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Bladder cancer angiogenesis and metastasis—translation from murine model to clinical trial

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Abstract In the majority of cases, death from bladder cancer results from metastatic disease. Understanding the closely linked mechanisms of invasion, metastasis and angiogenesis in bladder cancer has allowed us to develop new therapeutic strategies that harbor the promise of decisive improvements in patient survival. The essential link between cell based experiments and the translation of novel agents into human patients with bladder cancer is the animal model. With emphasis on the orthotopic xenograft model, this review outlines some key mechanisms relevant to angiogenesis and the development of metastasis in bladder cancer. We highlight especially pathways related to MMP-9, IL-8, VEGF and EGFR. Most commonly, expression patterns of these markers in patients have correlated to disease progression and patient survival, which has led to laboratory investigations of these markers and eventually novel targeted therapies that are translated back into the clinic by means of clinical trials. Although imperfect in their translatability into clinical efficacy, animal models remain a critical tool in bladder cancer research.

Keywords Bladder cancer · EGFR · MMP-9 · IL-8 · VEGF · Metastasis

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1 Introduction

Bladder cancer is the fourth most common cancer and the ninth leading cause of cancer death in the United States [1]. There will be an estimated 67,160 new cases and 13,750 deaths in men and women in 2007, the vast majority of which are transitional cell carcinoma (TCC). More than 70% of the incidence is due to papillary, noninvasive tumors that recur in more than half of patients but progress to invasive disease only infrequently. Most of the mortality, on the other hand, occurs in the other 20–30% of patients who present with non-papillary, invasive TCC. These tumors can penetrate deeply through the bladder wall and demonstrate a high propensity for lymphatic and distant metastasis. These two clinically distinguishable entities—invasive and noninvasive ("superficial") TCC—are reflected in two divergent molecular pathways [2, 3].

Invasive tumors require aggressive therapy. There is increasing evidence that neoadjuvant chemotherapy offers an incremental improvement in survival in conjunction with radical cystectomy in patients with invasive TCC [4]. Although the data is more limited, there may be a similar benefit to adjuvant chemotherapy [5]. The response to chemotherapy is, unfortunately, most often not durable and the majority of patients fail in the form of metastatic disease. Patients with metastatic disease can no longer be cured by conventional cytotoxic therapy, and there remains a need for novel therapies to improve survival. In order to identify more suitable targets and develop more efficacious agents directed at these targets, we will need to advance our understanding of metastasis by investigating the cellular properties and tumor–host interactions that direct metastasis.

Angiogenesis is intricately involved in and is prerequisite for growth and metastasis [6, 7]. It is regulated by a fine balance between stimulatory and inhibitory factors pro-

duced by the tumor and the surrounding stroma [8]. Bladder tumors produce high levels of several stimulatory factors, including VEGF [9, 10], bFGF [11, 12], and IL-8 [13]. Microvessel density, a surrogate marker for angiogenesis [14], has been shown to be predictive of progression and poor prognosis in invasive TCC [15–20].

The mechanisms of invasion, metastasis and angiogenesis in TCC of the bladder are the focus of this review. We will emphasize the role of animal models of TCC and attempt to demonstrate how these models bridge the gap between tissue culture and patient. We will also highlight our own work in targeting the epidermal growth factor receptor (EGFR) in bladder cancer as an example of the progress that has been made. The concepts and principles of this research are those championed by Dr. Isaiah J. Fidler in his 40 years of metastasis research.

2 Animal models of bladder cancer

Studying the molecular mechanisms of cancer development in tissue culture is limited by the artificial nature of the system and the isolation of the tumor cells from their microenvironment. *In vitro* techniques for the study of stromal–epithelial interactions are advancing rapidly, but animal models remain an essential extension of *in vitro* testing. Especially the study of metastasis, which is a carefully orchestrated process of multiple sequential steps, each of which is dependent on interaction with the microenvironment, requires animal models [21]. Testing novel agents in animal models is also important because it requires consideration of drug delivery, pharmacokinetics and potential toxicity. Findings made in the animal model are not always directly translatable into clinical practice, but they have provided important advancements in our understanding of tumor growth and metastasis [22].

