were fluorescent. C: representative cross section of the urinary bladders isolated from mice instilled with scVEGF/Cy5.5 indicates accumulation in the bladder urothelium and suburothelial cell layer (white arrows). Higher magnification pictures illustrate an intense accumulation of scVEGF/Cy5.5 in the urothelium in uroplakin-positive cells (D) and in the detrusor smooth muscle (D). Interestingly, a cross section of the mouse bladder containing the ureteral opening (white dotted line in E) serves as a landmark to demonstrate deeper distribution of scVEGF/Cy5.5 in the detrusor muscle, while white arrows indicate scVEGF/Cy5.5 accumulation.



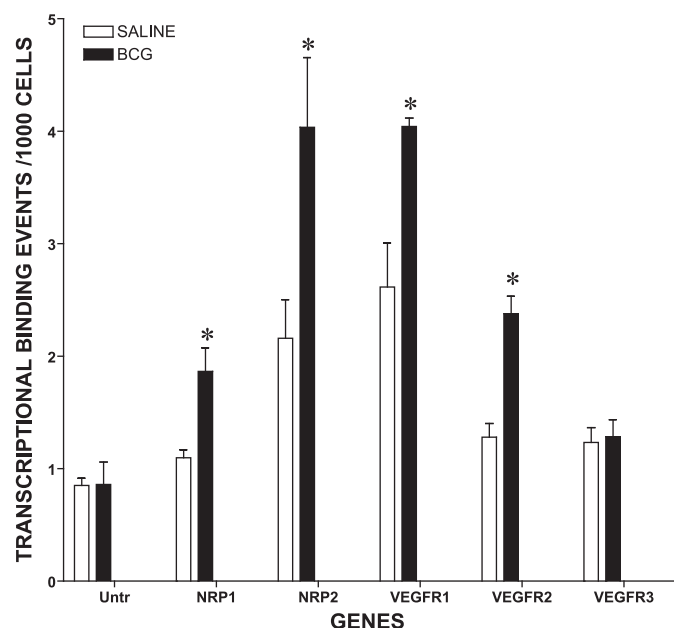


Fig. 6. Quantitative (Q)-PCR of chromatin immunoprecipitation (ChIP)-based assays. J82 cells ( $10^6$  cells) were treated with saline or bacillus Calmette-Guérin (BCG;  $8.1 \times 10^6$  colony-forming units) for 24 h and were used for extraction of chromatin (ChIP), and an antibody against RNA polymerase II (Abcam) was then used to precipitate the DNA transcriptome (44). Q-PCR was performed in triplicate to confirm using primer pairs described in Table 1. Results are presented as average and SE of transcription binding events detected per 1,000 cells. \*Statistically significant difference ( $P < 0.05$ ) for a specific gene between the group treated with saline and BCG.

of these genes (Fig. 6). Expression of VEGF-R1, VEGF-R2, NRP1, and NRP2 in J82 was further confirmed by immunostaining with corresponding antibodies (Fig. 7, A–H). To verify whether the IF stains were specific for the J82 cell line; we tested whether HeLa cells express these receptors. Figure 7, I and J, indicates that although the staining pattern for NRPs and VEGF-Rs are different between J82 and HeLa cells, the latter also express these receptors.

## DISCUSSION

This is the first report indicating that the bladder epithelium of IC patients exhibits an altered pattern of expression of VEGF-Rs and VEGF coreceptors, the neuropilins, compared with controls. The predominant expression of these receptors in the urothelium confirms our previous studies in the mouse and human bladders indicating that although some of these receptors are present in lymphatic and blood vessels, the urothelium represents a new and wealthy source of VEGF-Rs and neuropilins (43). Quantification of receptor and coreceptors expression by IF indicated that IC patients presented a significant downregulation of NRP2 and VEGF-R1. In addition, qualitative assessment indicated that, in control bladder, these receptors are localized primarily to the apical cell layer, whereas in IC some of these receptors are found throughout the urothelium, including the basal layer.

Although it is very unlikely that the short period of time between hydrodistension and biopsy would be enough to alter the expression of VEGF-Rs or NRPs, the present results have to be taken in light of the fact that the IC bladder specimens were obtained after hydrodistension, whereas control speci-

mens were obtained without hydrodistension. Nevertheless, in the mouse bladder, inflammation, but not the mechanical alteration induced by saline instillation, increased the expression and accessibility of these receptors (43).

Because of the limitations of performing studies in human subjects, we used a mouse model to clearly define whether the urothelial NRP and VEGF-Rs are functional. For this purpose, we used a fluorescent VEGF tracer which only internalizes in cells expressing active receptors (4) and is known to accumulate in the mouse bladder following systemic administration (43). In contrast, biotinylation of six to eight amino groups in scVEGF/Cy5.5 inactivates the tracer and prevents the internalization of the probe in the mouse bladder following systemic administration (43). Our present results obtained with the instillation of scVEGF/Cy5.5 into the mouse bladder raise the possibility that scVEGF/Cy5.5 interacts with urothelial VEGF-Rs and is transcytosed by the urothelial cells into deeper suburothelial layers. Alternatively, scVEGF/Cy5.5 could affect the permeability of the urothelium through mechanisms reminiscent of VEGF's effects on vascular permeability and resulting paracellular transport of VEGF. Indeed, the protein constituents comprising this highly effective urothelial barrier (tight junctions) have been recently studied in detail (1) and they include known targets of VEGF-mediated effects in vascular permeability: occludins (48) claudins (40), and zonula occludens-1 (33) proteins.

