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Profiling Bladder Cancer Organ Site-Specific Metastasis Identifies LAMC2 as a Novel Biomarker of Hematogenous Dissemination

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Little is known about which genes mediate metastasis in bladder cancer, which accounts for much of the mortality of this disease. We used human bladder cancer cell lines to develop models of two clinically common metastatic sites, lung and liver, and evaluated their gene expression with respect to human tumor tissues. Parental cells were injected into either the murine spleen to generate liver metastases or tail vein to generate lung metastases with sequential progeny derived by re-injection and comparisons made of their organ-specific nature by crossed-site injections. Both genomic and transcriptomic analyses of organ-selected cell lines found salient differences and shared core metastatic profiles, which were then screened against gene expression data from human tumors. The expression levels of laminin V gamma 2 (LAMC2) contained in the core metastatic signature were increased as a function of human tumor stage, and its genomic location was in an area of gain as measured by comparative genomic hybridization. Using immunohistochemistry in a human bladder cancer tissue microarray, LAMC2 expression levels were associated with tumor grade, but inversely with nodal status. In contrast, in node-negative patients, LAMC2 expression was associated with visceral metastatic recurrence. In summary, LAMC2 is a novel biomarker of bladder cancer metastasis that reflects the propensity of cells to metastasize via either lymphatic or hematogenous routes. (*Am J Pathol* 2009, 174:371–379; DOI: 10.2353/ajpath.2009.080538)

Metastasis represents a critical event in the natural history of disease for a cancer patient and is associated with significant reduction in patient survival.¹ However, although a significant body of research has shed light on this process, less is known about the nature of organ-specific metastasis.^{2,3} *In vivo* metastasis assays using cancer cell lines passaged through mouse models have been used to discover the role of specific genes relevant to metastatic behavior.⁴ For example, in breast cancer, recent reports have addressed the question of the molecular regulation of organ site-specific metastasis. Using the spontaneously metastatic MDA-MB-231 human breast cancer cell line, one group reported the *in vivo* selection of sublines of differing metastatic potential to bone, lung, and adrenal gland,^{5,6} and then evaluated in both animal models and metastatic tumor tissues from patients the gene expression profiles associated with site-specific metastasis.⁷

Bladder cancer is the fifth most commonly diagnosed cancer in the United States,⁸ and the molecular lesions characterizing bladder carcinogenesis and progression are beginning to be better elucidated.⁹ However, little is known about the pathways that regulate general metastatic propensity or organ site-specific tropism to lung or liver, which are two common sites of dissemination in this and other tumor types. Our prior work has focused on the discovery and investigation of genes that regulate lung metastasis, such as the metastasis suppressor *RhoGDI2*, by comparing metastatic T24T cells to their isogenic nonmetastatic relative, T24.^{10–12} We have extended this work by generating progressively more lung metastatic T24T-derivative cell lines to identify additional lung me-

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tastasis promoting or suppressing genes.¹³ Here, we further extend this latter model and additionally develop a new T24T isogenic liver metastasis model to identify genes associated with the general metastatic phenotype as well as those with specific organ tropism. Data derived from these models was then evaluated in the context of gene expression data from human bladder tumors, to identify genes with a high likelihood of clinical relevance in human urothelial cancer.

Materials and Methods

Derivation of Metastatic Cell Lines and Crossed Site Experiments

The T24T cell line and culture protocols have been described.^{10,11,14} Six-week-old nude mice were purchased from National Cancer Institute (Bethesda, MD) and were maintained under University of Virginia Animal Care and Use Committee guidelines. To generate cell lines with propensity for liver metastasis, mice were injected with 1×10^6 T24T cells in 100 μ l of serum-free Dulbecco's modified Eagle's medium into the splenic capsule. Mice were sacrificed when weight loss was >20% when compared with that for control animals. At necropsy, visible liver metastases were cultured *in vitro* as described.¹³ The first liver metastatic derivative cell line was named SL1 (spleen to liver), and cells were maintained in culture for as short a time as possible before splenic re-injection, usually <1 week. This protocol was repeated three times to yield SL2, SL3, and SL4. Sequential tail vein injections generated increasingly lung metastatic T24T derivatives FL1, FL2, and FL3 (from lung) as reported.¹³ Here we extended this protocol to generate a fourth-generation cell line, FL4, to enable comparison with the equivalent liver metastasis-derived line, SL4.

To examine whether these cell lines retained any organ site-specific growth properties, 1×10^6 FL4 or SL4 cells were injected into the tail vein of 6-week-old nude mice. At 5 weeks, mice were necropsied, organs harvested, and visible metastases in the lungs were counted. In a parallel experiment, the same cells were injected into the splenic capsule, with subsequent necropsy and evaluation for hepatic metastasis. These experiments were repeated and pooled metastasis counts averaged, with differences in average metastatic counts evaluated by Student's *t*-test, two tailed. To scale out any nonorgan-specific difference in metastatic ability between the cell lines, we then normalized the counts of metastases to either the liver or lung to the same cell line's average number of metastases across both the liver and lung sites. Lung and liver tissues were formalin-fixed for subsequent immunohistochemical study as described below.