2.1 Orthotopic xenografts

Xenograft modeling involves the implantation of human tumor cells into an immunodeficient animal, most commonly the athymic nude mouse. A prerequisite to xenograft modeling in TCC is the availability of multiple different TCC cell lines. Many different lines are being used by different groups for the study of urothelial carcinogenesis [23], most of which are derived from patients with invasive tumors. One question to be addressed is whether the cell lines are truly representative of typical human bladder cancer. Sanchez-Carbayo et al. performed cDNA microarray analysis on nine common TCC cell lines and validated resultant target genes on a tissue microarray of 193 patient tumors [24]. The necessary next step will be to analyze xenografts in a similar fashion to compare them to molecular profiles of human cancers, as has been done with comparative functional genomics for transgenic models of hepatocellular carcinoma [25].

Many researchers continue to report on findings from ectopic models, in which the tumor cells are injected subcutaneously. Fidler and others have emphasized the importance of orthotopic modeling, in which the tumor cells are injected into the organ from which they were derived [26–29]. The tumorigenic and metastatic potential of human tumors depends on inherent characteristics of the tumor cells, but also on the tumor environment and therefore the site of injection [30]. Fidler describes the heterogeneity of human tumors, whereby numerous clonal subpopulations arise within a tumor, all of which have differential abilities to invade and metastasize and different sensitivities to treatment [21]. All of these features—invasion, metastasis and response to treatment—are influenced by interactions between the tumor cells and the surrounding microenvironment, so that their study must be performed in models that mimic the human disease as closely as possible.

We established in 1995 the first reliable orthotopic model of bladder cancer by direct intramural implantation of the human 253J TCC cells into the bladder of athymic nude mice [30]. Subpopulations of the parental cell line were selected by *in vivo* recycling of bladder tumors and these were shown to have enhanced tumorigenic and metastatic potential. After five serial passages through the bladder, the resultant metastatic variant line, 253J B-V, was shown to have unique karyotypic alterations, increased expression of EGFR, increased anchorage-independent growth, increased expression of IL-8 and MMP-9, and an enhanced ability to migrate through matrigel [13, 30]. These molecular features of invasion and metastasis will be discussed in detail below.

The important differences between orthotopic and ectopic tumor inoculation were subsequently demonstrated using the same 253J B-V cells [31]. After 28 days of tumor growth either in the bladder or the subcutis, tumorigenicity and metastasis were evaluated, and markers of invasion, metastasis and angiogenesis were measured. The tumor size was similar in both sites, but only tumors growing orthotopically in the bladder developed metastasis to lymph nodes and lung. The orthotopic tumors, when compared to the subcutaneous tumors, had an increased microvessel density and a corresponding increase in VEGF and bFGF expression and MMP-9 activity. Together these findings demonstrate the importance of microenvironment on tumor cells and their ability to metastasize.

253J B-V is just one example of an orthotopic model. We are able to establish orthotopic models with multiple TCC cell lines, thereby allowing us to study molecular mechanisms in different tumors. In addition, we have added

luciferase imaging for monitoring tumor growth and the appearance of metastasis over time. There is also a second orthotopic model of bladder cancer established by intraluminal instillation of cells through a urethral catheter. A unique feature in the management of bladder cancer is the ability to deliver therapeutic agents into the lumen of the bladder, thereby avoiding systemic toxicity. This modality is used only for noninvasive tumors, since drugs delivered in this fashion will not penetrate deep into the bladder wall. This usually requires the application of electrocautery, trypsin or another chemical agent prior to cell instillation in order prepare the urothelium for implantation. Benedict has established this model using GFP for imaging tumor burden [32–36] and we have modified the same model to utilize luminescent imaging with luciferase [37].

2.2 Alternative bladder cancer models: syngeneic and transgenic

Other important models of bladder cancer include chemically induced cancer, syngeneic models and transgenic models. Chemically induced bladder cancer in rats and mice is utilized particularly for chemoprevention studies [38], although novel treatments and molecular mechanisms [39] can also be tested. The three most commonly used carcinogens are *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN), *N*-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide (FANFT) and *N*-Methyl-*N*-nitrosurea (MNU). Importantly, these agents are specific to the urothelium and they cause tumors in 100% of mice. Disadvantages in these models include the prolonged period required for tumor development (8–12 months) and the exposure of lab personnel to hazardous chemicals.

