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Identification of Genes Involved in Human Urothelial Cell-Matrix Interactions: Implications for the Progression Pathways of Malignant Urothelium

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ABSTRACT

Interactions between epithelial cells and the extracellular matrix are central to tissue homeostasis and have a dynamic role in tissue remodeling and repair. Regulation of these pathways is balanced by positive and negative feedback elements, many of which have been implicated in the pathways of malignant progression. We have used differential display to identify genes that are up-regulated in normal human urothelial cells in response to exposure to extracellular matrix proteins (Matrigel) *in vitro*. This approach has identified genes that have key roles in cell-cell and cell-matrix interactions and that have been implicated in the progression of carcinomas of urothelial or other epithelial cell origins. One confirmed but unknown differentially expressed sequence was used to isolate a full-length gene, *MIG-C4*, from a human urothelial cDNA library. This gene was found to encode a novel urokinase plasminogen-activator receptor-like member of the Ly-6 family of glycosyl-phosphatidylinositol-anchored glycoproteins, and was identified as the human homologue of the rat metastasis-associated *C4.4A* gene. By *in situ* hybridization, *MIG-C4* was expressed variably in normal urothelium and intensely in the tumor component of some noninvasive superficial lesions and in invasive and metastatic urothelial cancers. Thus, our approach has identified previously nonimplicated gene products involved in normal urothelium-matrix interactions that could be tumor-invasion or suppressor-gene targets in the development of invasive and metastatic tumor phenotypes.

INTRODUCTION

Understanding the mechanisms by which malignant cells spread both locally and distantly is critical to the development of new strategies for cancer treatment. Many tumor suppressor genes have roles in normal cell regulatory processes, and suppression of these functions in neoplasia confers a survival advantage to the transformed cells. In the same way, many of the genes associated with malignant progression have roles in normal cell-cell and cell-matrix interactions, particularly in the context of wound healing, which requires cell proliferation and motility, followed by reconfiguration of normal tissue architecture. Neoplastic cells may use these normal processes to spread and metastasize. Therefore, unraveling the pathways involved in tissue homeostasis and repair, and testing their relevance to neoplasia, provides a novel approach to unraveling mechanisms of tumor progression. Tissue-specific mechanisms would be of particular interest in the development of targeted therapies.

The excretory pathways of the urinary tract are lined by urothelium. TCC[2] of urothelial cell origin is the sixth most common cause of cancer deaths of men in the Western world. Prognosis is strongly correlated with the tumor stage, determined by the extent of local invasion and spread to nodes and distant sites. Investigation of normal and malignant urothelial cells provides some evidence that neoplastic cells usurp normal cell mechanisms to progress. Normal urothelium has an exceptionally low cell-turnover rate but a high capacity for regeneration after injury (1, 2). When isolated from the basement membrane and placed in monoculture, NHU cells become highly proliferative and migratory (3), as in a wound response. Analysis of integrin expression has shown that NHU cells in culture show *de novo* expression of the $\alpha_5\beta_1$ integrin and relocalization of the $\alpha_6\beta_4$ integrin away from hemidesmosomes (4). These phenomena have also been demonstrated in TCC *in vivo* (5, 6). We, therefore, proposed to pursue the hypothesis that TCCs use parts of the urothelial wound healing response in malignant progression.

We have previously shown that manipulation of culture conditions, and particularly of the culture substratum, alters the behavior of both normal and neoplastic urothelial cells. NHU cells, grown on plastic, form a monolayer and fail to stratify, but, when recombined with a de-epithelialized stroma in organ culture, they revert to a stratified, differentiated low-turnover urothelium (7). By contrast, TCC cell lines show invasive behavior and a degree of response to stromal signaling, which is dependent on the grade of the originating tumor (8).

These experiments suggest that the interactions between the cell and the extracellular matrix are important in mediating the normal wound response and have a role in modulating tumor behavior. To dissect the molecular response of NHU cells to stromal signaling, we used differential display to identify genes up-regulated when NHU cells were seeded onto a Matrigel matrix, by comparison to autologous cells grown on plastic. In this study, we identified genes that are integral to the response repertoire of normal cells and investigated expression of a novel gene in clinical tumor samples. We show that this approach can detect genes involved in normal tissue homeostasis that are also relevant to the invasive and metastatic phenotype of TCC.

MATERIALS AND METHODS

Tissues. Surgical specimens of ureter and renal pelvis were obtained from patients with no history of urothelial dysplasia or malignancy. Tissues were collected in HBSS containing 10 mM HEPES (pH 7.6) and 20 KIU aprotinin (Trasylol; Bayer plc, Newbury, United Kingdom), as described previously (9, 10). Representative pieces from each tissue sample were processed into paraffin wax for immunohistology and *in situ* hybridization. The remaining sample was cut into ~1-cm^2 pieces, placed into HBSS (Ca^{2+} and Mg^{2+} free) supplemented as above and containing 0.1% (w/v) EDTA, and incubated at 4°C overnight to release the urothelium. The isolated urothelium was either used to initiate NHU cell lines or used to extract RNA.

Cell Culture. NHU cell lines were established from ureter ($n = 3$) and renal pelvis ($n = 1$), and maintained in keratinocyte serum-free medium, containing bovine pituitary extract and epidermal growth factor at the manufacturer's recommended concentrations (Life Technologies, Inc., Paisley, United Kingdom) and 30 ng/ml Cholera toxin (Sigma Chemical Company, Poole, United Kingdom). These methods have been described in detail elsewhere (10). NHU cell lines were used for these studies between passages 4 and 8.