Comparative Genomic Hybridization (CGH) Analyses

CGH analysis with confirmation using a 99% confidence interval was performed as reported.¹⁵ Peripheral blood

from a healthy donor was used as a negative control, and a gastric tumor with known copy number changes was used as a positive control, with 1.17- and 0.85-fold cutoffs for gains or losses and a ≥ 1.5 -fold cutoff for high-level amplification.

Oligonucleotide Microarray Analysis of Cell Lines and Human Tumors

We used Affymetrix (Santa Clara, CA) HG-U133A to analyze mRNA expression in bladder cancer cell lines and human tissues including the T24T and FL1 to FL3 derivatives.^{11,13,16} Here, we used HG-U133A arrays in duplicate for each cell line, to analyze SL1 to SL4 cell lines. We also re-analyzed all of FL1 to FL3 and the newly generated FL4 derivative on the same platform, in parallel to the SL series. Ontological analysis of genes discovered in these models was performed using Ingenuity (Redwood City, CA) Pathway Analysis 5.5. HG-U133A profiling of human bladder cancers ($n = 65$) and normal bladder mucosa ($n = 15$), included 30 stage Ta, 5 Tis, 6 T1, 5 T2, 9 T3, and 10 T4 tumors as reported.¹⁷

Immunohistochemistry

Immunohistochemistry on human tumors was performed with approval of the University of Virginia Institutional Review Board. A tissue microarray of quadruplicate cores of 199 bladder cancers from patients who underwent radical cystectomy was constructed using standard techniques.¹³ This tissue microarray included 3 stage Ta, 7 stage T1, 61 stage T2, 93 stage T3, and 33 stage T4 samples. Tissue microarray sections were stained for Laminin V gamma 2 (LAMC2) using the monoclonal antibody clone D4B5 (Millipore, Billerica, MA) and enzymatic treatment with Subtilisin A (Sigma, St. Louis, MO), as described.¹⁸ This antibody detects dimeric LAMC2, monomeric LAMC2, and the processed protein.¹⁹ Staining was scored as low if $\leq 30\%$ of tumor cells stained positively or high if $>30\%$ cells stained positively. A superficial bladder tumor was used as a positive control, whereas normal bladder mucosa was used as a negative control for antibody workup. Associations between LAMC2 staining (high or low) on the tissue microarray and tumor grade (G2 to G4), nodal status (N0 versus N1+), and presence or absence of lymphovascular invasion as reported on the specimen's original pathology report were tested with χ^2 tests of association, and disease recurrence with the log-rank test. For immunohistochemical staining of *in vivo* metastases from FL and SL cells, formalin-fixed tissues from the murine metastasis experiments were paraffin-embedded, and the same LAMC2 staining procedure as above was used excepting the use of a mouse-on-mouse treatment kit (Vector Laboratories, Burlingame, CA) per the manufacturer's instructions.

Figure 1. **A:** Metastatic cell lines. T24T cells, a poorly metastatic derivative cell line of the transitional cell carcinoma cell line, T24,¹² were injected into the tail vein (**left**) or spleen (**right**) of nude mice, forming metastases in the lung or liver, each of which is the first capillary bed encountered by the venous drainage of the injection site. Animals were autopsied, and metastases were cultured *in vitro*, yielding the FL1- and SL1-derivative cell lines, respectively, which were each re-injected through a total of four passages yielding FL2 to FL4 (red) and SL2 to SL4 (green). **B** and **C:** Comparison of cell lines *in vivo*. FL4 and SL4 cell lines were injected into the tail vein (**B**) or spleen (**C**) in parallel animals to compare their organ site-specific metastatic ability. After 5 weeks, animals were necropsied and metastases counted as reported before. **D:** Specific and shared probes. A Venn diagram showing counts of microarray probes serially up- or down-regulated in the liver (green) or lung metastasis (red) models with both specific and shared expression profiles. Probes ($n = 93$) were implicated in the models, including 60 (48 up-regulated, 12 down-regulated) in the liver model, 23 (18 up-regulated, 5 down-regulated) in the lung model, and 10 (8 up-regulated, 2 down-regulated) shared between the two. The diagram shows numbers of probes, up- and down-regulated, overlapping between these models, and muscle invasive (stage T2 to T4) human bladder cancer tissues.¹⁷ **E:** CGH analysis. Both the FL4 and SL4 cell lines showed the same areas of chromosomal gains, compared with parental T24T cells. Areas of shared gains are shown shaded both red and green to highlight their being common to both models.