In syngeneic models, mouse tumors are inoculated in mice of the same strain from which the tumor cells were originally derived. The murine bladder tumor (MBT) cell lines are commonly used for syngeneic models. The advantages include low cost, reproducibility and an immunocompetent host, which is important, for example, in studying intravesical BCG treatment of bladder cancer [40]. The disadvantage is that the tumors are murine tumors and not of human origin. The tumor biology may be different, the therapeutic targets needed to be adapted to the human homologues and overall the models are one step further from clinical applicability than human xenograft models.

Transgenic tumor models are very important in bladder cancer research. Their use has been instrumental in elucidating the divergent molecular pathways of noninvasive ("superficial"), papillary and invasive, non-papillary bladder cancer [2, 3]. The most commonly used examples utilize mutation of H-ras to induce noninvasive tumors [41] and SV40 large T antigen expression to inactivate both Rb and p53 with resultant growth of invasive tumors [42]. Both are driven by a bladder specific uroplakin promoter, or less often a cytokeratin 19 promoter, so that tumors are seen only in the bladder [42]. The engineered molecular changes driving growth of bladder tumors in these models are the same as the predominant changes found in patient tumors of the same type [43]. Newer and more sophisticated techniques of genetic engineering, including site specific inducible mutagenesis are being developed [44].

The transgenic model facilitates the investigation of individual genetic defects in carcinogenesis. The invasive tumors also metastasize, so that metastasis can be studied in this model. The model is valuable for the study of stromal–epithelial interactions, although both components are of murine origin. Limitations include the requirement of an artificial promoter and the decreased cellular heterogeneity seen in human bladder tumors [45]. This lack of heterogeneity may have significant impact on tumor progression and metastasis.

3 Metastasis genes in bladder cancer

The orthotopic bladder cancer model revealed some important molecular characteristics important in bladder cancer invasion and metastasis [30]. These included increased expression of MMP-9, IL-8, VEGF and EGFR. The same markers in patient bladder tumors have proven to be correlate with stage and outcome [46, 47].

3.1 MMP-9 expression and progression of bladder cancer

The matrix metalloproteinase (MMP) family of extracellular proteinases plays a central role in normal physiologic processes, in benign disease and in malignancies. MMP-9 and potentially other MMPs are important for tumor-induced angiogenesis, tumor invasion and establishment of metastasis at the secondary site [48]. Once metastasizing cells have detached from the primary tumor, degraded the extracellular matrix (ECM), and invaded the stroma they must gain access to the circulation and lodge at a distant site [6]. There, they must induce their own blood supply if they are to become established at that site [49, 50]. Although invasion and angiogenesis are very different processes, both invasion and angiogenesis require alteration of the extracellular matrix and basement membrane by proteases such as MMP-9 [49, 51, 52]. In addition, it has become apparent over the last decade that MMPs do much more than simply break down the ECM. Their role in the proteolytic processing of different growth factors, growth factor receptors and cytokines has been recognized [53]. Furthermore, although MMP-9 can be detected in cancer cells, it is known to be possibly even more important

coming from the surrounding stroma [54] or bone marrow derived cells [55].

Increased expression of MMP-9 was one of the first detected differences identified in metastatic 253J B-V bladder cancer cells compared to their non-metastatic counterparts and normal mucosa [30]. In a different murine model, after intracardiac injection of bladder cancer cells, serial recycling of bone metastases revealed increasing levels of MT1-MMP, MT2-MMP, MMP-9 and TIMP-2 that correlated with metastatic potential [56]. Kawamata et al. demonstrated in a syngeneic orthotopic rat model that MMP-9 expressing, non-metastatic bladder cancer cells could be made metastatic by induced MMP-2 over-expression [57]. The use of MMP-2 [58] and MMP-9 [59] knockout mice has further consolidated the importance of both in metastasis. Especially in the MMP-9 knockout mice there was a marked reduction in spontaneous metastasis using different tumor types when compared to wild-type mice [59, 60].

Targeting MMP-2 or MMP-9 has only been tested in one animal study of bladder cancer. This study used Halofuginone, a specific inhibitor of MMP-2 at the level of gene transcription [61]. *In vitro* this agent inhibited invasion of mouse (MBT2) and human (5637) bladder cancer cells. *In vivo*, there was a reduced rate of lung metastasis after intravenous injection of MBT2 cells pretreated with halofuginone.