Three established human TCC cell lines were used, comprising RT4,

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[2] The abbreviations used are: TCC, transitional cell carcinoma; NHU, normal human urothelial; EST, expressed sequence tag; RPA, RNase protection assay; ddRT-PCR, differential display reverse transcription-PCR; poly(A), polyadenylic acid; HTGS, high-throughput genome sequence; uPAR, urokinase plasminogen activator receptor.

Table 1 *Primer sequences used in ddRT-PCR*

Primer	Oligonucleotide sequence
Anchored primer	
$HT_{11}C$	AAGCTTTTTTTTTTTC
$HT_{11}A$	AAGCTTTTTTTTTTTA
$HT_{11}G$	AAGCTTTTTTTTTTTG
Arbitrary primer	
AP1	AAGCTTAACGAGG
AP4	AAGCTTTGGCTCC
AP6	AAGCTTCTCCGTC
AP9	AAGCTTGTCATAG
AP11	AAGCTTCTAAGCG
AP14	AAGCTTCAAGTCC
AP17	AAGCTTCTGACAC
AP20	AAGCTTCAATCGC
AP21	AAGCTTCTAACCG
AP22	AAGCTTCGCATTG

RT112, and EJ. These well-characterized cell lines show a range of phenotypes reflecting the urothelial origin and the different grades of cancer from which the lines were derived (11) and have been shown to have different invasive potentials on bladder stroma (8). For some experiments, the TCC-derived COLO232 cell line was also included. All of the TCC cell lines were maintained in a 1:1 mixture of RPMI 1640 and DMEM with 5% fetal bovine serum, as described previously (8).

Culture of NHU Cells on Matrigel. Matrigel (Becton Dickinson, supplied by Stratech Scientific, Luton, United Kingdom) was thawed on ice to coat tissue culture dishes at 100 μl/cm^2 and was polymerized at 37°C for 30 min. NHU cells were seeded onto Matrigel at an equivalent density of 5×10^4 to 1×10^5 cells/cm^2 and left for 1 h to attach before flooding with growth medium. Parallel cultures were seeded at equivalent densities onto plastic tissue culture dishes. Cultures were harvested for RNA extraction at 24- and 48-h time points.

Extraction of RNA. Isolated sheets of urothelium were homogenized in Ultraspec, and total RNA was extracted following the manufacturer's protocol (Biogenesis, Bournemouth, United Kingdom).

To extract RNA from Matrigel and control cell cultures, the growth media were aspirated and replaced with 8 ml of Ultraspec. Scrapers were used to detach the cells, and the suspensions were transferred to polypropylene centrifuge tubes. Total RNA was extracted following the manufacturer's protocol. At this stage, impurities from the Matrigel were still present, and it was found necessary to precipitate the RNA in 4 M lithium chloride, followed by additional ethanol precipitation and washes (12). The RNA from cells grown on plastic was treated in exactly the same way. DNA contamination was removed by treatment with a Message Clean kit (GenHunter Corporation, Nashville, TN) according to the manufacturer's protocol. All of the RNA preparations were assessed for quality on formaldehyde agarose gels and by A_{260}:A_{280} ratios.

RNA isolated from human brain, kidney, heart, liver, and lung tissues was obtained from Clontech Laboratories UK Ltd. (Basingstoke, United Kingdom).

ddRT-PCR. Differential display was performed with three one-base anchored oligodeoxythymidylate ("downstream") and 10 arbitrary 13-bp ("upstream") primers (see Table 1), as described previously (13). For each cDNA reaction, 200 ng of total RNA was heated to 65°C for 10 min with 50 pmol of $HT_{11}X$ (where H is a *Hin*dIII site and X is nucleotide A, C, or G), cooled on ice and subsequently reverse-transcribed in a 20-μl reaction containing $1 \times$ GeneAmp PCR buffer (Perkin-Elmer, Warrington, United Kingdom), 8 mM $MgCl_2$ (Perkin-Elmer), 20 μM each deoxynucleotide triphosphates (Pharmacia Biotech, St. Albans, United Kingdom) and 5 units of M-MuLV reverse transcriptase (Pharmacia Biotech) at 37°C for 60 min. Control reactions were included from which the reverse transcriptase was omitted. The reverse transcriptase was inactivated by heating at 95°C for 10 min. PCR amplification of 2 μl of each of the three first-strand cDNAs was performed in a 20-μl reaction volume containing 1× GeneAmp PCR buffer [1.5 mM $MgCl_2$, 20 μM dNTPs, 1 μCi [^{32}P]dATP (Amersham Pharmacia Biotech UK Ltd, Little Chalfont, United Kingdom)] and 1.25 units of AmpliTaq DNA polymerase (Perkin-Elmer). The corresponding $HT_{11}X$ primer and a single upstream primer were added to final concentrations of 2.5 μM each. PCR cycling conditions were 95°C for 2 min, followed by 40 cycles at 95°C for 30 s, 32°C for 2 min, and 72°C for 30 s, with a final incubation at 72°C for 5 min. Separation of the multiple PCR products was performed on 6% denaturing polyacrylamide gels. Gels were dried, marked with phosphorescent tape, and exposed overnight to X-ray film. Alignment of the X-ray film with the gel allowed each band of interest to be cut out with a clean scalpel and eluted by placing in 100 μl of TE [10 mM Tris, 1 mM EDTA (pH 8.0)] at 65°C overnight.