Results

Organ-Selected T24T-Derivative Cell Lines Maintain Tropic Metastatic Behavior

After generation of increasingly metastatic T24T-derived liver (SL1 to SL4) and lung (FL1 to FL4) metastatic cell

lines by *in vivo* selection (Figure 1A), we sought to determine whether such cells could retain their organ-specific growth properties. We tested their metastatic competence side by side in the liver or the lung to see if the cell line not selected for growth in a specific organ would have reduced numbers of metastases at that site. These experiments involved injecting 1 million FL4 or SL4 cells via tail vein or splenic capsule of 10 mice, with animals necropsied at 5 weeks and visible metastases counted.¹³ Comparing the lung metastatic abilities of FL4 and SL4, we found that FL4 is 10.1-fold more lung metastatic than SL4 ($P = 0.004$). At the liver site, we found that FL4 and SL4 showed no difference in numbers of metastases ($P = 0.86$), both consistent with our observation that *in vivo* lung selection has resulted in a FL4 line that is, on average, 2.7-fold more metastatic than SL4 ($P = 0.02$). To correct for the different general metastatic behaviors of these cell lines and begin to evaluate the fold of organ-specific ability of these cell lines, we normalized average metastases for a cell line at a particular organ site by the cell line's average over both lung and liver sites. Although this calculation does not explain why serial lung metastatic selection of FL4 resulted in a derivative cell line that has gained 2.7-fold in metastatic ability at either site compared with SL4, it does allow scaled comparison of the organ-specific metastatic ability of these cell lines. We thus found that FL4 is 3.8-fold more lung-specific than SL4, whereas SL4 is 2.4-fold more liver-specific than FL4. Figure 1, B and C, illustrates these comparison experiments, whereas Supplementary Figure 1, see <http://ajp.amjpathol.org> plots the metastasis experiment data.

Transcriptional Profiling of Organ-Specific Metastatic Human Bladder Cancer Cell Lines

To determine whether differential expression of genes was associated with the *in vivo* selection and derivation of these cells, we evaluated our series of lung-selected FL and liver-selected SL cell lines, using the Affymetrix HG-U133A microarray platform.¹¹ Proceeding from the hy-

Table 1a. Top Probe Sets Progressively Up-Regulated in the Liver Model Only

Probe	Symbol	T24T	SL1	SL2	SL3	SL4	Fold [†]	P value [‡]
201858_s_at	PRG1	10.4	196.4	2589.5	3087.5	1577.5	152.4	0.03
209942_x_at	MAGEA3*	7.8	91.8	334.6	672.8	1136.4	145.7	<0.00001
214612_x_at	MAGEA6*	12.2	70.7	214.6	526.0	918.7	75.6	0.0002
211506_s_at	IL8	21.7	374.4	1783.7	1605.8	1009.2	46.5	0.003
210118_s_at	IL1A	13.4	109.8	1474.4	1461.9	559.4	41.9	0.0003
201859_at	PRG1	29.3	264.8	1890.5	2153.9	1202.9	41.1	0.01
209774_x_at	CXCL2	24.7	187.6	2361.9	2134.6	863.1	34.9	0.03
221730_at	COL5A2*	16.3	30.8	200.3	419.1	549.6	33.7	0.0004
210248_at	WNT7A	5.0	36.2	159.2	123.0	123.9	24.8	0.0004
204470_at	CXCL1	261.4	1618.3	7118.8	7313.8	5657.0	21.6	0.0002
221729_at	COL5A2*	23.9	40.0	155.7	480.3	507.7	21.2	<0.00001
210298_x_at	FHL1	17.4	85.4	311.8	411.8	300.1	17.3	0.002
202859_x_at	IL8	118.6	619.3	3594.3	3605.1	2004.7	16.9	0.003
219630_at	PDZK1IP1	20.8	74.1	1466.3	1046.3	318.2	15.3	0.007
201508_at	IGFBP4	49.0	192.1	1220.4	1125.5	615.1	12.6	0.05

*Indicates probes also overexpressed in muscle invasive stage T2+ bladder cancers, $P = 0.05$.

[†]Calculated as average expression in SL4 cells divided by average expression in T24T cells.

[‡]t-Test for comparison of duplicate measures of mRNA expression in T24T cells versus SL4 cells.

