

Mesenchymal-to-Epithelial Transition Facilitates Bladder Cancer Metastasis: Role of Fibroblast Growth Factor Receptor-2

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Abstract

Epithelial-to-mesenchymal transition (EMT) increases cell migration and invasion, and facilitates metastasis in multiple carcinoma types, but belies epithelial similarities between primary and secondary tumors. This study addresses the importance of mesenchymal-to-epithelial transition (MET) in the formation of clinically significant metastasis. The previously described bladder carcinoma TSU-Pr1 (T24) progression series of cell lines selected *in vivo* for increasing metastatic ability following systemic seeding was used in this study. It was found that the more metastatic sublines had acquired epithelial characteristics. Epithelial and mesenchymal phenotypes were confirmed in the TSU-Pr1 series by cytoskeletal and morphologic analysis, and by performance in a panel of *in vitro* assays. Metastatic ability was examined following inoculation at various sites. Epithelial characteristics associated with dramatically increased bone and soft tissue colonization after intracardiac or intratibial injection. In contrast, the more epithelial sublines showed decreased lung metastases following orthotopic inoculation, supporting the concept that EMT is important for the escape of tumor cells from the primary tumor. We confirmed the overexpression of the IIIc subtype of multiple fibroblast growth factor receptors (FGFR) through the TSU-Pr1 series, and targeted abrogation of FGFR2IIIc reversed the MET and associated functionality in this system and increased survival following *in vivo* inoculation in severe combined immunodeficient mice. This model is the first to specifically model steps of the latter part of the metastatic cascade in isogenic cell lines, and confirms the suspected role of MET in secondary tumor growth. (Cancer Res 2006; 66(23): 11271-8)

Introduction

Cancer metastasis accounts for ~90% of cancer deaths (1), yet the mechanisms facilitating progression from benign to invasive, and finally to metastatic carcinoma, remain largely elusive. A greater understanding of those mechanisms holds much therapeutic and diagnostic promise. Extraordinary demands are placed on epithelium-derived carcinoma cells to successfully metastasize, including separation from the epithelial collective, degradation of the surrounding matrix, migration and invasion through the

basement membrane, intravasation and survival in the circulation, extravasation at a secondary site, survival as a micrometastasis, and finally growth into overt metastases (1). To successfully complete these complex steps, cancer cells exhibit both mesenchymal- and epithelial-like properties at different times, or even at the same time (2). Indeed, commitment to lineage differentiation in normal and tumor cells is more pliant than first thought, and cellular transition is emerging as a major mechanism of adult tissue homeostasis (3).

Cellular plasticity is fundamental to embryologic development. The emergence of the primitive mesoderm during gastrulation constitutes the first epithelial-to-mesenchymal transition (EMT), resulting in the formation of highly motile cells that are critical to the development of the body plan (4). By commandeering developmental EMT pathways, sessile epithelial carcinoma cells are transformed into cells with migratory and invasive capability, metastatic potential, and a resistance to anoikis and chemotherapy (5). In both normal developmental EMT and pathologic EMT during tumor formation and progression, the cell adhesion protein E-cadherin is critical to the differentiation and maintenance of the epithelial phenotype, and loss of this protein is rate-limiting for EMT (4, 5). The transcription factors Twist, Snail1, and Snail2 can independently drive this inductive morphogenesis by directly binding to E-boxes in the *E-cadherin* gene promoter and repressing its expression (5). EMT in both contexts is further characterized by a switch in β -catenin location and function, changing from an integral junctional complex component in epithelial cells to forming an active bipartite complex with members of the lymphoid enhancer factor family of DNA-binding proteins. That complex then translocates to the nucleus and transactivates target genes that include oncogenes and tumor suppressors (6). Although EMT is clearly important to tumor progression, it is inconsistent with the observation that metastatic lesions share the epithelial nature of their primary tumor counterparts. Accordingly, primary colon carcinomas and their corresponding liver metastases display a mixed epithelial-mesenchymal phenotype, where in both sites cells in the tumor center retain cytoplasmic β -catenin and junctional E-cadherin, and tumor cells at the periphery lose surface E-cadherin and up-regulate fibronectin and vimentin expression (7). These findings indicate that the metastatic cascade is multifaceted, with EMT critical to the initial transformation from benign to invasive carcinoma, and mesenchymal-to-epithelial transition (MET) critical to the latter stages of metastasis.

MET is a fundamental embryologic process, with examples including somitogenesis and nephrogenesis (8). The adult mammalian kidney derives from the metanephric mesenchyme that condenses and then undergoes MET to form epithelial tubules that ultimately develop into nephrons (9). The signaling cascades seminal to kidney organogenesis have particular

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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relevance to bladder carcinoma, as the trigone region of the bladder and the kidney develop from a common embryologic origin (10). Several growth factor families are critical regulators of kidney MET, including the wnt/wingless and bone morphogenetic protein families. In particular, fibroblast growth factors (FGF), FGF receptors (FGFR), and proteoglycans that modulate FGF signaling are essential modulators of nephrogenic MET. There are five FGFRs and 23 known FGFs that exhibit considerable overlap between their ligand specificities (11, 12). FGF1 and FGF2 are detected in mesenchymal condensates and in the epithelial vesicles that form from mesenchymal condensates (13). FGF2 plays a significant inductive role in mesenchyme condensation (14), whereas FGF7 signaling influences the number of nephrons that form in the developing kidney (15). Discernable kidney defects were not observed in FGF2, FGF3, or FGF5 transgenic mice, demonstrating that a certain level of redundancy exists (13, 16–19). In contrast, broad midgestational expression of a soluble kinase-deficient FGFR2IIIb, blocking FGF1, FGF3, and FGF7, severely affected kidney organogenesis, including the absence of early nephron development markers that indicates disrupted MET (13, 20). FGFR3 immunostaining is found in mesenchymal condensates, whereas FGFR1, FGFR2, and FGFR4 immunoreactivity emerges as the condensates convert into epithelium. In addition, proteoglycans (syndecans and glypicans) that function as signaling reservoirs or coreceptors for FGFs and other heparin-binding growth factors are also implicated in kidney organogenesis, and glypican-3 in particular is a key morphogenic mediator (21, 22).