Five μl of each eluted DNA was reamplified using 40 pmol of the corresponding primers in a 100-μl reaction containing 1× GeneAmp PCR buffer II (1.5 mM $MgCl_2$, 200 μM dNTPs and 5 units of AmpliTaq).

Cloning and Sequencing of PCR Products. Reamplified PCR products were separated on 1% agarose gels, excised, and purified using β-Agarase 1 (BioLabs, Beverly, MA) according to the manufacturer's instructions. Approximately one-half of each product was TA-cloned into the *Sma*I site of pGEM 3Z vector. Up to 10 clones generated from each band were purified using a Plasmid Mini kit (Quiagen Ltd, Crawley, United Kingdom). Ten μl of each DNA was digested with *Hin*DIII followed by analysis on 2% agarose gels to estimate insert sizes. When all of the inserts from one ligation were the same size, initially two clones were sequenced. When more than one insert size could be seen, clones representing the alternative products were sequenced. Sequencing reactions were carried out by BigDye Terminator Cycle Sequencing (Perkin-Elmer, Warrington, United Kingdom) and the products were analyzed on an ABI Prism 377 DNA sequencer (ABI, Warrington, United Kingdom). Sequence homologies were determined by BLAST searches against DNA and EST databases.

RPAs. ^{32}P-antisense transcripts of the cDNAs of interest were generated from linearized plasmids using an *in vitro* transcription kit (Promega, Southampton, United Kingdom), according to the manufacturer's protocol. After DNase treatment, riboprobes were purified either by phenol-chloroform extraction or by passage through Chromaspin 30-DEPC columns (Clontech).

A 152-bp portion of the coding region of human GAPDH (accession no. M33197) cloned into *pGEM-T Easy* (Promega) was used as an internal riboprobe control (14). Approximately 2 fmol of each test and GAPDH control probe were mixed and hybridized to 5 μg of total RNA (test or yeast control) using an RPAII kit [Ambion; supplied by AMS Biotechnology (Europe) Ltd., Abingdon, United Kingdom], according to the manufacturer's protocols. Products were separated on 5% denaturing polyacrylamide gels (Sequagel; Flowgen, Lichfield, United Kingdom) and visualized either by autoradiography or analyzed by means of a phosphorimager (Bio-Rad GS-525 Molecular Imager System; Hemel Hempstead, United Kingdom).

Urothelial cDNA Library Screening. EST sequences were obtained as I.M.A.G.E. Consortium [LLNL] cDNA clones (Ref. 14a) from the United Kingdom Human Genome Mapping Project Resource Center, Cambridge, and DNA prepared from the clones was sequenced to confirm that they contained the expected sequences. The inserts were prepared by digestion with restriction enzymes *Eco*RI and *Not*I, followed by gel electrophoresis and purification using a Qiagen Gel Extraction kit (Qiagen). Radioactive probes were prepared from 25 ng of insert by incorporating [^{32}P]dCTP using the Rediprime kit (Amersham) according to the manufacturer's instructions. Each probe was used to screen a human urothelial cDNA library cloned in *pCDM8* (14).

The library was grown on 15-cm plates at a density of $\sim 5 \times 10^4$ colonies per plate. Colonies were lifted onto Hybond N+ membranes (Amersham) followed by denaturation and fixing according to the manufacturer's protocols. The membranes were prehybridized for 1 h at 65°C in 10 ml of Rapid-hyb buffer (Amersham), followed by the addition of probe and hybridization for 2.5 h. Posthybridization washes were carried out according to Amersham protocols. Colonies identified as positive by autoradiography were scraped into broth, plated at a lower density, and subjected to a second round of screening. Isolated, single positive colonies were amplified and sequenced on an ABI 377 automated sequencer.

Northern Blot. Aliquots (15 μg) of RNA were electrophoresed on a 1.3% (w/v) agarose/formaldehyde gel and blotted onto Hybond N membrane (Amersham). ^{32}P-probe was prepared using the Rediprime II random prime labeling system (Amersham), according to the manufacturer's instructions. Ten % (10^7 cpm) of the prepared probe were hybridized to the blot in ULTRAhyb buffer (Ambion), according to the manufacturer's instructions and the hybridization signal visualized by autoradiography.

RNA Dot Blot Hybridization. A human RNA master blot containing normalized loading of poly(A) + RNA from 50 different tissues was obtained from Clontech. Radioactive probe was prepared by incorporating [^{32}P]dCTP into 25 ng of gel-purified plasmid insert cDNA using the Rediprime II kit

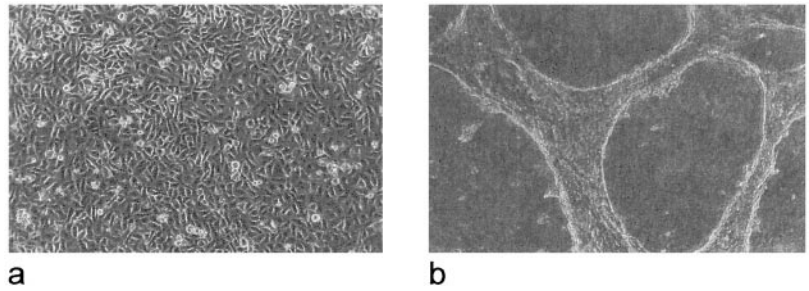


Fig. 1. Phase contrast microscopy of NHU cell cultures. On plastic, NHU cells displayed a typical epithelioid morphology and grew as a monolayer, forming a tightly packed cobblestone pavement at confluence (*a*). By contrast, NHU cells seeded onto Matrigel formed a stratified lattice over the matrix surface (*b*).

