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

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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...
(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 examination of VEGF-Rs and NRPs in bladder urothelium (43).

Specimen collection. Patients with IC underwent cystoscopy and hydrodistension at 90 cmH₂O 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.

Table 1. Primers used for ChIP-Q-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Alternative Name</th>
<th>Accession No.</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRP1</td>
<td></td>
<td>NM_003873</td>
<td>tccagattttttgacacattca</td>
<td>tccctttgcaacttgctttgta</td>
</tr>
<tr>
<td>NRP2</td>
<td></td>
<td>BC177413</td>
<td>tccctttcgaggacaccttc</td>
<td>ggctttttggctttcttttc</td>
</tr>
<tr>
<td>VEGF-R1</td>
<td>Flt1</td>
<td>DQ836395</td>
<td>cttttttttttgaccttatt</td>
<td>tgacctttcttccaaaaaatttca</td>
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<tr>
<td>VEGF-R2</td>
<td>Flk-1</td>
<td>AF063658</td>
<td>gacccacagactgccacagag</td>
<td>cttgctttaggtaatgacttacttga</td>
</tr>
<tr>
<td>VEGF-R3</td>
<td>Flt4</td>
<td>NM_182925.3</td>
<td>gaggtcaggccacactttact</td>
<td>catccacactctgctcttgtgct</td>
</tr>
<tr>
<td>Untr12</td>
<td></td>
<td></td>
<td>7GAACCTTTACCCGCTT2XCA</td>
<td>AGCAAGGGAAGTGGATGACGAGAA</td>
</tr>
</tbody>
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ChIP, chromatin immunoprecipitation; Q-PCR, quantitative PCR.
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 multiacquisitions 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 40 objective (0.75 NA) and used for quantitative analysis that was 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 ± 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 ×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

Table 2. Primary antibody characteristics

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host Species</th>
<th>Species Against</th>
<th>Dilution Code</th>
<th>Supplier Address</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUM (uroplakin polyclonal)</td>
<td>Rabbit</td>
<td>Bovine AUM</td>
<td>1:10,000</td>
<td>Dr. X.-R. Wu</td>
</tr>
<tr>
<td>Neurophilin 1 (NRP1)</td>
<td>Goat (P)</td>
<td>Mouse</td>
<td>1:750</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Neurophilin-2 (NRP2)</td>
<td>Goat (P)</td>
<td>Rat</td>
<td>0.5625</td>
<td>R&amp;D Systems</td>
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<tr>
<td>VEGF-R1 (Flt-1)</td>
<td>Rabbit</td>
<td>Human (NH2 terminus)</td>
<td>1:100</td>
<td>Zymed Laboratories, Inc.</td>
</tr>
<tr>
<td>VEGF-R2 (Flk-1)</td>
<td>Rat</td>
<td>Mouse</td>
<td>1:50</td>
<td>NeoMarkers</td>
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</table>

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 μM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 2× MEM Vitamin Solution. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO2 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 μl media 62.5 μl cells 1 ml cells 1 ml), whereas for chromatin immunoprecipitation (ChIP)/quantitative real-time PCR (Q-PCR) analysis, the cells were grown in 75-mm² flask (10 ml media 1 ml cells 1 ml). All cells were serum starved overnight and challenged for 24 h with BCG [8.1 × 10⁶ 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 (10⁶ 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 ~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). 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
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 μl 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 ± 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 ± 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

![Polarized Stain Apical Cells](image_url)

![Nuclear Stain Apical Cells](image_url)

![Nuclear Stain Intermediate Cells](image_url)

![Stained Througout Urothelium](image_url)

Fig. 3. Qualitative analysis of the immuno-fluorescence (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 intermedium 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 ± 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 μm²) 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 ± 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.

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**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 ± 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...
of these genes (Fig. 6). Expression of VEGF-R1, VEGF-R2, NRP1, and NRP2 in J82 was further confirmed by immuno-staining with corresponding antibodies (Fig. 7A–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 specimens 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-

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**Fig. 6.** Quantitative (Q)-PCR of chromatin immunoprecipitation (ChIP)-based assays. J82 cells (10⁶ cells) were treated with saline or bacillus Calmette-Guérin (BCG; 8.1 × 10⁶ 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.
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

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.
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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17. Freeman MR, Schneck FX, Gagnon ML, Corless C, Sokrer S, Niknejad MA. vectoring 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.

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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