We have generated a progression series of the T24 transitional cell carcinoma (TCC) bladder carcinoma cell line TSU-Pr1, consisting of the TSU-Pr1, TSU-Pr1-B1 (B1), and TSU-Pr1-B2 (B2) cell lines, which show sequentially increasing metastatic potential following intracardiac inoculation (23). MET underpins this model, as shown by direct characterization of the cell lines; to our knowledge, this is the first carcinoma model to capture this component of the metastatic cascade. Given their role in regulating MET in kidney development, the expression of FGFR1 to FGFR4 was assessed. The TSU-Pr1 series predominantly expressed IIIc-type isoforms of FGFR1 to FGFR3 in addition to FGFR4. Moreover, FGFR2IIIc was greatly up-regulated across the TSU-Pr1 series and plays a key role in determining the epithelial or mesenchymal phenotype in these cell lines.

Materials and Methods

Cell lines and reagents. TSU-Pr1 cells were obtained from Dr. Dan Djakiew (Georgetown University, Washington, DC; ref. 24). B1 and B2 cell lines were generated as previously described (23). All cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS). Antibodies used were anti-pan-actin (Neomarkers, Fremont, CA), antivimentin (Cell Marque, Hot Springs, AR), and anticytokeratin (Dakocytomation, Glostrup, Denmark). Rhodamine phalloidin was a kind gift from Dr. Don Newgreen (Murdoch Children's Research Institute, Melbourne, Australia).

Immunocytochemistry. Cells were cultured on eight-well Lab-Tek II chamber slides (Nalge Nunc International, Rochester, NY). Cells were fixed with 10% buffered formalin, permeabilized with 0.2% Triton X-100, then blocked with 10% normal goat serum or protein block (Dakocytomation) for 30 minutes. All antibodies were incubated with cells at 4°C overnight. Antibodies were detected by immunofluorescence (AlexaFluor secondary antibodies; Invitrogen) or diaminobenzidine chromagen (Dakocytomation) and digitally captured (Carl Zeiss AG, Oberkochen, Germany) and processed with Axiovision Rel 4.2 software (Carl Zeiss).

Quantitative reverse transcription-PCR. RNA extraction and quantitative real-time PCR (qRT-PCR) were done as previously described (25) and a list of primers used in this article are provided in Supplementary Table S1.

Western blot analysis. Cell extracts were prepared as previously described (26) and were run with Laemmli buffer in 4% to 12% Bis-Tris NuPAGE gels with MES buffer (Invitrogen). Protein was visualized with anti-IRDye800CW-conjugated affinity-purified anti-goat IgG (Rockland, Gilbertsville, PA) or Alexafluor 680 anti-mouse IgG (Invitrogen). Membranes were scanned on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) and quantitated with Odyssey Infrared Imaging system version 1.2 software.

Migration and invasion assays. Chemoinvasion analyses were done as previously described (27) using Matrigel coating (25 µg/filter) and 10% FBS chemoattractant, and were incubated for 7 hours. Migration assays were done on uncoated, 20 µg/mL collagen type I-coated (Vitrogen100, Cohesion, CA) or collagen type IV-coated (Sigma-Aldrich, St. Louis, MO) membranes for 5 hours with 20% FBS chemoattractant. The cells that had traversed the filter and spread on the lower surface were fixed with methanol, stained with Quick Dip (HistoLabs, Riverstone, NSW, Australia), and counted. Experiments were repeated thrice and the data presented are mean ± SE of cells per 10 representative microscopic fields in each experiment.

Wound healing assay. Assay was done as previously described (25).

Soft agar colony formation assay. Cells were plated in 1% agar (2,500 per plate) and maintained in DMEM containing 10% FBS. The cells were incubated at 37°C for 10 days, after which time they were stained with 0.5 mL crystal violet (0.05%) for 1 hour. Colonies were photographed using the MCID system (Linton, England) and then counted. The data represents three individual experiments done in triplicate.