(Amersham) according to the manufacturer's instructions. Unincorporated nucleotides were removed by applying the probe to a Chromaspin100-TE column (Clontech). The RNA blot was prehybridized for 2 h at 65°C, hybridized overnight and washed using the recommended protocol (Clontech). The membranes were subjected to autoradiography and the density quantified on a phosphorimager (as above).

***In Situ* Hybridization.** *In situ* hybridization on paraffin wax-embedded sections using ^{35}S-labeled UTP-riboprobes was performed essentially as detailed previously (14). Briefly, 500 ng of linearized template was used to transcribe a ^{35}S-antisense riboprobe using an *in vitro* transcription kit (T7 Riboprobe System from Promega) and 2×10^6 cpm were hybridized against dewaxed, proteinase K-treated, and acetylated sections. After removal of nonbound probe, slides were dehydrated, coated in autoradiographic emulsion, and left for up to 20 days before development. A β-actin riboprobe was used to determine RNA transcript integrity within each tissue.

RESULTS

Morphology of NHU Cells Grown on Plastic and Matrigel. NHU cells grew on plastic as a pavemented epithelioid monolayer and remained evenly distributed over the surface of the substratum. At confluency, the cells remained nonstratified and adopted a typical epithelioid cobblestone morphology. NHU cells seeded onto a Matrigel matrix attached evenly over the substratum within 15 min of plating but within hours had coalesced to form a stratified lattice over the matrix surface (Fig. 1).

ddRT-PCR. The ddRT-PCR was performed on mRNA isolated from two independent NHU cell lines grown on plastic and Matrigel for 24 h (one cell line) or 24 and 48 h (one cell line). When a primer pair resulted in a differentially expressed band in both cell lines, the differential display reaction was repeated on a third independent cell line grown on plastic or Matrigel for 24 and 48 h. This strategy resulted in the identification of 24 bands, originating from 14 primer pairs, that showed consistent, increased intensity in cells grown on Matrigel relative to the plastic control. Several primer pairs produced more than one distinct differentially expressed band, and in several cases, bands were present as doublets or multiplets, separated by a few bp. The individual differentially expressed bands and multiplets were excised and cloned.

Up to six clones from each band or multiplet were sequenced and compared for homogeneity. The majority of bands produced clones that contained a single sequence. Clones from three bands were heterogeneous: two bands were each represented by two independent transcribed sequences, and two bands were contaminated by se-

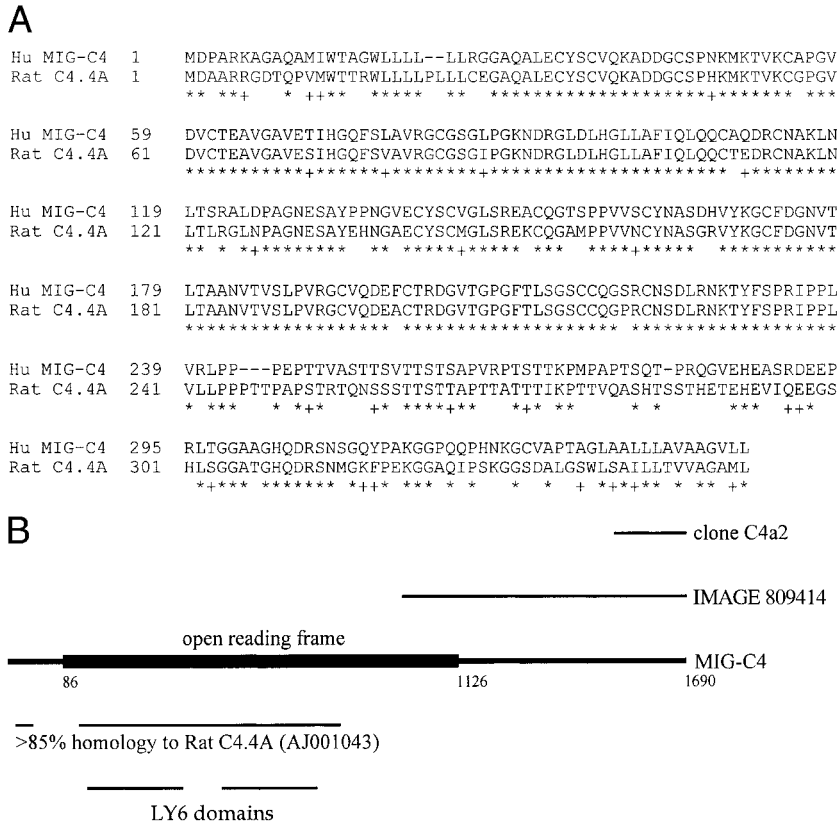
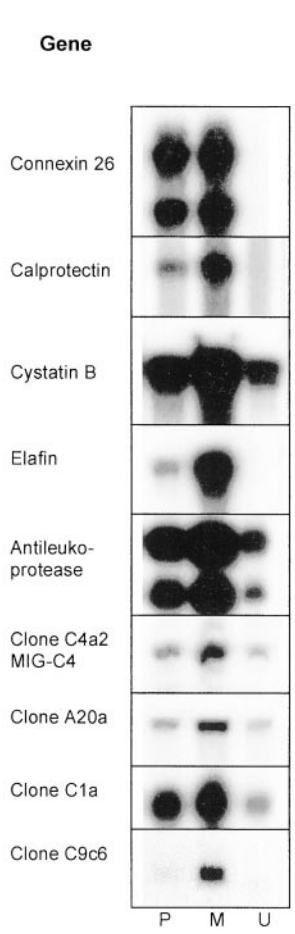