Table 1b. Top Probe Sets Down-Regulated Progressively in the Liver Model Only

Probe	Symbol	T24T	SL1	SL2	SL3	SL4	Fold [†]	P value [‡]
205081_at	<i>CRIP1</i>	317.9	240.7	11.7	7.5	5.1	-62.9	0.02
221501_x_at	<i>LOC339047*</i>	940.7	345.1	265.6	341.3	90.8	-10.4	0.001
220779_at	<i>PADI3</i>	270.3	143.5	30.0	51.2	39.3	-6.9	0.006
212233_at	<i>3'UTR*</i>	414.3	135.8	55.4	44.0	65.1	-6.4	0.002
209081_s_at	<i>COL18A1</i>	951.5	415.9	157.6	144.7	169.4	-5.6	0.0009
209082_s_at	<i>COL18A1</i>	727.7	365.5	169.3	165.0	180.1	-4.0	0.001
209118_s_at	<i>TUBA3</i>	671.4	401.0	172.3	154.2	170.7	-3.9	0.004
209784_s_at	<i>JAG2</i>	453.7	280.3	117.5	148.9	131.7	-3.4	0.007
212484_at	<i>FAM89B</i>	207.1	165.8	123.9	95.7	77.7	-2.7	0.01
209485_s_at	<i>OSBPL1A</i>	235.2	205.6	105.7	88.0	88.7	-2.7	0.01
215016_x_at	<i>DST*</i>	377.9	303.0	192.7	184.4	150.1	-2.5	0.0005
201467_s_at	<i>NQO1</i>	157.8	151.3	91.2	133.3	66.5	-2.4	0.02

*Indicates probes also underexpressed in muscle invasive stage T2⁺ bladder cancers, *P* = 0.05.

[†]Calculated as negative reciprocal of average expression in SL4 cells divided by average expression in T24T cells.

[‡]t-Test for comparison of duplicate measures of mRNA expression in T24T cells versus FL4 cells.

pothesis that progressive changes in gene expression as a function of progressive rounds of *in vivo* passage are likely to be biologically relevant, we searched for genes increasingly up-regulated or down-regulated through the four generations of *in vivo* metastatic selection that produced the FL and SL series. Using the criteria that probes are expressed at the end of the *in vivo* selection process (microarray expression value >100 in either FL4 or SL4); are differentially expressed after the first round of *in vivo* metastatic selection compared with the parental cells (FL1 or SL1 > or <T24T); and are further changed more than twofold in the same direction throughout the selection process (FL4/FL1 >2 or <0.5 and or SL4/SL1 >2 or <0.5), we found a union of 93 probes for the two models. This set included a shared core metastatic differential expression profile between the liver- and lung-selected cells (10 probes: 8 up, 2 down) as well as organ site-specific probes (48 up, 12 down in the SL series; 18 twofold up and 5 twofold down in the FL series). Tables 1a, Table 1b, Table 2a, Table 2b, Table 3a, and Table 3b list the top 15 probes with the largest fold changes and *P* ≤ 0.05 (for complete list see Supplementary Table 1, see <http://ajp.amjpathol.org>).

Using Ingenuity Pathway Analysis of probes associated with either liver or lung metastasis, we found significant enrichment of several ontological classes of genes, including those involved in cell movement, signaling, and growth. When evaluated separately, the liver model showed enrichment of genes related to hematological, lymphatic, and immune response, whereas the lung model showed enrichment of genes related to cellular motility, proliferation, or suppression of apoptosis.¹³ Table 4 provides a comparison of the top 10 ontological classes for the liver-specific probes with comparison with lung-specific probes, indicating that, in some cases, similar types of (although not the same) genes were present in both models.

Comparative Genomic Analysis of Liver-versus Lung-Selected Cell Lines

To determine whether similarities or differences existed between genomic changes present in the SL and FL models compared with their parental T24T cell lines, we performed chromosomal CGH analysis. We reported pre-

Table 2a. Top Probe Sets Progressively Up-Regulated in the Lung Model Only

Probe	Symbol	T24T	FL1	FL2	FL3	FL4	Fold [†]	P value [‡]
201839_s_at	<i>TACSTD1*</i>	15.4	17.4	191.8	221.9	243.6	15.8	0.05
205428_s_at	<i>CALB2</i>	89.5	223.4	1682.4	853.9	1259.1	14.1	0.02
215856_at	<i>CD33L3</i>	19.0	26.0	111.3	113.5	154.9	8.2	0.02
209873_s_at	<i>PKP3*</i>	89.5	317.9	578.1	602.7	708.4	7.9	0.0009
209872_s_at	<i>PKP3*</i>	24.3	72.4	185.8	147.2	175.8	7.2	0.01
212062_at	<i>ATP9A</i>	27.2	66.4	144.5	144.0	147.6	5.4	0.01
212012_at	<i>PXDN</i>	467.3	880.5	2629.7	1595.9	2135.2	4.6	0.003
212013_at	<i>PXDN</i>	209.8	318.3	1030.5	580.0	895.6	4.3	0.02
209135_at	<i>ASPH</i>	85.8	114.7	335.5	342.0	329.7	3.8	0.01
211003_x_at	<i>TGM2*</i>	208.5	229.4	528.1	922.5	800.0	3.8	0.0009
201042_at	<i>TGM2</i>	710.3	941.8	2178.9	3257.2	2616.5	3.7	0.05
209457_at	<i>DUSP5</i>	208.5	276.2	768.1	478.4	587.2	2.8	0.008
206665_s_at	<i>BCL2L1*</i>	83.7	110.8	296.0	253.7	222.2	2.7	0.008
212473_s_at	<i>MICAL2</i>	506.0	512.4	1024.8	844.2	1331.6	2.6	0.05
212472_at	<i>MICAL2</i>	376.6	425.0	739.2	633.0	918.1	2.4	0.04

*Indicates probes also overexpressed in muscle invasive stage T2⁺ bladder cancers, *P* = 0.05.