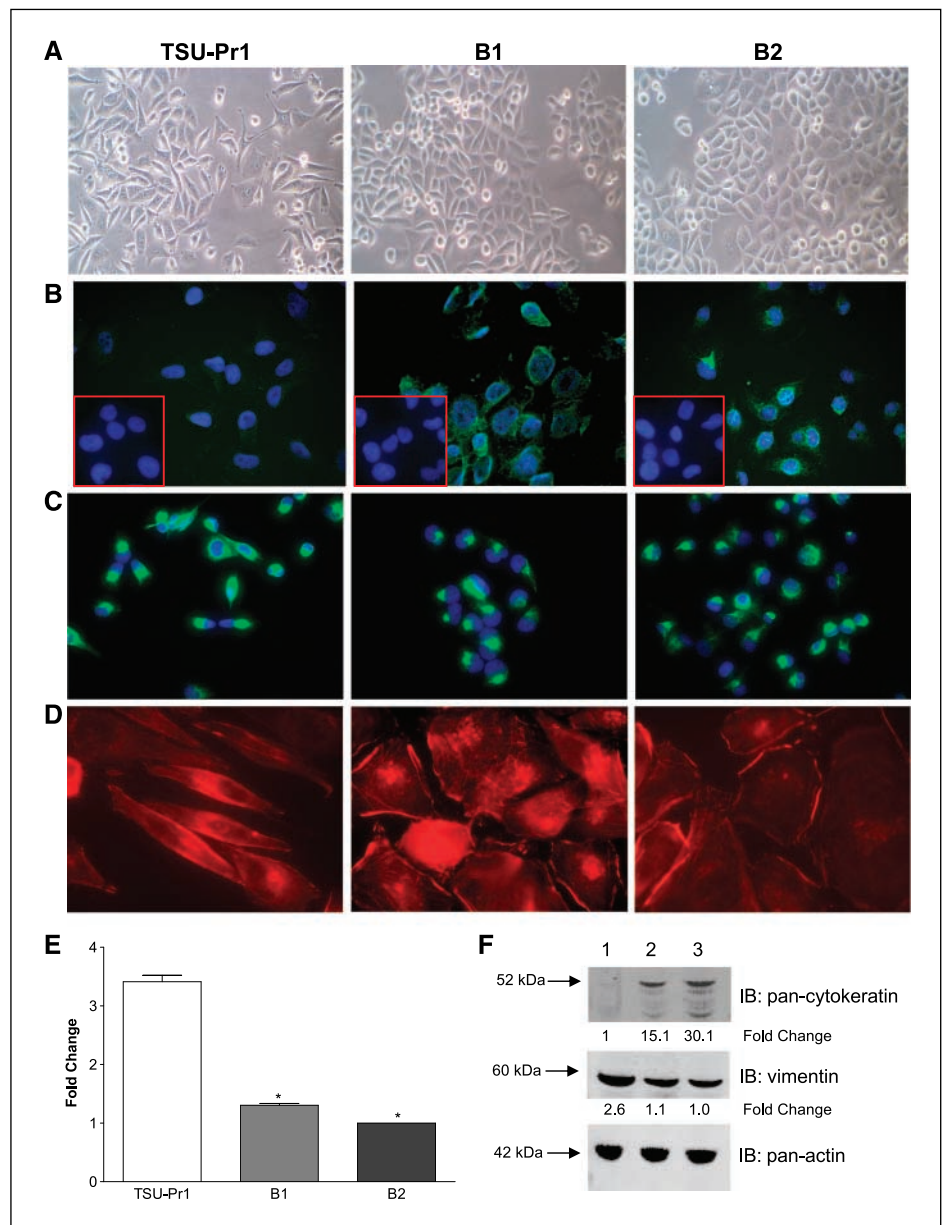
Short hairpin RNA knockdown of FGFR2 in B2 cells. FGFR2 short hairpin (shRNA) constructs (sequences provided in Supplementary Table S2; Ambion, Austin, TX) in pSilencer 4.1-CMV puromycin (0.8 µg; Ambion) were transfected into the B2 cells (1:2 ratio of DNA/LipofectAMINE 2000 Transfection Reagent; Invitrogen). Cells positive for pSilencer were selected using puromycin (0.5 µg/mL). Pooled puromycin-resistant cell populations were single-cell cloned and each clone was analyzed for FGFR2 expression.

In vivo inoculation and determination of metastasis. Intracardiac inoculation and determination of metastatic bone lesions was done as previously described (23). Intratibial injections were done as previously described (25). For orthotopic inoculations, 5×10^5 cells in 0.05 mL PBS were injected into the bladder wall. Alu-PCR was carried out as previously described (28).

Results

Epithelial characteristics of B1 and B2. We previously reported increased tumor metastases in mice following intracardiac inoculation of the TSU-Pr1 bladder carcinoma progression series (TSU-Pr1, B1 and B2 cell lines), where the number of mice with metastatic deposits increased progressively in association with a shortened onset of radiologically detectable lesions (23). We noted marked morphologic differences between these cells *in vitro* (Fig. 1A) that associated with increased metastatic potential *in vivo*. TSU-Pr1 cells have a spindle-like fibroblastoid cell shape reminiscent of mesenchymal cells, whereas the B1 and B2 cell lines have a cuboidal appearance and closely apposed cell-to-cell junctions typical of epithelial cells. Epithelial and mesenchymal cells are structurally divergent, reflecting their different functions and capabilities, manifest in their differing cellular composition (29). Indeed, the epithelial cytokeratin intermediate filaments were notably absent in TSU-Pr1 cells, but were markedly up-regulated in B1 and were further up-regulated in B2 cells (Fig. 1B and F). Commensurately, TSU-Pr1 cells express large amounts of the mesenchymal intermediate filament vimentin (Fig. 1C), which was down-regulated in the B1 and B2 cells (Fig. 1E

Figure 1. B1 and B2 cells have acquired epithelial characteristics. **A**, phase-contrast photographs of the TSU-Pr1 series. Magnification, $\times 20$. TSU-Pr1 cells exhibit fibroblastoid morphology, whereas B1 and B2 cells exhibit a cuboidal epithelial morphology. **B**, epithelial cytokeratin filaments were not detected in TSU-Pr1 cells; in contrast, there was a marked up-regulation of cytokeratins in B1 and B2 cells. Magnification, $\times 40$. *Inset*, nonimmune (IgG) control immunostaining. **C**, vimentin immunostaining was detected in all three cell lines of the TSU-Pr1 series. Magnification, $\times 40$. **D**, cortical actin staining, which is a characteristic of epithelial cells, is evident in the B1 and B2 cells but not in TSU-Pr1 cells. Actin was visualized by rhodamine-conjugated phalloidin. Magnification, $\times 100$. **E**, qRT-PCR illustrating the significant decrease in vimentin expression in B1 and B2 cells compared with TSU-Pr1 cells. *Columns*, mean of three biological replicates done in triplicate; *bars*, SD. Statistical significance was determined by one-way ANOVA followed by Tukey's posttest. *, $P < 0.001$, significantly different from TSU-Pr1. **F**, Western blot analysis of total protein levels of cytokeratins or vimentin in the TSU-Pr1 series normalized to actin loading control. *Lane 1*, TSU-Pr1 cells; *lane 2*, B1 cells; *lane 3*, B2 cells. Representative of three replicate experiments.



and *F*). The implicit MET was further reinforced by the presence of actin stress fibers typical of mesenchymal cells in TSU-Pr1 cells and cortical actin fibers, which are characteristic of epithelial cells in B1 and B2 cells (Fig. 1*D*). Although much emphasis has been placed on the role of EMT in metastatic progression, these morphologic findings strongly indicate that the TSU-Pr1 series has instead benefited from MET.