The preclinical evidence supporting a role of MMP-9 in metastasis has been validated in a variety of human malignancies, including bladder cancer, where MMP-9 expression correlates directly with tumor grade, invasion and metastasis [48, 62–66]. Kawamura et al. have shown an association between *in situ* gelatinolytic activity in patient tumor samples and tumor grade, stage, vascularity and disease specific survival [67]. This activity, however, was demonstrated to correlate best with MMP-2 and not MMP-9 expression. The balance in the expression of MMP-9 and its natural tissue inhibitor TIMP-2 appears to be important for predicting the metastatic potential of TCC [68]. We have also found that the balance between MMP-9 and E-cadherin is highly predictive of disease-specific mortality after neoadjuvant chemotherapy and radical cystectomy in patients with locally advanced tumors, indicating that the MMP-9:E-cadherin ratio is a key feature of the metastatic phenotype [46]. Most recently we have shown that polymorphisms in the promoter of MMP-9 are associated with bladder cancer invasiveness [69]. The functional importance of these polymorphisms remains to be determined.

Although the MMPs appear to be critical in many aspects of carcinogenesis and metastasis, specifically targeting them for therapy in bladder cancer has not, to our knowledge, been attempted, and has largely failed in other malignancies up to this point [70]. A complicating factor in targeting MMPs is that numerous MMPs work in concert at different stages of the metastatic process and in part oppose each other in their effects. MMP-7, for example, may have a protective role in tumorigenesis [48]. In addition, MMPs are involved in homeostasis in virtually all normal tissues in the body, so that the potential for adverse effects is high. The numerous functions of MMPs need to be better understood before we will be able to target them effectively in cancer therapy.

3.2 Interleukin 8

IL-8 was originally described as a neutrophil chemoattractant [71] but was subsequently found to possess mitogenic, motogenic and angiogenic properties [72]. The mechanism of IL-8 activity is believed to be related to autocrine and paracrine loops [13, 73, 74]. IL-8 produced by tumor cells stimulates both tumor and stromal cells to express angiogenesis-related factors, thus promoting endothelial cell proliferation, tumor growth, and metastasis [13, 73]. IL-8 has also been shown to act directly on vascular endothelial cells to promote survival [75, 76].

Over-expression of IL-8 correlates with tumor stage as well as disease progression and recurrence in multiple different cancers [77]. In some of the same cancers, there is also a direct correlation between IL-8 and both angiogenesis and metastasis in xenograft models [78, 79]. In patients with bladder cancer, IL-8 expression was increased in muscle-invasive tumors and carcinoma *in situ* when compared with noninvasive papillary tumors [47].

Inoue et al. characterized IL-8 in the orthotopic bladder cancer xenograft model [13]. The non-tumorigenic 253 J-P was stably transfected with IL-8 and was subsequently found to be tumorigenic and to develop spontaneous metastases. Furthermore, silencing IL-8 with a specific anti-sense construct in the highly metastatic and IL-8 over-expressing 253J B-V diminished this cell line's tumorigenicity and metastatic potential [13]. Expression levels of VEGF and bFGF were not altered with manipulation of IL-8. The level of MMP-9, however, increased with sense IL-8 transfection and decreased with antisense IL-8 transfection. This is similar to findings by Luca et al. linking IL-8 to MMP-2 in melanoma [73].

IL-8 was subsequently evaluated as a therapeutic target using a fully human anti-IL-8 antibody (ABX-IL8, Abgenix, Inc., Fremont, CA) [80]. ABX-IL8 binds and neutralizes IL-8. It had no effect on growth *in vitro*, even in cells with high levels of IL-8 expression, but did inhibit invasion through a matrigel coated membrane. This was associated with a decrease in MMP-2 and MMP-9 expression and activity, which, in turn, was associated with diminished expression and transcriptional activity of NF-κB. NF-κB acts as a mediator between IL-8 and MMP expression. *In vivo*, there

was a significant decrease in tumor growth with ABX-IL8 treatment. The differential effect on tumor, stromal and endothelial cells was not elucidated in this study and requires further investigation. These experiments are a useful example of how a targeted agent may inhibit growth *in vivo* but not *in vitro*, where the tumor cells are isolated from their microenvironment. Unfortunately, Abgenix discontinued development of ABX-IL8 after its poor performance in clinical trials treating psoriasis and rheumatoid arthritis.