Fig. 2. Sequence homologies. *A*, amino acid sequence alignment illustrating homology between human MIG-C4 [346 amino acids (aa)] and the rat metastasis-associated glycosyl-phosphatidylinositol-anchored protein, C4.4A (352 aa). *, identical amino acids; +, conservative substitutions. *B*, DNA sequence. Alignment of differential display clone C4a2 against IMAGE 809414 used to isolate the full length *MIG-C4* gene from the human urothelial cDNA library. The regions are marked in the 5′ untranslated and in the open reading frame in which >85% homology was found between *MIG-C4* and the rat *C4.4A* metastasis-associated gene. Also shown are the positions of the cysteine loop domains characteristic of the Ly-6 protein family.



Gene	NHU on Plastic	NHU on Matrigel	Freshly isolated	RT4	RT112	EJ
Connexin 26	++	+++	-	(+)	(+)	-
Calprotectin	++	+++	-	-	-	-
Cystatin B	++	+++	++	++	++	+
Elafin	+	+++	-	-	-	-
Antileuko-protease	++	+++	+	(+)	++	-
Clone C4a2 MIG-C4	+	+++	+	+	+	-
Clone A20a	+	++	+	+	+	+
Clone C1a	++	+++	+	++	++	++
Clone C9c6	(+)	++	-	-	++	-

Fig. 3. Summary of mRNA expression in urothelial tissue and cell lines of gene sequences identified by differential display. Data were obtained by RPA. *P*, plastic; *M*, Matrigel; *U*, freshly isolated urothelium.

quences derived from genomic DNA. Analysis of the resultant clones for homology on the GenBank database showed them to represent 14 independent cDNA sequences. The apparent discrepancy of numbers was attributable to six genes being differentially amplified by more than one primer combination. For example, sequences homologous to Cystatin B were generated from four primer combinations using two upstream primers and either the A- or G-anchored dT11 primer. In each case the anchored primer had primed close to the poly(A) tail, but the upstream priming had occurred in different positions, giving overlapping sequences of between 150 and 330 bp. Similarly, an unknown sequence that matched EST AA459897 was isolated from three different primer combinations.

Confirmation of Differential Gene Expression. Five of the 14 independent sequences matched known genes. These were *connexin 26*, *calprotectin* (also known as macrophage migration inhibition factor, *MRP-14*, *L1*, or *calgranulin A*), *cystatin B*, *elafin*, and *antileukoprotease*. The other four clones showed homologies to sequences with unknown functions in human cells. Clone A20a showed homology to transcript ch138 previously isolated by differential display from stomach cancer cell lines (accession no. S77393). No function has been described for this transcript and a BLAST search on the GenBank database of HTGSs assigned it to a contig on chromosome 4 (accession no. AC021860). Clone C1a showed homology to a hypoxia-inducible gene (*HIG1*; accession no. AF145385) isolated from cervical cancer cells. Screening of the HTGS database showed homology to contigs on several chromosomes. Clone C9c6 showed homology to several EST clones that have been assigned to a contig (AC024502) on chromosome 9. These EST clones show homology to the adipophilin gene, and it is possible that our sequence lies within the 3′ untranslated region of adipophilin. Clone C4a2 showed good homology to the 3′ end of a rat GPI-anchored protein associated with metastasis (Ref. 15; Fig. 2*B*).

RPA analysis of the nine identified sequences confirmed differential gene expression in NHU cells grown on Matrigel *versus* plastic. The results of hybridization with these riboprobes on RNA extracted from freshly isolated urothelium and the RT4, RT112, and EJ cell lines grown on plastic or Matrigel are shown in Fig. 3. Four of the genes, *connexin 26*, *calprotectin*, *elafin*, and *clone C9c6*, were not expressed by freshly isolated urothelium and showed only limited expression on TCC-derived cell lines. Transcripts for *antileukoprotease* and *clone C4a2* were detected in freshly isolated urothelium and were differentially expressed in the three TCC cell lines (Fig. 3).

Isolation of a Novel Urothelial Gene. Screening of a human urothelial cDNA library with the ESTs that showed homology to clones A20a, C9c6, and C1a failed to identify positive clones. This may reflect the fact that the human urothelial cDNA library was constructed from mRNA pooled from normal quiescent urothelium, in which there was low expression of these transcripts (see Fig. 3).

EST AA459897, corresponding to IMAGE clone 809414, which showed identity at its 5′ end to clone C4a2, identified three positive clones after two rounds of screening. Sequencing showed that two of the clones were virtually identical but that the third had an insert of 300 bases toward the 5′ end that probably represented an unspliced intron, because it had consensus donor and acceptor sites at its ends. The full consensus sequence of this Matrigel-induced gene, *MIG-C4*, was submitted to GenBank (accession no. AF082889).