The behavior of the TSU-Pr1 series *in vitro* is consistent with MET. Mesenchymal cells are typically more motile and invasive than epithelial cells and, thus, *in vitro* assays assessing those criteria are used to classify phenotypes (2, 29). We examined the migratory potential of these cell lines through uncoated, collagen type I-coated, and collagen type IV-coated filters (Fig. 2*A*). In accordance with their epithelial morphology, B1 and B2 cells showed significantly slower migration compared with TSU-Pr1 cells on all substrates, whereas B1 cells were not significantly different from B2 cells on any substrate examined. B1 and B2 cells

also migrated more slowly than TSU-Pr1 cells from a leading edge in the monolayer wound-healing assay (Fig. 2*B*), and B1 and B2 cells were less invasive than TSU-Pr1 cells through Matrigel-coated filters (Fig. 2*C*). Mesenchymal cells are also more capable than epithelial counterparts of colony formation following suspension as single cells in soft agar (30), and the B1 and B2 sublines showed a stepwise decrease in colony formation compared with TSU-Pr1 cells (Fig. 2*D*).

FGFR3IIIc variants are expressed in the TSU-Pr1 series. As mentioned above, FGF family members play important roles in mediating nephrogenic MET. We examined the expression of *FGFR1* to *FGFR3*, including splice variants of these receptors involving the third immunoglobulin-like domain (III) that arise from the inclusion of exon 8 (IIIb) or exon 9 (IIIc), and *FGFR4* in the TSU-Pr1 series. By qRT-PCR, we determined that although *FGFR1IIIc* was not differentially expressed, *FGFR3IIIc* was significantly up-regulated in B2 cells compared with TSU-Pr1 or

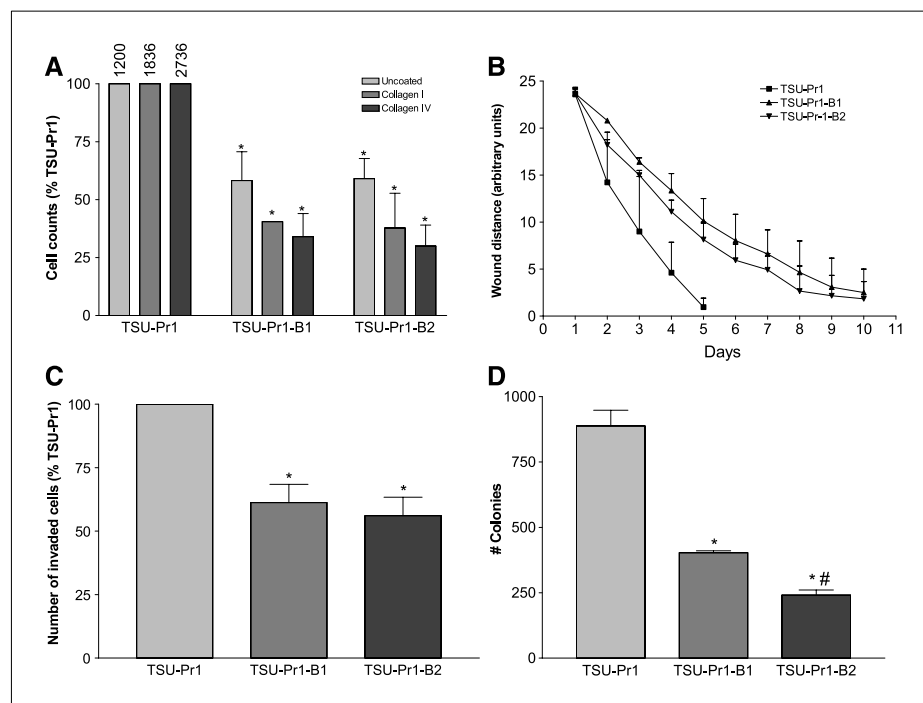


Figure 2. The epithelial nature of B1 and B2 is confirmed by a series of *in vitro* assays.

A, migratory potential of the TSU-Pr1 series through uncoated, collagen type I-coated, and collagen type IV-coated filters. *Numbers above the TSU-Pr1 columns*, average number of cells counted for an individual well. *Columns*, mean; *bars*, SE. Percentage of TSU-Pr1 cells from two to three independent experiments done in triplicate and analyzed by one-way ANOVA followed by Tukey's posttest. *, $P < 0.05$, significantly different from TSU-Pr1. **B**, invasive capacity of the TSU-Pr1 series through Matrigel. *Points*, mean; *bars*, SE. Three independent experiments done in triplicate and analyzed by one-way ANOVA followed by Tukey's posttest. *, $P < 0.01$, significantly different from TSU-Pr1. **C**, migratory potential of the TSU-Pr1 series in the monolayer wound-healing assay. *Columns*, mean; *bars*, SE. Two independent experiments done in triplicate and analyzed by one-way ANOVA followed by Tukey's posttest. *, $P < 0.01$, significantly different from TSU-Pr1; #, $P < 0.05$, significantly different from B1.