3.3 VEGF

Rapid tumor growth is thought to cause local hypoxia which triggers the release of pro-angiogenic factors, of which VEGF is likely the best studied in a broad spectrum of tumor types. Evidence from both patient tumors and preclinical models indicates that VEGF is also a key mediator of angiogenesis in bladder cancer.

Crew et al. investigated VEGF expression at the RNA level in patients with low or intermediate grade T1 bladder cancer [9]. T1 tumors are often referred to as "superficial" but they are invasive into the lamina propria and are biologically more similar to muscle-invasive disease than lower stage papillary disease (Ta). They are usually treated with bladder preservation, however, so that the ability to predict progression to deeper invasion and/or metastasis is particularly relevant. Crew et al. found that higher levels of VEGF RNA expression predicted earlier recurrence and an increased risk of progression. In another study with a broad spectrum of tumor stages, VEGF expression by immunohistochemistry increased with increasing stage [81]. Similarly, serum levels of VEGF in 58 patients with invasive and noninvasive bladder cancer were found to correlate with stage, grade, vascular invasion and the presence of carcinoma *in situ* [82]. A VEGF level in the serum of ≥400 pg/ml was found to be highly predictive of metastatic disease.

We evaluated the expression of VEGF and other metastasis-related genes in patients with advanced bladder cancer who had undergone neoadjuvant combination chemotherapy, investigating whether these genes could predict nonresponders [46]. The pretreatment expression levels of VEGF and E-cadherin but not IL-8, MMP-9 or bFGF correlated with disease-specific survival after chemotherapy and cystectomy. This led us to the conclusion that VEGF may be an important target in bladder cancer.

VEGF, however, is not the only target in the VEGF signaling pathway. VEGF acts on two principle tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR), both of which are over-expressed in most tumor vasculature and therefore represent attractive targets . VEGFR3 (Flt-4) appears to be more important in lymphangiogenesis. More recent evidence indicates that VEGFR2 is expressed also in urothelial carcinoma cell lines [83] and bladder tumors [84], where its expression level correlate to pathologic stage. Targeting VEGFR2 therefore has the potential to affect both the tumor cells and the blood vessels.

We investigated the effects of a neutralizing monoclonal antibody targeted at murine VEGFR2 (DC101, ImClone Systems, New York, NY) in the orthotopic xenograft model [85]. In combination with paclitaxel this agent was found to impair tumor growth and vascularity markedly, as well to prevent metastatic spread to the lymph nodes and to prolong mouse survival. DC101 affected primarily the smaller immature vessels at the periphery of the tumors and not the larger established vessels in the center of the tumors [86]. Enhanced apoptosis was observed in both tumor and the endothelial cells. This study provides proof of principle for this treatment strategy. A phase I clinical trial with the equivalent antibody (IMC-1C11) targeting human VEGFR2 was subsequently successful in patients with metastatic colorectal carcinoma [87] but has not been developed further.

Another method of targeting the VEGFR is through a fusion protein of VEGF121, one splice variant of VEGF-A, and gelonin (rGel), a plant toxin that is cytotoxic at nanomolar concentrations. This VEGF121/rGel construct had little effect on the highly metastatic 253J B-V cells *in vitro* but it did suppress tumor growth in orthotopic xenografts [88]. On immunohistochemical analysis, a very high expression of VEGFR2 was detected in the tumor vasculature. Immunofluorescent studies demonstrated colocalization of the rGel and CD31, and there was increased apoptosis of the endothelial cells. The efficacy of this fusion protein has also been shown in the prevention of metastasis in prostate [89] and breast [90] cancer and is pending phase I clinical investigation at MD Anderson (M. Rosenblum).

The most widely investigated agent targeting the VEGF/VEGFR pathway is bevacizumab, a humanized monoclonal antibody that binds to and neutralizes all isoforms of VEGF-A. It has been proven effective in multiple phase III clinical trials at different tumor sites including colorectal [91] and renal cell carcinoma [92], and has been FDA approved since 2004. At MD Anderson we have designed a phase II clinical trial (A.O. Siefker-Radtke) investigating the use of neoadjuvant high-dose MVAC plus bevacizumab in patients with locally advanced but resectable TCC. The neoadjuvant trial design will allow us to investigate biological markers of response in the cystectomy specimens. This clinical trial stems directly from preclinical and retrospective clinical data on the important role of VEGF in bladder cancer.