Analysis of the cDNA sequence of *MIG-C4* showed it to be 1690-bp long excluding the poly(A) tail, with an open reading frame

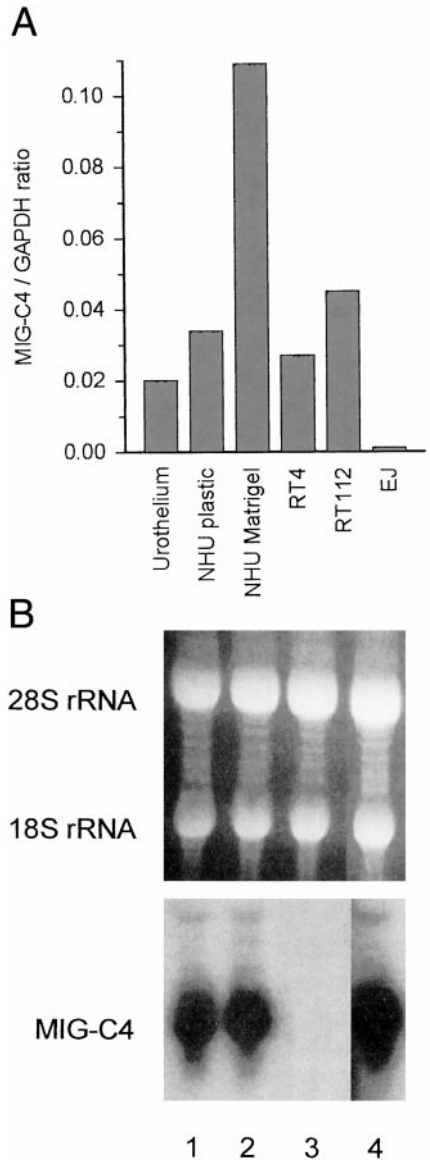


Fig. 4. Expression of MIG-C4 by normal and malignant urothelial cells. *A*, RPA of MIG-C4 expression in normal urothelium isolated from the stroma, compared with NHU cells grown on plastic or Matrigel and the RT4, RT112, and EJ bladder cancer cell lines. The results were quantified by phosphorimager and corrected for loading against the GAPDH hybridization signal. *B*, Northern blot showing ethidium bromide staining of rRNA as loading control (*upper panel*) and autoradiograph of hybridized MIG-C4 probe for RT4 (*Lane1*), RT112 (*Lane 2*), EJ (*Lane 3*), and NHU (*Lane 4*) cells (*lower panel*). MIG-C4 was absent from EJ cells, but present as a single mRNA species of about 1.9 kb in the other cells tested.

between bases 86 and 1123. This would produce a 346-amino acid sequence (calculated molecular weight of 35,971), with a polyadenylation signal sequence at base 1663, a hydrophobic COOH terminus, and six possible glycosylation sites. Signature analysis of the amino acid sequence revealed two tandem copies of the Ly-6 cysteine loop domain, the first of which lacked the fourth disulfide bond (Fig. 2).

A BLAST search against GenBank databases found homology with the previously described rat metastasis-associated GPI-anchored protein C4.4A (15). The homology was 85% at the nucleotide level, with 72% identity and 78% similarity at the amino acid level (Fig. 2).

Expression of the *MIG-C4* Gene by Cells and Tissues. A 774-bp *Pst*I-*Kpn*I fragment of the full length urothelial *MIG-C4* cDNA clone was subcloned into the equivalent sites in *pGEM3Z*, which facilitated production of a 588-bp riboprobe after linearization of the construct using the internal *Acc*I site. This riboprobe was used in RPA and confirmed the original analysis of expression using clone C4a2, namely that the gene was expressed in NHU, RT4, and RT112 cells; up-regulated in NHU cells on Matrigel; and absent from EJ cells (Fig. 4*A*). This was confirmed by Northern hybridization of the 774-bp fragment of *MIG-C4* cDNA. The transcript ran at a position close to 18S rRNA, which would agree with its estimated size of 1700-bp minus the poly(A) tail (Fig. 4*B*).

RPA analysis using RNA derived from urothelium, brain, kidney, heart, liver, and lung showed expression restricted to urothelium (Fig. 5). Hybridization of a dot blot of RNA from 50 human tissues with the 774-bp cDNA probe revealed high expression in placenta and trachea. Weaker signals were also seen with salivary gland, prostate, uterus, thymus, and whole bladder (not shown).

By *in situ* hybridization, the 588-bp *MIG-C4* riboprobe hybridized specifically to urothelium in sections of human ureter from four donors (Fig. 6). There was some variation in the hybridization signal on different specimens of normal urothelium, which ranged in intensity from weak to moderate. The *MIG-C4* probe did not hybridize against human liver, included as a negative control.

In five cases of noninvasive (pTa) TCC specimens, the *MIG-C4* riboprobe hybridized intensely and specifically to the tumor component of two grade 3 and one grade 2 TCCs, and was weak or negative, respectively, on two additional grade 2 TCCs.

In four cases of invasive TCC with local ($n = 1$) or distant ($n = 3$) lymph node metastases, the *MIG-C4* riboprobe showed a positive, tumor-specific reaction in both the primary and metastatic deposit (Fig. 6). The intensity was consistent between the primary tumor and metastatic deposits, and ranged from weak to strong intensity. In one of the cases, in which there was a concurrent prostatic adenocarcinoma, the *MIG-C4* riboprobe hybridized only against the TCC component. Two additional primary/metastatic tumor pairs did not provide interpretable results because of a high nonspecific background.