B1 cells, and *FGFR4* was significantly down-regulated in B1 and B2 cells compared with TSU-Pr1 cells (Fig. 3A). Furthermore, we observed a dramatic incremental increase in *FGFR2IIIc* expression across the TSU-Pr1 series (Fig. 3A). The IIIb subtype of *FGFR1* and *FGFR2* were not detected in the TSU-Pr1 series, and *FGFR3IIIb* was detected in B2 cells only (data not shown).

FGFR2IIIc is a critical determinant of the epithelial phenotype. To establish the role of *FGFR2IIIc* in the determination of the epithelial phenotype, we transfected B2 cells with a scrambled shRNA sequence, or one of three shRNA sequences targeting *FGFR2*. Six to nine single-cell clones were generated for each sequence and the morphology was assessed, followed by analysis of *FGFR2IIIc* levels by qRT-PCR and *FGFR2* protein levels by Western blot. shRNA-expressing clones with significant *FGFR2IIIc* reduction were selected (Supplementary Table S3). We determined that the scrambled shRNA clones maintained high levels of *FGFR2IIIc* expression and an epithelial phenotype. We observed a series of clones with significantly reduced *FGFR2IIIc* expression that maintained an epithelial phenotype, and we observed that the two clones with the greatest reduction in *FGFR2IIIc* expression had acquired a mesenchymal phenotype.

To highlight the relationship between *FGFR2IIIc* levels and morphology, clones were grouped into three groups. The control group comprised two clones expressing the scrambled shRNA. Single-cell clones with significant *FGFR2IIIc* reduction were grouped according to their morphology as either epithelial (two shRNA-1-expressing clones and one shRNA-2-expressing clone with significant *FGFR2IIIc* knockdown that retain their epithelial morphology) or mesenchymal (one shRNA-2-expressing clone and one shRNA-3-expressing clone with significant *FGFR2IIIc* knockdown that have acquired mesenchymal-like morphology; Fig. 3B). We observed a striking similarity in *FGFR2IIIc* expression between TSU-Pr1 cells and the mesenchymal group, between B1 cells and the epithelial group, and between B2 cells and the control group, illustrating that reducing *FGFR2* expression in B2 cells to the level

that is expressed in TSU-Pr1 cells causes a switch from an epithelial-like to a mesenchymal-like morphology.

We further assessed the *in vitro* behavior of the single-cell clones in the soft agar colony-forming assay (Fig. 3C), which were compared with the original TSU-Pr1 series data (data repeated here for comparison from Fig. 2D). These results illustrate that the large reduction in *FGFR2IIIc* expression and subsequent reversion to mesenchymal-like morphology translates into a significant increase in performance in this assay, confirming that the clones have acquired mesenchymal-like behavior.

Reduced *FGFR2* expression increases survival following *in vivo* inoculation. Mice were injected (intracardiac) with a pool of clones expressing low (*FGFR2_{low}*) or high (*FGFR2_{high}*) *FGFR2* levels (Supplementary Fig. S1). Mice were harvested when symptoms of disseminated disease were observed (including >10% loss of body weight). Survival was significantly increased in the *FGFR2_{low}* mice (Fig. 4).

***In vivo* inoculation shows that an epithelial phenotype is favorable in the latter stages of the metastatic cascade.** It is possible to model various stages of the metastatic cascade by varying the site of inoculation (Fig. 5A). Monitoring the growth and development of metastases from the orthotopic site demands that tumor cells are able to complete all components of the metastatic cascade, including migration away from the primary tumor, invasion of the surrounding matrix, intravasation, survival in the circulation, extravasation into the secondary site, and then survival and development into an occult metastasis at the secondary site. Alternatively, intracardiac inoculation bypasses the initial stages of the metastatic cascade and measures the ability of a cell to survive in the circulation, extravasate, and to grow as a metastatic deposit at a secondary site. Furthermore, tumor cells can be directly seeded into a secondary site where their ability to develop into a metastatic deposit is assessed independently of survival in the vasculature and extravasation. We compared the metastatic ability of the TSU-Pr1 series