The group at the Medical University of South Carolina is recruiting patients to a different neoadjuvant clinical trial (NCT00268450). In this single-arm phase II trial for patients with locally advanced but resectable bladder

cancer, all patients receive 4 cycles of bevacizumab in addition to cisplatin and gemcitabine prior to cystectomy [93]. Patients with residual disease at the time of surgery are given adjuvant therapy with paclitaxel and bevacixumab. Three additional trials are investigating the use of bevacizumab in more advanced disease. One of these (NCT00098592) is interesting because it combines a VEGF-neutralizing agent (bevacizumab) with a receptor inhibitor (sorafenib).

VEGFR2 is often one of the key receptors targeted by a group of inhibitors that are directed at multiple tyrosine kinases. These agents are better able to overcome the cross-talk and redundancy in downstream signaling that can lead to resistance to an agent targeting a single receptor. The most extensively studied of this group are sunitinib and sorafenib, both of which have attained FDA approval. Little preclinical work has been performed in bladder cancer with these agents, but the accumulation of evidence is compelling that they warrant investigation, and several clinical trials in advanced bladder cancer are currently underway. Sunitinib is particularly promising because it also targets PDGFR-β, which we have demonstrated to be important in bladder cancer [94].

4 EGFR in bladder cancer

4.1 Rationale for targeting EGFR

Clinical studies evaluating the significance of EGFR expression in human bladder cancer have shown that more than 50% of tumors over-express EGFR and that the level of expression directly correlates with tumor grade, stage, and survival [95–97]. In noninvasive TCC, EGFR expression also predicts disease progression to muscle invasive or metastatic TCC and is an independent prognostic factor for death in a multivariate analysis [95]. In muscle-invasive TCC, EGFR expression also has prognostic significance, such that patients with tumors over-expressing EGFR have only a 20% probability of long term cancer-specific survival. The majority of metastases from patients with TCC over-express EGFR, and this expression is not decreased by chemotherapy or radiation [98]. These studies establish the importance of EGFR over-expression in the development and progression of human urothelial carcinoma and offer compelling rationale to pursue EGFR as a target of treatment.

The relevance of EGFR in bladder cancer is also evident in preclinical models. One of the genes over-expressed in the invasive and metastatic 253J B-V cell line after serial recycling in the mouse was EGFR [30]. Further studies of this and other cell lines both *in vitro* and in the orthotopic xenografts have confirmed that EGFR regulates cell proliferation, angiogenesis, invasion and metastasis. In transgenic models of bladder cancer, EGFR over-expression under the control of the uroplakin promoter induces urothelial hyperplasia. Crossing EGFR over-expressing mice with mice that have p53 and Rb inactivated induces high-grade but noninvasive tumors [99].

4.2 Targeting EGFR: preclinical investigation

Most urothelial cell lines express abundant EGFR [94] and a subset of these cells are sensitive to EGFR inhibition with either the small-molecule tyrosine kinase antagonist gefitinib or the humanized blocking anti-EGFR antibody cetuximab [94, 100]. Both agents have been shown to inhibit tumor growth in the orthotopic bladder xenograft model [101, 102], strongly suggesting that EGFR inhibitors could have significant clinical activity in patients with bladder cancer.

The anti-neoplastic effect of EGFR has been shown to be due at least in part to an inhibition of angiogenesis [103]. VEGF, IL-8 and MMP-9 are all downstream effectors of EGFR stimulation, and, as has been described above, all three have been shown to be closely correlated to bladder cancer progression. This has led us to focus on the mechanisms by which EGFR blockade inhibits angiogenesis and subsequent tumor growth and metastasis.

We measured VEGF, IL-8 and bFGF production by 253J B-V grown in culture with and without cetuximab [102]. All three pro-angiogenic markers were reduced with EGFR blockade. In the orthotopic xenograft model, the expression of the same markers was reduced, as assessed by immunohistochemistry and *in situ* hybridization, and there was a concomitant reduction in microvessel density. By harvesting some tumors at an intermediate time-point, we were able to show that down-regulation of the pro-angiogenic factors preceded the involution of blood vessels. Overall tumor growth was suppressed and the rate of nodal metastasis was decreased from 100% to 0%. These studies made it clear that the growth inhibitory effect of EGFR blockade in bladder cancer was at least in part due to an inhibition of angiogenesis. These findings were augmented by combination of EGFR blockade with paclitaxel [104].