We also have shown by Q-PCR and IF the expression of NRPs and VEGF-Rs in a human bladder cancer cell line, suggesting that this cell line should be added to a list of cancer cells expressing these receptors (32). In addition, treatment of the human bladder cancer cell line with BCG significantly altered the message for these receptors. In an effort to find possible cell lines to be used as a control for nonspecific binding of the specific antibodies used in this experiment, we tested the HeLa cell line. However, our results indicate that these receptors are also expressed in HeLa cells.

One of the physiological roles of VEGF-Rs in the lower urinary tract may be to control the expression of tight junction proteins, thereby modulating urothelial permeability. VEGF signaling may also be important for homeostasis and survival of various cells in different tissues, including the vasculature (28), particularly kidney epithelial cells (24), and neuronal tissue (9). In the urinary tract, VEGF is also involved in renal epithelial cell morphogenesis in a NRP1-dependent fashion, and blockade of VEGF-R and NRP prevents the morphogenic response (24). These reported roles of VEGF on epithelial cells warrant future research on the possibility that urothelial VEGF-Rs and NRP receptors enable VEGF to act as a survival factor for the urothelium. Our results call attention to the rich expression of these receptors in the urothelium and shift the paradigm toward investigating their role outside of the vasculature.

We went further to confirm our previous results obtained in mice, which indicated that exposure to BCG led to increased access of VEGF to NRP and VEGF-Rs in the mouse bladder (43). Taking into consideration that at least a subset of IC patients seems to benefit from BCG therapy and it is known that BCG increases the urinary levels of VEGF (35), it is reasonable to raise the intriguing question of whether disorders that alter urothelial integrity and permeability, such as inter-