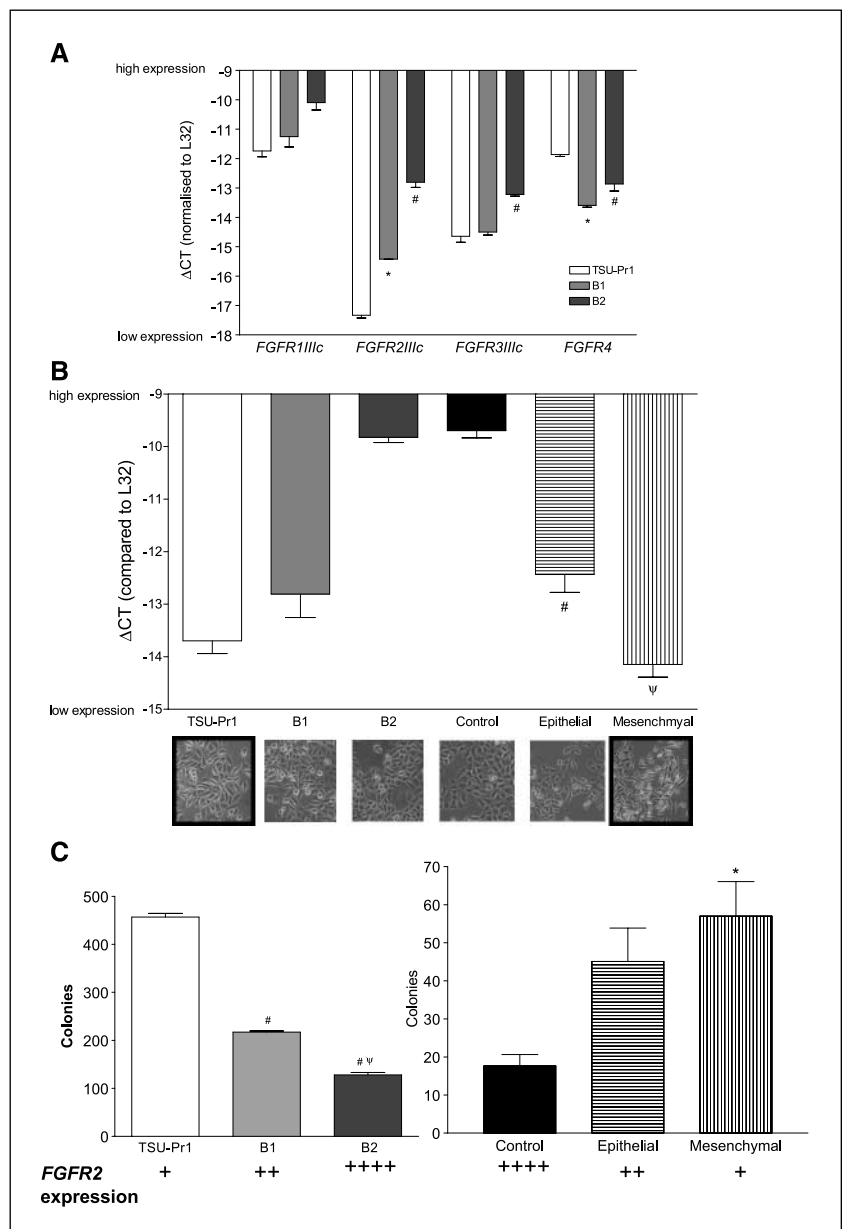
following orthotopic, intracardiac, and intratibial inoculation (Fig. 5B-D). The incidence of micrometastatic deposits in lung tissue following orthotopic inoculation was higher for TSU-Pr1 cells than for B1 or B2 cells (Fig. 5B), consistent with multiple EMT reports associating a higher incidence of metastasis with mesenchymal phenotype (5). In contrast, the incidence of metastasis following intracardiac or intratibial inoculation increased incrementally from TSU-Pr1 to B1 to B2 cells (Fig. 5C-D), confirming that an epithelial phenotype favors the latter stages of metastasis. Indeed, once established in bone, TSU-Pr1, B1, and B2 tumors all showed a similar epithelial morphology (Fig. 5E). The dissection of these steps in a single model is unique.

Discussion

It has previously been shown that EMT and the implicit migratory and invasive attributes aid the initial transformation

from organ-confined to invasive carcinoma. MET has been invoked as an explanation for the discordance between migratory and invasive carcinoma cells with mesenchymal features, and the carcinomatous, epithelioid macrometastatic deposits that present clinically, although functional evidence for this has not been forthcoming to date (31). MET has been evidenced with the identification of nuclear β -catenin at the invasive front of colon carcinoma and its subsequent reversion to the membrane in distant metastases (32). Also, lesions of the ovarian surface epithelium, which are mesothelially derived, remain premalignant unless E-cadherin expression is acquired (33). The TSU-Pr1 series is the first model to show the spontaneous reversion to epithelial characteristics in a mesenchymal-like carcinoma cell line and its association with increased metastatic ability in advanced malignancy. We have shown through morphology, the expression of epithelial markers and performance in a series of *in vitro* and *in vivo* assays, that B1 and B2 cells have reacquired epithelial

Figure 3. *FGFR2IIIc* determines epithelial morphology in the TSU-Pr1 series. **A**, by qRT-PCR analysis, *FGFR1IIIc* was not differentially expressed; however, *FGFR2IIIc* expression was significantly up-regulated in B1 cells compared with TSU-Pr1 cells and in B2 cells compared with B1 cells ($P = 0.0002$; *, $P < 0.01$, significantly different from TSU-Pr1; #, $P < 0.01$, significantly different from B1). *FGFR3IIIc* expression was significantly up-regulated in B2 cells compared with TSU-Pr1 and B1 cells ($P < 0.05$), whereas *FGFR4* expression was significantly down-regulated in B1 and B2 cells compared with TSU-Pr1 cells ($P = 0.0087$; *, $P < 0.01$; #, $P < 0.05$). Columns, mean; bars, SE. Three biological replicates done in triplicate and analyzed by one-way ANOVA followed by Tukey's posttest. **B**, association of cellular morphology with *FGFR2IIIc* expression in the TSU-Pr1 series and in the single-cell clones of B2 cells with reduced *FGFR2IIIc* expression. Bottom, typical morphology of a clone in each group. Magnification, $\times 20$. B2 single-cell clones were grouped into "Control" (two clones expressing a scrambled shRNA sequence) and either "Epithelial" (three clones) or "Mesenchymal" (two clones) according to their morphology. The epithelial group had significantly reduced *FGFR2IIIc* mRNA compared with the control group (#, $P < 0.001$). The mesenchymal group display significantly reduced *FGFR2IIIc* expression (ψ) compared with epithelial ($P < 0.01$) and control ($P < 0.001$) groups. Columns, mean; bars, SE. Analyzed by one-way ANOVA followed by Tukey's posttest. **C**, colony formation in soft agar by the TSU-Pr1 series (data repeated from Fig. 2D) and by the *FGFR2*^{-/-} B2 clones. Columns, mean; bars, SE. Analyzed by one-way ANOVA followed by Tukey's posttest. ψ , $P < 0.001$ and $P < 0.001$, significantly different from B1 and TSU-Pr1, respectively; #, $P < 0.01$, significantly different from TSU-Pr1; *, $P < 0.05$, significantly different from control.