We have established that inhibition of EGFR is accompanied by destruction of mature tumor vessels in 253J B-V xenografts. On the basis of reports indicating that recruitment of pericytes to the endothelial cells of tumor vessels is required to maintain vessel survival, we investigated vessel integrity after gefitinib treatment *in vivo*. Interestingly, the architectural structure of the tumor vessels was significantly disturbed and pericycte coverage of vessels was lost after EGFR blockade in sensitive tumors. However, EGFR-resistant tumors such as UM-UC13 and UM-UC3 did not display these modifications (unpublished data).

In anticipation of clinical trials of EGFR-targeting agents in bladder cancer we have attempted to define markers *in vitro* that predict response to EGFR inhibition. Some of the disappointment related to the early clinical trials using gefitinib was related to patient selection. Mutations in the tyrosine kinase domain of EGFR have been found to correlate with increased sensitivity to EGFR-targeted therapy in patients with non-small cell carcinoma of the lung [105], which has led to renewed interest in this treatment strategy and has focused researchers on seeking markers of response to better select patients for future clinical trials.

We have shown that EGFR mutations are not found with a meaningful frequency in bladder tumors or cell lines [106]. We have identified alternative growth factor receptors, especially PDGFR-β [94] and HER4 (unpublished), that are expressed only in cell lines resistant to the anti-proliferative effects of cetuximab, and we are investigating the mechanisms related to these associations. PDGFR-β signaling appears to short circuit the EGFR/MAPK pathway for mitogenic stimuli in a subset of resistant cell lines [94]. Downstream activation of GSK-β and degradation of its target cyclin D1 were indicative of sensitivity to gefitinib. These changes were observed in the PDGFR-β-dependent cell lines only after treatment with a chemical inhibitor of PDGFR-β. In separate studies, p27(Kip1) accumulation and decreased CDK2 activity were observed only in cell lines that were sensitive to the anti-proliferative effects of gefitinib [100]. For both cetuximab and gefitinib, loss of E-cadherin expression correlated with a poor response to inhibition [100]. These markers require validation in clinical trials.

These observations also provide strong support for the idea that a better understanding of the basic biological effects of these drugs in solid tumors will enable us to prospectively identify subsets of patients who will obtain the most clinical benefit from EGFR inhibitors and, by extension, from other biological therapies.

4.3 Targeting EGFR: clinical translation

The EGFR has been a high-priority biological target in cancer therapy for several years. Cetuximab, gefitinib and erlotinib, another small-molecule inhibitor of the receptor tyrosine kinase, have been evaluated in several phase I–III clinical trials for disease at various sites. Results have been mixed but all three drugs have received approval from the FDA. Subsequent poor performance by gefitinib in large phase III clinical trials [107] has led to its restricted approval by the FDA whereby it may only be used within a clinical trial or in patients currently receiving benefit from treatment with the drug. All three drugs are being tested in clinical trials involving patients with bladder cancer.

The effects of combined EGFR blockade and paclitaxel therapy on angiogenesis and down-regulation of VEGF and MMP-9 in the xenograft studies compelled us to pursue this combination in patients with minimal residual metastatic bladder cancer after frontline systemic chemotherapy. A phase II trial was started in 2004 in which patients with metastatic or unresectable local disease who responded to initial cytotoxic chemotherapy are randomized to consolidative treatment including docetaxel with or without gefitinib. The objectives of this trial are to compare progression-free survival rates at 9 months after the start of consolidation therapy. Time to progression and overall survival will also be analyzed. Additional translational studies will use patient tumor tissue, urine and serum to quantify target inhibition and molecular markers of response (e.g., urinary VEGF or MMP-9 and tissue expression of EGFR, p-EGFR, p-MAPK, pAKT, GSKβ, p27) before, during, and after therapy.