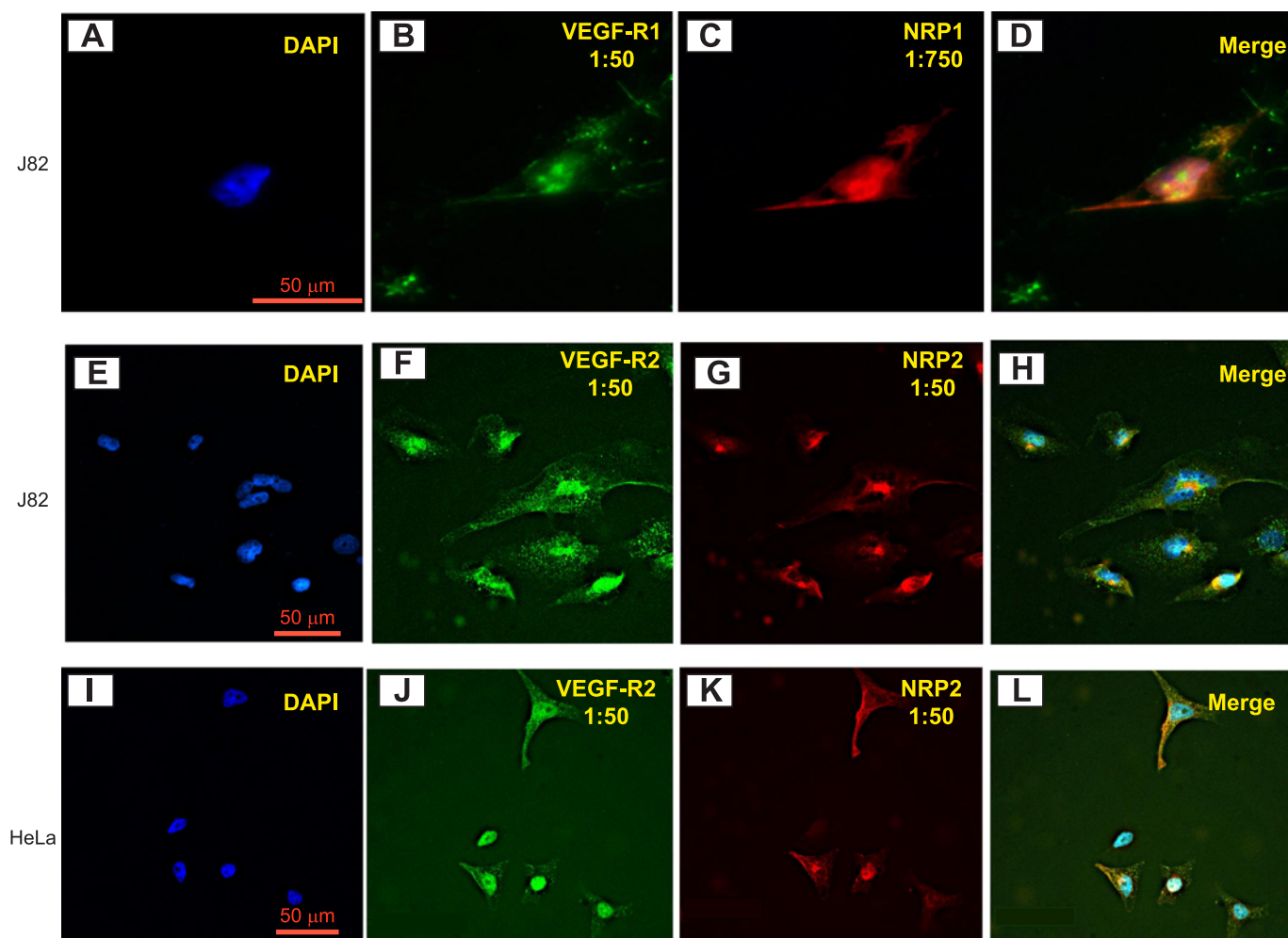


Fig. 7. Expression of VEGF-Rs and NRPs in human urothelial carcinoma cell line (J82) and HeLa cells. Representative photomicrographs of the J82 cell line indicate that the urothelial cells express VEGF-R1 (B), VEGF-R2 (F), NRP1 (C), and NRP2 (G) and that HeLa cells express VEGF-R2 (J) and NRP2 (K). A, E, and I are representative of 4',6-diamidino-2-phenylindole (DAPI) highlights of the cell nuclei and D, H, and L are merged pictures.

stitial cystitis, infection, and cancer, can be diagnosed and/or treated via modulation of VEGF-Rs and NRPs.

Finally, the use of a targeted tracer for whole-animal NIRF imaging followed by immunohistochemical analysis of cells tagged *in vivo* appears to be a promising approach for elucidating the mechanisms during physiological and pathological states of the urinary bladder. Major advantages of this approach are 1) opportunities to monitor uptake of the tracer in real time and 2) opportunities to identify cells with accessible and active receptors that bind and internalize the tracer *in vivo*. Since immunohistochemical analysis does not discriminate between “working” and “idle” receptors, *in vivo* tagging with an appropriate tracer might significantly enrich our understanding of temporal and spatial distribution of signal transduction activity. Furthermore, since scVEGF/Cy5.5 is retained within the cell after the targeting protein is degraded (24), there are opportunities for longitudinal studies of tagged cells. In view of these advantages, it would be interesting to use a fluorescent endoscope to perform a bladder examination with the scVEGF/Cy5.5 tracer under conditions of inflammation and cancer.

This paper opens a new field of research that will be devoted to the study of the possible functions of these receptors in the

bladder urothelium and their role in IC. Besides a possible role in permeability highlighted above, it needs to be taken into consideration that these receptors are important survival factors for the vascular and neuronal systems. Indeed, two related NRP receptors, NRP1 and NRP2, are expressed in both the nervous and vascular systems and are at the heart of the cross talk between these systems (15). NRP2, which is specifically down-regulated in IC, seems to modulate the responses of both VEGF (51) and VEGF-C under normal circumstances (16). As NRP2 was initially identified as a semaphorin receptor and mediator of axon guidance (10), it is possible that alterations in NRP2 expression in the urothelial cell layer would impact the distribution of suburothelial nerves or would be responsible for alterations in bladder capillaries. Reciprocally, it seems that nerve growth factor can alter the expression of VEGF-Rs and NRPs in the mouse bladder, as indicated by a recent work that correlates the expression of this growth factor in the bladder urothelium with the expression of VEGF-Rs and NRPs (11). In addition, recent studies have also emphasized the potential role of VEGF as a neuronal protective factor (27), suggesting that antiapoptotic VEGF functions might be important for certain cell types. It remains to be determined whether NRPs and

VEGF-Rs also play a survival role in urothelial cells. In this context, this paper provides two additional systems, the mouse and J82 cell lines, that can be used to test such a hypothesis.

Interestingly, a substantial fraction of the NRP receptor is a glycosaminoglycan (GAG) modified with either heparan sulfate or chondroitin sulfate (49). Evidence was recently presented indicating that GAG modification of NRPs plays a critical role in modulating VEGF/NRP signaling (8, 49). The latter is particularly important in IC since previous studies have shown a deficit of chondroitin sulfate on the bladder luminal surface in IC (21, 22), suggesting a possible connection between the GAG deficiency and the functionality of this signaling system.

To date, there is only one report showing strong NRP2 mRNA expression in bladder smooth muscle cells during development (10), and another report showing correlation of NRP2 expression with advanced bladder cancer stage and grade (47). Thus our findings of widespread expression of both NRP1 and NRP2 in the control and IC urothelia are the first indications that these rather promiscuous receptors might play a prominent role in both control and diseased urinary bladder. Our finding, therefore, is an important addition to the literature, especially since use of anti-VEGF therapies are increasing in clinical application.

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## DISCLOSURES

J. Backer is a shareholder and an employee in SibTech, Inc., and M. Backer is an employee of SibTech, Inc.

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