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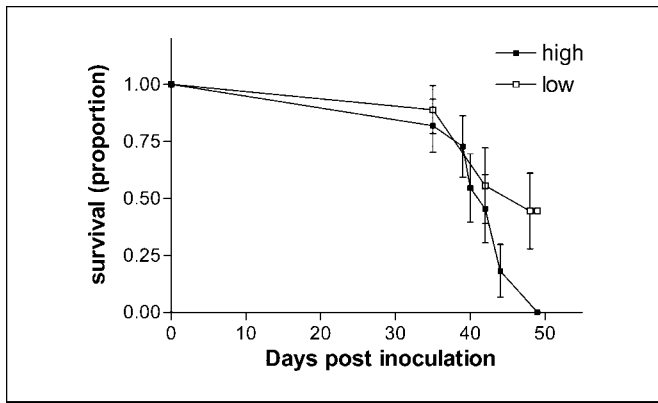


Figure 4. Reduced FGFR2 levels increase survival. Mice were injected (intracardiac) with a pool of clones expressing low (FGFR2_{low}) or high (FGFR2_{high}) FGFR2 levels. Significance was determined by comparing survival curves using the log-rank test ($P = 0.0435$).

characteristics. Furthermore, we have shown that high FGFR2IIIc expression is a critical determinant of the epithelial phenotype in the TSU-Pr1 series, and that reducing FGFR2IIIc expression in B2 cells transforms the B2 cells into cells with mesenchymal-like properties. Moreover, the acquired mesenchymal-like phenotype of FGFR2_{low} B2 clones, with FGFR2 levels reduced comparable with

that of TSU-Pr1 cells, translates into increased survival *in vivo*. This is consistent with the association of the epithelial phenotype with poor outcome. Together, these findings show that MET enhances the ability of a cell to complete the latter stages of the metastatic cascade.

FGFs and FGFRs are critical modulators of nephrogenic MET (14, 20). There is evidence to suggest that the FGF family plays a fundamental role in cellular differentiation and tumor phenotype in bladder carcinoma. *FGFR2IIIb* is expressed in normal urothelium and is down-regulated in bladder transitional cell carcinoma. Furthermore, loss of this receptor is accompanied by a loss of E-cadherin and the epithelial phenotype, along with a gain in migratory and invasive potential in bladder carcinoma (34). In models of bladder carcinoma, an FGF2 or FGF1 ligand-receptor autocrine loop elicits profound effects on cellular phenotype reminiscent of EMT, and these effects are associated with an increase in matrix metalloproteinase and urokinase activity, resulting in increased invasive potential and tumor volume following s.c. inoculation (35, 36).

Alternate splicing of *FGFR1* to *FGFR3* generates IIIb or IIIc splice variants that have traditionally been considered to exhibit epithelial- or mesenchymal-specific tissue expression, respectively (37, 38); however, it is becoming increasingly evident that this tissue-specific expression is not as strict as once thought. There are now several reports of FGFR2IIIc expression in normal