Also in an effort to translate xenograft studies into the clinic, we have designed a prospective randomized trial of neoadjuvant EGFR blockade in patients with bladder cancer requiring cystectomy but not conventional neoadjuvant chemotherapy due to low risk of progression. An important component of this trial will again be to test the effects of EGFR inhibition on pharmacodynamic markers in the primary tumors. Thus, we are in a unique position to directly test the validity of the hypotheses we have generated in our preclinical studies and to exploit them in the design of more effective, EGFR-based therapeutic strategies.

We have two overall hypotheses that will be tested in the studies proposed as part of this trial. The first is that quantification of changes in the expression of active EGFR, cyclin D1, p27, and GSK-β will allow us to identify tumors that are dependent on EGFR-mediated signaling for cell cycle progression. The second is that quantification of tumor proliferation (PCNA) and cell death *in vivo* can be used to identify tumors that are responding from those that are not. Tumor cell death may result either from direct effects on tumor cells or indirect effects of angiogenesis inhibition, so we will also monitor measurements of angiogenesis inhibition (VEGF, IL-8, microvessel density and endothelial cell apoptosis) to determine whether changes in angiogenesis factor expression correlate with decreases in microvessel density, and occur independently of changes in the proliferation-associated markers. Ultimately, we hope to use the knowledge gained from this descriptive study to identify appropriate patients for EGFR-directed or other selective targeted therapies.

5 Conclusions

Understanding the mechanisms of invasion, metastasis and angiogenesis in bladder cancer sets the stage for the development of novel targeted agents that are better able

to inhibit these molecular pathways and thereby deliver a therapeutic benefit to patients with advanced disease. Animal models are at the center of the laboratory research designed to elucidate these mechanisms, and are at the same time the bridge to the clinic. The complex biological processes involved in metastasis cannot be studied adequately in isolation outside the living organism. At the same time, animal studies are not completely representative of the human disease and there are still challenges in translating knowledge gained from the models in to clinical applicability.

In this review we have concentrated on a only few molecules involved in metastasis in an attempt to highlight the use of orthotopic xenograft models. The route of progress is often one of discovering features in patient tumors that are brought into the lab for study in controlled models. These models, including primarily cell culture and animal models, lead to the design of new targeted agents that are then returned to the bedside in the form of clinical trials. This route is perhaps best exemplified in bladder cancer by EGFR, where an association between EGFR expression and patient outcome led to interest in EGFR as a target. This target was validated in TCC cell lines and orthotopic xenografts. Studies were done to characterize predictors of response *in vitro* and now the targeted agents are being utilized in clinical trials. The principles are similar for the VEGF/VEGFR pathway, for which multiple inhibitors are entering clinical trials. IL8 is a less established target that may receive more attention in the future, and targeting MMP-9 remains promising but is currently fraught with confounding problems. In each case, however, the findings in xenograft models have been essential to advancing the development of the target.

Focusing on a few molecules and pathways necessarily neglects other important molecules and pathways. P53 alterations, for example, are one of the hallmarks of invasive bladder cancer and are intimately linked with multiple facets of invasion [108], angiogenesis [109] and metastasis [110]. Even VEGF is a downstream mediator of the effects of p53 tumor suppression [9]. P53, like the other factors reviewed here, has become an important target of novel drug development [43]. E-cadherin is another molecule vitally important to progression of bladder cancer [111]. Reduced levels of E-cadherin have been shown to be associated with poor prognosis in invasive bladder cancer [111–114]. Gene profiling studies have confirmed loss of E-cadherin as a predictor of higher stage and grade and of poorer survival [24, 115]. E-cadherin is itself not a targetable molecule, but mediators of its expression may be.

Moving forward, both xenograft and transgenic models will require validation with molecular profiling techniques to ensure that they are representative of human bladder cancer. There appears to be a shift towards greater acceptance of transgenic models which may in the future gain wider use in mechanistic and therapeutic studies. Also, high throughput technologies related to proteomics, genomics and kinomics may enhance the utility of preclinical models, not only in their validation, but also in their applicability. Patient response to chemotherapy in bladder cancer, for example, can be predicted based on cDNA profiling of bladder cancer cell lines exposed to the same cytotoxic drugs [116]. Most elementary of all is perhaps the concept of combined therapy to target multiple cellular signaling pathways. Ultimately there is reason for optimism that our understanding of metastasis will continue to grow at a rapid pace and our therapeutic choices will allow us to control if not cure most cases of advanced bladder cancer.

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