[†]Calculated as average expression in FL4 cells divided by average expression in T24T cells.

[‡]t-Test for comparison of duplicate measures of mRNA expression in T24T cells versus FL4 cells.

Table 2b. Top Probe Sets Progressively Down-Regulated in the Lung Model Only

Probe	Symbol	T24T	FL1	FL2	FL3	FL4	Fold [†]	P value [‡]
203789_s_at	<i>SEMA3C</i>	751.8	726.8	341.3	333.9	246.8	-2.9	0.003
204474_at	<i>ZNF142</i>	159.3	127.9	76.6	111.7	58.6	-2.2	0.05
218726_at	—	996.4	961.4	502.0	578.8	479.1	-2.0	0.02
213258_at	<i>TFPI*</i>	591.9	449.1	229.1	248.3	224.1	-2.0	0.006

*Indicates probes also underexpressed in muscle invasive stage T2⁺ bladder cancers, $P = 0.05$.

[†]Calculated as negative reciprocal of average expression in FL4 cells divided by average expression in T24T cells.

[‡]t-Test for comparison of duplicate measures of mRNA expression in T24T cells versus SL4 cells.

viously that the lung metastasis-derived FL cell lines exhibited similar baseline changes compared with T24T, with progressive accumulation of additional gains and high-level amplifications, including gains at 1q, 3q, 7q, 8q, 9q, 12q, 17q, and high-level amplification of 20q by the third generation FL3 derivative.¹⁵ By comparison, the liver metastasis-derived SL cell lines showed fewer total changes to that for the T24T cell line than the FL series, lacking gains at 8q and 9q. However, in several instances the same genomic changes occurred in the SL series, including gains at 1q, 3q, 7q, 12q, 17q, and 20q amplification. Figure 1E shows the gains shared between the models.

We previously showed that the 17q gain reported in the lung metastasis model was associated with the differential expression of eight genes within the region, constituting a gene expression hotspot.¹⁵ Here, we found that several of the core metastatic genes common to both the liver and lung models (see Table 3a) were located in regions of chromosomal gains, including the laminin V gamma 2 subunit (*LAMC2*) and the KiSS-1 metastasis suppressor (*KISS1*) in the 1q region, as well as zinc finger, BED-type containing 2 (*ZBED2*) at the 3q locus.

Expression of Metastasis-Associated Probesets in Human Bladder Tumors

To focus on genes likely to be functionally relevant in human bladder cancer, we screened the candidate probesets discovered in this study against microarray expression data from 24 stage T2 to T4 muscle invasive bladder cancers.¹⁷ Using the criterion that probes discovered in either the liver or lung metastasis models must also be significantly differentially expressed in the same direction for invasive bladder cancers as compared with normal bladder mucosa ($P < 0.05$), we found that 16 of

the 48 probes up-regulated in the SL model and 3 of the 12 probes down-regulated were also significantly up- or down-regulated in muscle invasive bladder cancer. In the FL model, 5 of 18 probes up-regulated and 2 of 5 probes down-regulated were also similarly expressed in human tissues. Finally, of the eight probes up-regulated in both the liver and lung models, three probes met the aforementioned criterion for overexpression in invasive bladder tumors: annexin A3 (*ANXA3*), vesicle-associated membrane protein 8 (*VAMP8*), and *LAMC2*. Figure 1D shows the overlaps between the liver and lung model probes and human tumors.

Immunohistochemical Evaluation of *LAMC2* Expression in Human Bladder Cancer

The convergence of data from CGH and gene expression analysis in both xenograft models and human tumors identified *LAMC2* as a potential biomarker of metastasis in human bladder cancer. Hence, we used immunohistochemistry¹⁸ to compare *LAMC2* protein expression with salient clinicopathological parameters in human bladder cancer, including metastatic recurrence. First, we examined FL4 and SL4 lung and liver metastases and found that in all cases, xenograft tumors strongly expressed *LAMC2* protein (Figure 2, A and B). In our tissue microarray composed of 199 human bladder cancers (Figure 2, C and D), we found that *LAMC2* staining was significantly associated with tumor grade ($P = 0.0003$). Interestingly, despite our discovery of *LAMC2* as an overexpressed gene shared between liver and lung metastasis, we found that *LAMC2* staining was inversely associated with nodal metastasis ($P = 0.005$). Given this finding, we evaluated the association of *LAMC2* staining level from the tissue microarray with presence or absence of lymphovascular invasion on the case's original pathological

Table 3a. Top Probe Sets Progressively Up-Regulated in Both Liver and Lung Models