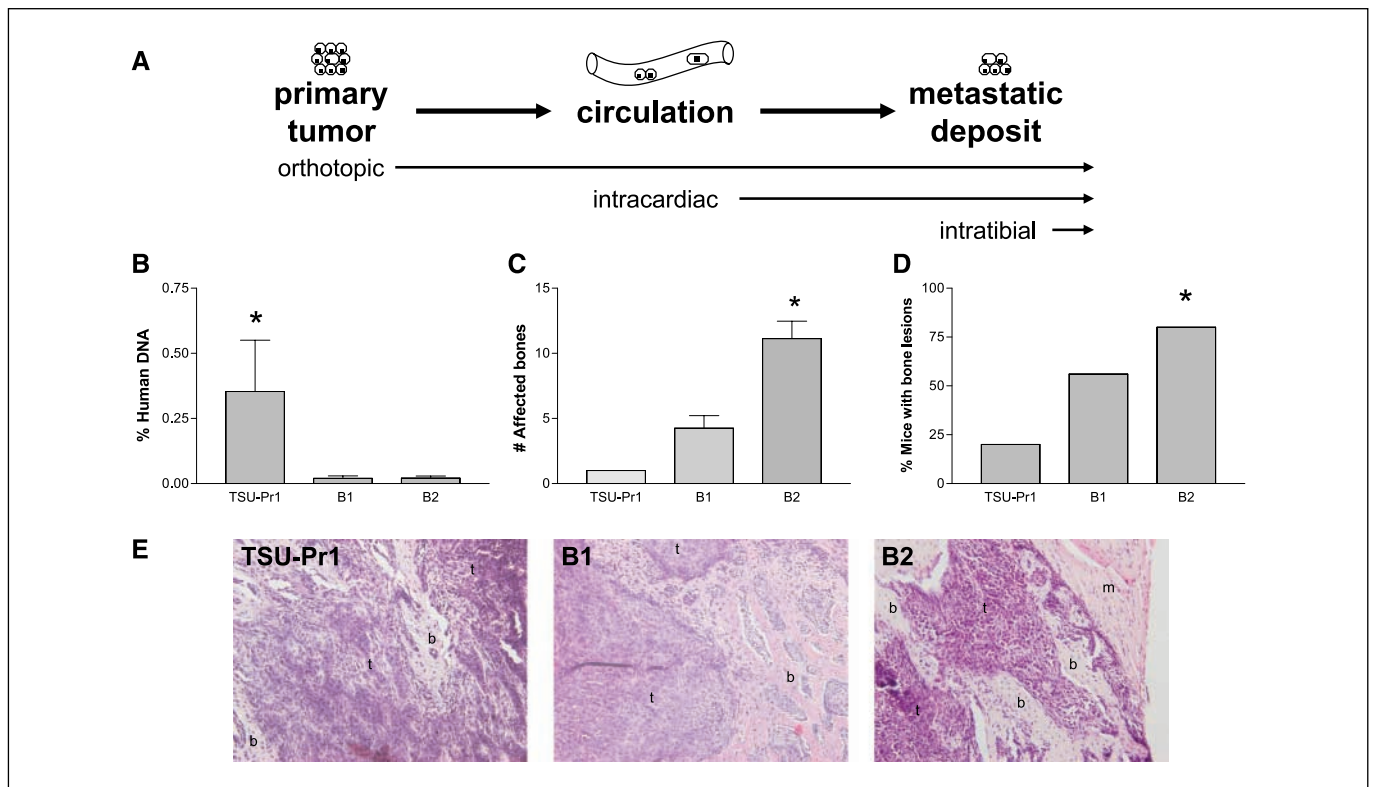


Figure 5. An epithelial phenotype is advantageous in the latter stages of the metastatic cascade. **A**, major stages of the metastatic cascade analyzed in this article and the corresponding modes of inoculation that model each stage. **B**, orthotopic inoculation of the TSU-Pr1 series ($n = 14-15$ per group). Micrometastatic deposits in lung were measured by Alu-PCR. Columns, mean; bars, SE. *, significant difference from B1 and B2 cells [nonparametric ANOVA ($P = 0.0461$) followed by Kruskal-Wallis *post hoc* test ($P < 0.05$ compared with TSU-Pr1)]. **C**, intracardiac inoculation of the TSU-Pr1 series ($n = 8-16$ per group). Lesions were monitored by X-ray and the number of affected bones was determined at the completion of the experiment. Columns, mean; bars, SE. Results were analyzed by one-way ANOVA and Tukey's *posttest*. *, $P < 0.001$, significantly different from TSU-Pr1 and B1. **D**, intratibial inoculation of the TSU-Pr1 series ($n = 9-10$ per group). The number of bone lesions was determined by X-ray at 7 weeks postinoculation. Results are expressed as percentage of mice with bone lesions. *, significant difference analyzed by χ^2 test ($P = 0.0262$) and χ^2 test for trend ($P = 0.0073$). **E**, hematoxylin and eosin-stained sections ($5 \mu\text{m}$) of TSU-Pr1, B1, and B2 tumors in bone following systemic inoculation. Magnification, $\times 20$. b, bone; m, muscle; t, tumor.

epithelia (39, 40). In addition, a switch from *FGFR2IIIb* to *FGFR2IIIc* expression has been reported in a small subset of prostate tumors (41), and both *FGFR2IIIb* and *FGFR2IIIc* are expressed in normal and malignant human oral squamous cells (40, 42, 43). Furthermore, independent studies show that up-regulation of either *FGFR2IIIb* or *FGFR2IIIc* is associated with malignancies of the ovarian surface epithelium, which is particularly interesting as these carcinomas exhibit properties of complex epithelial structures, including induction of the epithelial marker E-cadherin (42, 43). Clearly, there is a foundation supporting the expression of *FGFR2IIIc* in epithelial cells and epithelial carcinomas, which now includes the TSU-Pr1 series. Further work is needed to elucidate the function of *FGFR2IIIc* in epithelial cells.

Systemic (intracardiac) inoculation of the TSU-Pr1 series, together with the results from direct inoculation of these cell lines into a secondary site, illustrates that the B1 and B2 cells that exhibit epithelial characteristics have an enhanced ability to complete the latter stages of the metastatic cascade compared with the mesenchymal TSU-Pr1 cell line. In this study, we also showed that orthotopic inoculation of the TSU-Pr1 series resulted in behavior expected of an EMT model, in that the mesenchymal TSU-Pr1 cells exhibited the highest metastatic potential to lung, whereas the B1 and B2 cells were less capable of metastasizing from the orthotopic site. This is consistent with our hypothesis that secondary tumor formation is enhanced by the ability of epithelial cells to grow together as a cohesive mass, whereas escape from the primary tumor requires more invasive mesenchymal attributes. The underlying biology that permits epithelial-like cells to be more capable of forming macrometastatic deposits than mesenchymal-like cells is intriguing. Although further work is needed to elucidate this mechanism, it is worth noting that B1 and B2 cells still retain several mesenchymal markers, including vimentin expression, and that they have an up-regulated membrane type 1-matrix metalloproteinase/tissue inhibitor of metalloproteinase-2 axis compared with TSU-Pr1 cells (23). In addition, it has been shown in other

model systems that extravasation at the secondary site is not an essential prerequisite for the development of a metastatic deposit. Rather, tumor cells can remain in the blood vessel, clumping and sometimes dividing until they disrupt the vessel and grow into the surrounding tissue (44). Given the strategy for derivation of these cell lines, it is possible that the B1 and B2 cells do not have the necessary traits to perform the earlier stages of the metastatic cascade. In light of the importance of EMT in the early stages of cancer progression, and the potential therapeutics aiming to prevent EMT, our findings provide a further level of complexity where selection of an epithelial phenotype could actually be deleterious in patients with advanced malignancy. This could be balanced, however, with advances in strategies to block epithelialization of metastases, as residual metastatic disease is an important target of adjuvant therapy.

We have generated a model of carcinoma progression that shows an association between increasing metastatic ability from systemic inoculation and the acquisition of an epithelial phenotype. The TSU-Pr1 series is the first carcinoma model to capture the MET component of the metastatic cascade and shows that cellular plasticity is a critical factor in cancer progression and metastasis. Furthermore, we have shown that *FGFR2IIIc* is a critical determinant of the epithelial phenotype in this series. The identification of further key molecules driving MET in this system holds promise for the development of preventative and therapeutic strategies to minimize metastatic disease.

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