DISCUSSION

This study has illustrated that cell-matrix interactions can influence gene expression in NHU cells, and that these genes are not only relevant to urothelial tissue repair and remodeling but also have a potential role in malignant disease progression. Thus, studying normal tissue repair mechanisms has implicated six genes, *connexin 26*, *calprotectin*, *cystatin B*, *elafin*, *antileukoprotease*, and a novel gene (*MIG-C4*) in urothelial cell-matrix interactions.

The protein coded for by the *MIG-C4* gene has a hydrophobic COOH terminus common to GPI-anchored proteins (16). Analysis of the amino acid sequence revealed two conserved signature domains:

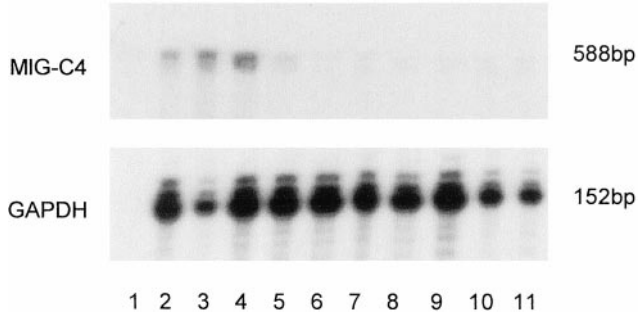


Fig. 5. Expression of MIG-C4 in cells and tissues. RPA was performed using a combination of probes to MIG-C4 and GAPDH. After RNase treatment, protected fragments were run on a 5% polyacrylamide gel. Autoradiograph shows hybridization to MIG-C4 (*upper panel*) and GAPDH (*lower panel*) with yeast total RNA control (*Lane 1*), NHU cells (*Lane 2*), bladder cancer cell lines (RT112, *Lane 4*; COLO232, *Lane 5*; EJ, *Lane 6*) and human tissues (urothelium, *Lane 3*; brain, *Lane 7*; kidney, *Lane 8*; heart, *Lane 9*; lung, *Lane 10*; liver, *Lane 11*).