Probe	Symbol	T24T	SL4	SL fold [†]	P value [‡]	FL4	FL fold [†]	P value [‡]
205563_at	<i>KISS1</i>	4.1	336.8	82.1	0.0049	130.5	31.8	0.01
202267_at	<i>LAMC2*</i>	44.4	1465.6	33.0	0.0004	1327.0	29.9	0.003
209369_at	<i>ANXA3*</i>	18.0	368.4	20.5	0.1735	263.1	14.7	0.005
219836_at	<i>ZBED2</i>	56.2	676.0	12.0	0.0717	570.1	10.1	0.01
202546_at	<i>VAMP8*</i>	20.7	237.2	11.5	0.0088	460.5	22.2	0.002
207111_at	<i>EMR1</i>	35.0	257.6	7.4	0.0026	227.7	6.5	0.004
210896_s_at	<i>ASPH</i>	73.2	479.2	6.5	0.0167	373.6	5.1	0.006
203438_at	<i>STC2</i>	75.3	431.0	5.7	0.0110	233.7	3.1	0.005

*Indicates probes also overexpressed in muscle invasive stage T2⁺ bladder cancers, $P = 0.05$.

[†]Calculated as average expression in FL4 or SL4 cells, as indicated, divided by average expression in T24T cells.

[‡]t-Test for comparison of duplicate measures of mRNA expression in T24T cells versus SL4 or FL4 cells as indicated.

Table 3b. Probe Sets Progressively Down-Regulated in Both Liver and Lung Models

Probe	Symbol	T24T	SL4	SL fold*	P value [†]	FL4	FL fold*	P value [†]
218718_at	<i>PDGFC</i>	926.8	228.2	−4.1	0.0013	206.1	−2.6	0.003
206765_at	<i>KCNJ2</i>	436.4	46.3	−9.4	0.0202	56.2	−7.6	0.01

*Calculated as negative reciprocal of average expression in SL4 or FL4 cells, as indicated, divided by average expression in T24T cells.
[†]t-Test for comparison of duplicate measures of mRNA expression in T24T cells versus SL4 or FL4 cells as indicated.

examination, finding that it was also inversely associated, approaching statistical significance ($P = 0.08$). Lympho-vascular invasion is considered a harbinger of nodal metastasis,¹⁹ thus further supporting the inverse correlation of LAMC2 staining with nodal status. Finally, for 23 of the 199 tumors, tissue samples of nodal metastases were available, and LAMC2 staining of these revealed that 19 of 23 (83%) exhibited low levels of LAMC2 (Figure 2E). This was again consistent with the inverse relationship of LAMC2 expression with nodal metastasis in primary tumors.

In contrast to nodal metastases, liver or lung metastases of human bladder cancers are not routinely excised and thus are not available for analysis. However, given the aforementioned findings coupled with the methods by which we derived the metastasis models, we sought to test the hypothesis that LAMC2 expression is a marker of hematogenous rather than lymphatic dissemination. Thus we examined the relationship between LAMC2 staining and metastatic recurrence in patients without nodal metastases at cystectomy, in an effort to determine whether LAMC2 staining could help risk stratify this otherwise lower metastasis risk patient population. Consistent with the *in vivo* model's findings of high LAMC2 expression in lung and liver metastases (Figure 2, A and B), we found that high LAMC2 staining was significantly associated with visceral metastatic recurrence of bladder cancer in patients after cystectomy ($P = 0.040$) (Figure 2F). Supplementary Table 2, see <http://ajp.amjpathol.org> shows microarray data for LAMC2 staining stratified by original tumor grade (A) or nodal status (B) (assigned at pathological review of the tissue block from which the tissue microarray cores were obtained).

Discussion

Despite the paucity of tissues available for molecular investigation of bladder cancer metastases, clinically relevant biological patterns can emerge from comparative analysis of xenograft models of liver and lung metastases. We found that these models selected for cell lines exhibiting organ-specific growth properties, as best illustrated by the significantly reduced lung metastatic potential of the liver metastatic SL4 cell line. Interestingly, however, we found that the FL4 lung metastatic cell line gained through four generations of *in vivo* selection increased metastatic competence at both lung and liver sites (~2.7-fold on average), although there was a preponderance of selection for lung metastatic ability (~10-fold as compared with SL4). One could simply attribute this phenomenon to the fact that genes specifically associated with lung model exhibited many of the same ontological classes as genes from the liver model (Table 4). However, this is a compelling finding, potentially even clinically important, given both the pervasiveness of tumor cell dissemination through systemic circulation from even small tumors²¹ and the fact that cells growing (and shedding into circulation) from a patient's lung metastasis have already been extensively selected and enriched for metastatic competence.²² Future experiments should examine whether this phenomenon is of any generality to bladder or other cancers that metastasize to the lung; speculatively, such a process could beg the question of more aggressive management of patients with limited pulmonary metastasis to prevent dissemination elsewhere.

On the other hand, liver metastasis selection of parental T24T cells though might have resulted in a less strin-

Table 4. Ingenuity Pathway Analysis of Top Classes of Liver and Lung Model Genes

Ontology, functions and diseases	Liver model			Lung model*		
	P value [†]	No. genes	% Genes [‡]	P value [†]	No. genes	% Genes [‡]
Cellular movement	1.50E-10	18	47			
Cellular growth and proliferation	2.59E-08	21	55	1.65E-04	5	38
Cell-to-cell signaling and interaction	2.91E-08	22	58			
Tissue development	2.91E-08	21	55			
Cell death	4.25E-07	24	63	1.35E-04	4	31
Cancer	5.40E-07	27	71	1.65E-04	12	92
Tumor morphology	8.60E-07	9	24			
Hemat. system development and function	1.08E-06	16	42			
Imm./lymphatic system development and function	1.08E-06	13	34			
Immune response	1.08E-06	14	37			

*Only three classes of genes enriched in the liver model genes were also significantly enriched in the lung model-specific genes ($P < 0.001$).

[†]P value from the Ingenuity Pathway Analysis 5.5 application for enrichment of ontological class of gene.

[‡]Percentage of total liver- or lung model-specific genes belonging to the indicated ontological class.

Figure 2. Immunohistochemical expression of LAMC2 in metastases of human bladder cancer xenografts and primary human bladder tumors. **A** and **B**: Two examples of FL4 *in vivo* mouse metastases, in lung (**A**) and liver (**B**) showing intense staining for LAMC2 in xenograft metastases, adjacent to mouse lung (**A**) or liver (**B**) parenchyma. **C** and **D**: Representative examples of LAMC2 staining in human bladder cancer. **E**: Bladder cancer cells from a bladder cancer nodal metastasis showing no staining for LAMC2. **F**: Kaplan-Meier estimation of metastatic recurrence of bladder cancer by LAMC2 staining. At 24 months, high staining with LAMC2 was significantly associated with increased recurrence by the log rank test. Original magnifications: $\times 200$ (**A**, **B**); $\times 100$ (**C**, **D**, **E**).

gent selection than for the lung. The liver has been classically considered to be a receptive parenchyma,²³ favorable to metastatic seeding from many tumor types. Speculatively, this might be associated with the liver's capacity to regenerate, and at least one recent report has actually experimentally demonstrated this phenomenon in a mouse model of colorectal cancer metastasis.²⁴ In this study, the authors found that *a priori* partial hepatectomy actually stimulated formation of hepatic (and even extrahepatic) experimental metastases. Although obviously our experiments did not use hepatectomy as part of the protocol, it is important to consider this unique property of liver parenchyma, and its signaling milieu,²⁵ as part of the process of liver metastasis.

We found substantial differences between genes up-regulated or down-regulated as a function of several rounds of *in vivo* metastatic selection, including liver or lung selection-specific and overlapping genes between the two models. The comparative ontological analysis suggested that there were cases in which similar broad ontological classes of genes (but not the same genes) were enriched among liver or lung model genes (cellular movement, proliferation, and cancer). However, the differences observed suggest that the host organ microen-

vironment²⁶ participates in selection of clones expressing substantially different patterns of transcripts.

Always, an important caveat for this model (and all experimental, as opposed to spontaneous metastasis models) is that the model only recapitulates the last stage of metastasis, the arrest and colonization of the second parenchyma, missing potentially important (or targetable) genes regulating processes of invasion, intravasation and survival in lymphatic and venous draining of the primary tumor. The technique itself bears consideration; based on the experiments done, we would be unable to discriminate between genes irrelevant to liver metastasis but that support survival through injection, or more specifically, splenic injection and venous drainage (an anatomical route not traveled, at least directly, by disseminated bladder cancer cells). At a minimum, such cells are then subsequently and terminally selected for growth in the liver parenchyma, just as are their counterparts through the tail vein lung metastasis model.

Importantly, as regards clinical relevance of these targets, we identified a significant overlap between genes identified by the models and genes similarly dysregulated in invasive bladder cancer. It bears discussion that this is a very stringent secondary screen for candidate

metastasis genes: individual genes contributing to metastasis or organ-specific metastasis do not perforce have to be differentially expressed on average among all cells in the tumor bulk (as is required to be detected in microarray studies of human tumor tissues). Indeed, recent reports suggest that even rare subpopulations of cancer cells may exhibit differential metastatic tropism because of their unique gene expression,⁶ although our screening strategy was designed to identify targets (ie, LAMC2) potentially amenable to use as immunohistochemical biomarker in gross tissue.