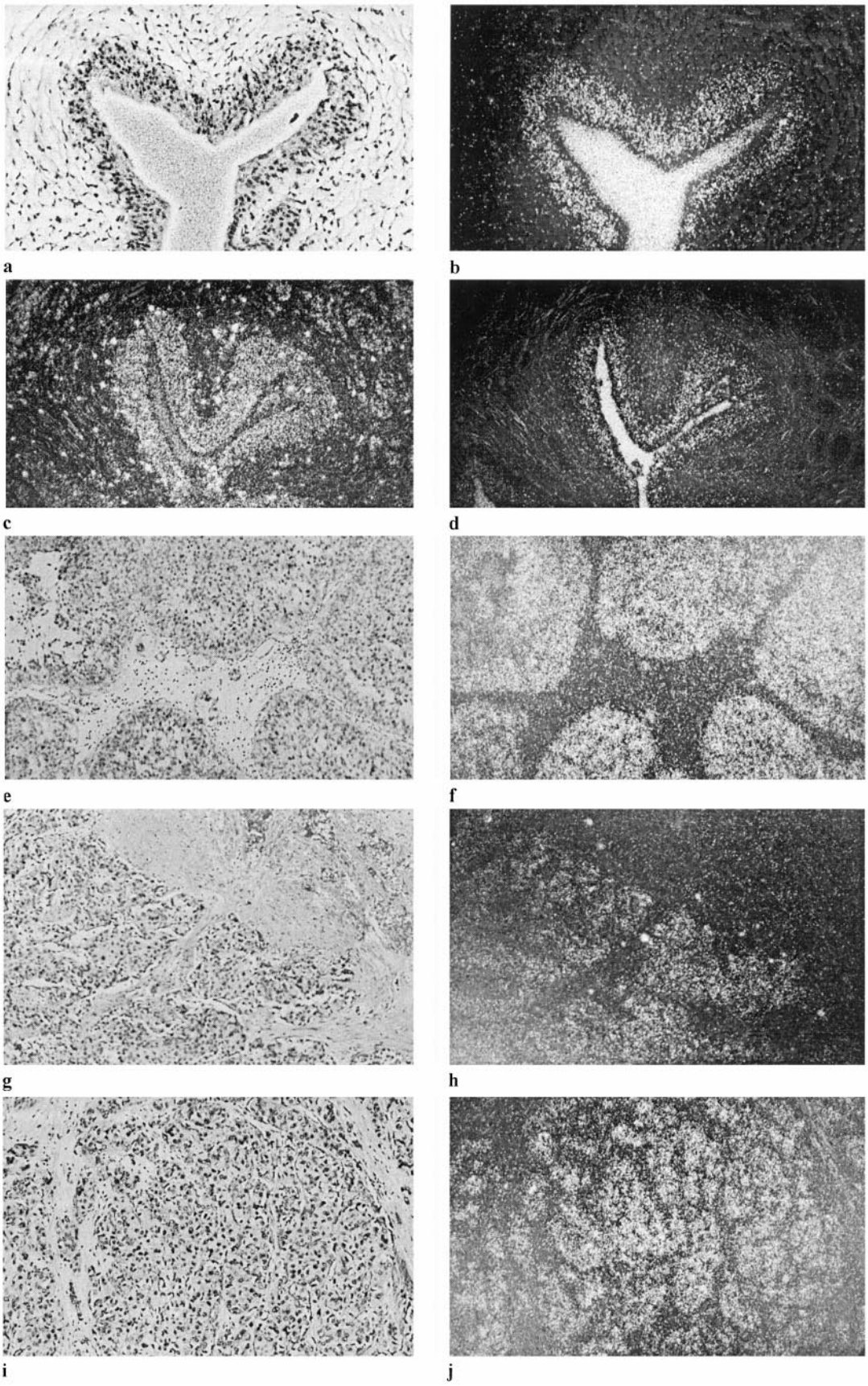


Fig. 6. *In situ* hybridization of MIG-C4 transcripts. Sections of human ureter (*a–d*) and TCC (*e–j*) were hybridized against ^{35}S-riboprobes and subjected to autoradiography. Sections were visualized by transmitted light to show histology (*a*, *e*, *g*, and *i*) and by epipolarized illumination to visualize the hybridization signal (*b*, *c*, *d*, *f*, *h*, and *j*). In normal urothelium, MIG-C4 transcripts were located throughout the urothelium, but considerable variation was found between specimens (*b* and *d*), which was not related to the quality of RNA, as shown by the 74-actin hybridization signal (*c*). In TCC, an intense and specific signal was found throughout the tumor component of some noninvasive superficial lesions (*e* and *f*), and in invasive (*g* and *h*) and metastatic (*i* and *j*) cancers.

an 85 amino acid domain distinct from the extracellular portion of the transforming growth factor β receptor and a 100-amino acid region containing two tandem copies of a cysteine loop domain. This latter identifies the protein as a member of the Ly-6 family of GPI-linked cell surface glycoproteins (17). The absence of the fourth disulfide bond from the first of the two cysteine loop domains is characteristic of the human uPAR, which is itself a member of the Ly-6 family. However, unlike the MIG-C4 protein, uPAR has three, not two, tandem copies of the cysteine loop domain (18).

The identified signature domains support a role for MIG-C4 in cell-matrix interactions, and the variable expression of MIG-C4 in sections of normal urothelium supports the likelihood that expression of the gene is inducible *in vivo*. The massively increased expression of the gene seen in the majority of primary and metastatic TCCs cannot alone be taken to imply a direct causal association between overexpression and TCC progression, because it may simply reflect the presence of a high concentration of inducing factor. Nevertheless, it may provide a valuable disease marker. Indirect evidence for a causal link in tumor progression can be found from studies with the homologous rat *C4.4A* gene, which encodes a GPI-anchored protein of 352 amino acids (15). *C4.4A* expression in rat pancreatic carcinoma cells was associated with enhanced migration through Matrigel and decreased encapsulation of metastases in rats (15).

In quiescent urothelium *in situ*, *connexin 26*, *calprotectin*, and *elafin* transcripts were absent, and the other genes identified by ddRT-PCR were detectable only at a low level. This suggests that our strategy has identified genes that constitute part of the repertoire of inducible wound response genes in urothelium. Of the identified genes, only *connexin 26* has previously been reported to be expressed by urothelium (19). Nevertheless, several of the identified genes have recognized roles in cell-cell and cell-matrix interactions, including *connexin 26*, *cystatin B*, and *elafin*.

Interactions between epithelial cells and the stroma during wound repair are highly coordinated processes resulting in tissue remodeling and epithelial restitution. We suggest that malignant cells use the same mechanisms as normal cells to remodel the stromal matrix but lack some of the normal feedback constraints. Thus, identification of gene expression changes associated with normal urothelial cell-matrix interactions may reveal the mechanisms of TCC tumor invasion and/or provide markers of tumor progression. In addition to the possible role of *MIG-C4*/*C4.4A* in tumor spread discussed above, several other of the genes identified as differentially up-regulated by NHU cells on Matrigel have been implicated as invasion or metastasis suppressor genes in carcinomas. This includes *connexin 26*, loss of which has been reported in invasive TCC tumors and cell lines (19, 20). Cystatin B is a cysteine-proteinase inhibitor, which mainly inhibits cathepsin L. The expression of cystatin B is markedly decreased in esophageal carcinomas, and this loss has been associated with advanced stage and lymph node metastasis (21). By contrast, increased activity of cathepsin L has been reported in a number of human carcinomas (22–27). Elafin is an inhibitor of elastase and is also known as skin-derived antileukoproteinase (SKALP). Elafin is not expressed by normal epidermis but is expressed by keratinocytes under hyperproliferative conditions, such as wound healing, psoriasis, and in cell culture. The role of elafin may be to prevent proteolytic degradation of elastic tissues during tissue remodeling (28), and loss of elafin expression has been associated with carcinomas of skin and breast (29, 30), which suggests a possible role in tumor spread. One study has shown elastase activity in human bladder cancer cell lines derived from high-grade invasive tumors (31).

In conclusion, we have identified previously nonimplicated gene products involved in normal urothelium-matrix interactions that could be tumor invasion or suppressor gene targets in the development of invasive and metastatic tumor phenotypes. Furthermore, the identification of cystatin B (a cysteine protease inhibitor) and the serine proteinase inhibitors, elafin and antileukoprotease, raises the possibility that this approach may be useful in identifying novel protease inhibitors.

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