Surprisingly, one of the largest increases in gene expression in both liver and lung models was the KISS1 metastasis suppressor gene,²⁷ whose expression was observed to be induced after multiple rounds of *in vivo* cell line selection. One potential mechanism for this phenomenon might be that *in vivo* selection of cells might have resulted in selection of clones with down-regulation of the KISS1 receptor (KISS1R/GPR54), whose expression is not monitored on the HG-U133A microarray platform, or another necessary downstream signaling component in this metastasis-suppressor pathway. A similar observation has been made for the RhoGDI2 metastasis suppressor gene, in which repeated pulmonary metastatic selection of breast cancer cells resulted in the re-expression of the RhoGDI2 transcript.⁷

At the genomic level, chromosomal CGH analysis showed many changes, including gains at 1q, 3q, 7q, 12q, and 17q, and amplification of 20q, that were shared between the liver and lung models. To our knowledge, there is no published report of CGH performed on metastatic bladder cancer tissue for comparison with our results. However, this is not the case in colorectal cancer, in which metastases are routinely excised, and two recent reports show interesting parallels between our cell line CGH gains in lung and liver metastasis and actual primary human colon cancer lung and liver metastases. Although gain at 1q is rather common in metastasis across several tumor types,²⁸ gains at 3q, 7q, 12q, 17q, and 20q have all been observed in colorectal cancer lung metastases, although the 7q, 12q, and 20q lesions were particularly noted to arise during the transition between the primary tumor and lung met.²⁹ Another report actually compared CGH changes in patient liver and lung metastases from colorectal cancer, finding, just as we did in our *in vivo* selected bladder cell lines, a phenomenon of generally less total CGH changes in liver compared with lung metastases, although they did not identify in their lung metastases the 8q and 9q gains that we found in our lung selected cell lines.³⁰ For the 1q and 3q loci, these regions appeared to contain genes that were differentially expressed as a function of *in vivo* selection, including LAMC2 and two others of the eight core genes discovered to be overexpressed in both metastatic models. Given the similarity of CGH changes evinced between our model and these prior colorectal cancer reports and the lack of genes identified in all of the areas of CGH gain by our microarray platform, we believe that analysis of our metastasis models by high-density array CGH or SNP arrays might uncover other targets that are associated

our causally related to metastasis, potentially relevant to both of these diseases.

The metastasis-associated genes that emerged from our models suggest some potential therapeutic targets. For example, monoclonal antibodies against TACSTD1 (EpCAM), discovered here as a lung metastasis-specific target gene (Table 2a) and recently shown to be a marker for cancer stem cell populations,³¹ have been used in the clinical trial setting.³² Our findings, in concert with a recent report of associating TACSTD1 IHC with poor prognosis for human bladder cancer³³ might suggest the preclinical evaluation of such a therapy for patients with lung metastatic bladder cancer. In the case of LAMC2, its EGF-like repeats have been shown to be processed from the protein by matrix metalloproteases and they may agonize the EGFR,¹⁹ a receptor tyrosine kinase now targeted via several therapeutic modalities. Moreover, our models can serve as platforms for validation of the potential preclinical efficacy of therapeutic modalities targeting these genes and their pathways *in vivo*, as we have recently shown for Atrasentan, a drug targeting endothelin-1, which is a downstream transcriptional target of the RhoGDI2 metastasis suppressor.³⁴

A novel finding in our study was the observation that LAMC2 expression is a marker or even possibly a mediator for hematogenous dissemination of cancer cells as opposed to lymphatic metastasis, at least in bladder cancer. High LAMC2 expression was inversely correlated with lymph node metastasis despite being detected as a core metastatic gene whose elevated expression was shared between muscle invasive bladder cancer and the liver and lung metastatic cell. Yet, LAMC2 staining was associated with development of metastatic recurrence at visceral sites after surgery in patients without lymph node metastases. This observation highlights the importance of validation and interpretation of findings from animal models in the context of clinical outcomes observed in patients.^{3,6}

In conclusion, we have developed an *in vivo* model system for the discovery and future study of genes implicated in lung and liver metastases in bladder cancer. We found genes whose expression differed based on the metastatic organ site from which they were derived, but also include a core metastatic signature shared between the sites. Several of these suggest therapeutic targets, and some, such as LAMC2, provide novel insights regarding the different routes of metastasis.

Future studies using these models should not only generalize the findings of this T24T-based system to other cell lines but also use dissociated cells from primary human bladder tumors. Because of the marked heterogeneity of tumors of even the same stage, grade, and histology and concomitant epigenetic and genetic instability, regulation of organ tropism is likely affected by large constellations of genes (as opposed to the few we have started to characterize herein), which larger numbers of cell lines or dissociated tumor cells could more appropriately represent. Such cells have also not been subjected to the vagaries of *in vitro* culture adaptation. Given new protocols to prospectively identify highly tumorigenic stem cell-like subpopulations of cells within

tumor bulk³⁵ and an unknown relationship between these stem cell-like populations and populations leading to metastatic colonization, such studies will hopefully provide further insights into this most morbid and mortal stage of disease.

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