Epithelial Plasticity, Cancer Stem Cells, and the Tumor-Supportive Stroma in Bladder Carcinoma

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Abstract

High recurrence rates and poor survival rates of metastatic bladder cancer emphasize the need for a drug that can prevent and/or treat bladder cancer progression and metastasis formation. Accumulating evidence suggests that cancer stem/progenitor cells are involved in tumor relapse and therapy resistance in urothelial carcinoma. These cells seem less affected by the antiproliferative therapies, as they are largely quiescent, have an increased DNA damage response, reside in difficult-to-reach, protective cancer stem cell niches and express ABC transporters that can efflux drugs from the cells. Recent studies have shown that epithelial-to-mesenchymal transition (EMT), a process in which sessile, epithelial cells switch to a motile, mesenchymal phenotype may render cancer cells with cancer stem cells properties and/or stimulate the expansion of this malignant cellular subpopulation. As cancer cells undergo EMT, invasiveness, drug resistance, angiogenesis, and metastatic ability seem to increase in parallel, thus giving rise to a more aggressive tumor type. Furthermore, the tumor microenvironment (tumor-associated stromal cells, extracellular matrix) plays a key role in tumorigenesis, tumor progression, and metastasis formation. Taken together, the secret for more effective cancer therapies might lie in developing and combining therapeutic strategies that also target cancer stem/progenitor cells and create an inhospitable microenvironment for highly malignant bladder cancer cells. This review will focus on the current concepts about the role of cancer stem cells, epithelial plasticity, and the supportive stroma in bladder carcinoma. The potential implications for the development of novel bladder cancer therapy will be discussed. Mol Cancer Res; 10(8); 995–1009. ©2012 AACR.

Bladder Cancer

Worldwide, an estimated number of 386,300 new cases and 150,200 deaths from bladder cancer occurred in 2008, with the majority of cases (77%) occurring in men. Thereby, bladder cancer is the seventh most common cancer and the ninth most common cause of cancer-related death in men. Bladder cancer is most common in developed countries, where 63% of the cases occurred in 2002. In Europe and the United States, for example, bladder cancer is the fourth most common cancer in males. Overall, the 5-year survival rate of bladder cancer is approximately 80%, and it varies with the stage of the tumor, ranging from 97% for carcinoma in situ to 6% for metastatic disease.

Urothelial carcinoma is a heterogeneous disease and multifocal tumors can develop in the same patient. Multifocality can be explained by the monoclonal theory, that is, multiple tumors arise from a single transformed cell or by the field cancerization theory (1). In the latter, exposure of the urothelium to important risk factors for bladder cancer, such as occupational carcinogens induces changes at different sites of the urothelium. As described by Cheng and colleagues (1), urothelial carcinoma in situ is a well-established precursor of invasive bladder cancer and may be caused by field cancerization. Depending upon the genetic analyses used such as evaluation of LOH using microsatellite markers, one can find evidence for either clonal origin or the field effect (2, 3). However, these 2 theories are not mutually exclusive, both field cancerization and monoclonal tumors may coexist in the same patient.

Urothelial carcinoma can arise through 2 independent pathways, the noninvasive, papillary pathway and the invasive pathway (4). Approximately 80% of bladder cancers arise through the papillary pathway, in which hyperplastic urothelium grows toward the bladder lumen. These tumors can be successfully treated by transurethral resections but have a tendency to recur and therefore patients require intensive surveillance after treatment, making bladder cancer one of the most expensive cancers to manage. Despite the frequent recurrence (occurring in up to 80% of cases), the majority of papillary bladder tumors are not lethal because only approximately 15% will progress into invasive, metastatic tumors (5). The muscle-invasive bladder cancers, which make up the resulting 20% of the bladder tumors, arise from severe dysplasia or carcinoma in situ. These tumors are more aggressive, and approximately half the patients will progress to metastatic disease of which the 5-year survival rate is only 5% (5, 6).
Genetic alterations occurring in papillary tumors are most frequently caused by activating mutations of proto-oncogenes, of which fibroblast growth factor receptor 3 (FGFR3) and HRAS are most prevalently mutated, with mutations in respectively up to 75% and 30% of the papillary tumors (4, 7, 8). Because both oncogenes activate the RAS/MEK/ERK signaling pathway, they appear to be mutually exclusive (8).

In contrast, the majority of invasive carcinomas arise through inactivation of the tumor suppressor pathways of TP53, RB1, and PTEN (4, 9). These mutations result in genomic instability and an antiapoptotic phenotype, which enables tumor progression through accumulation of mutations (7).

Other mutations that are observed in both papillary and invasive tumors are mutations of phosphoinositide 3-kinase (PI3K, >10%) and deletion of the tumor suppressor genes Tuberous Sclerosis 1 (TSC1, 10%), Patched (PTCH, 40% LOH), CDKN2A, and Deleted in Bladder Cancer 1 (DBC1, 50%; reviewed in ref. 7).

Chan and colleagues identified a subpopulation of cells with a unique bladder cancer stem/progenitor-like gene signature, which was defined by its enriched ability to induce xenografts tumors in vivo (10). Using this signature, it is possible to distinguish between non–muscle-invasive (superficial) cancer and muscle-invasive bladder cancer (10). The functional and molecular differences between papillary and invasive bladder cancer could be correlated to the frequency of mesenchymal, bladder cancer stem/progenitor-like cells in these tumors (1).

In addition, it is believed that the transition from an epithelial phenotype into a mesenchymal phenotype, the epithelial–mesenchymal transition (EMT), increases the invasiveness, metastatic ability, and drug resistance of tumors, therefore giving rise to a more aggressive tumor type.

This review will describe the biologic and molecular insights in the pathogenesis of bladder cancer, including the relevance of cancer stem cell (CSC) and epithelial plasticity (EMT). Furthermore, the potential implications of EMT and CSCs for bladder cancer therapy will be discussed.

**CSCs/Tumor-Initiating Cells**

The CSC hypothesis is an important concept arising in cancer research. This hypothesis postulates the existence of a subgroup of cancer cells, the CSCs, which has the ability to self-renew and to differentiate into all cell types of the original heterogeneous tumor, thus resembling the function of normal epithelial stem cells (Fig. 1; refs. 11–13).

Since the discovery of CSCs, research has been focused on the identification of CSC markers. Although many markers have been described that are useful for the enrichment of the often relatively small CSC subpopulation, none of these markers have been proven to be exclusively expressed by CSCs (11). Therefore, CSCs are often identified functionally, by serial xenotransplantation of a small number of cells in immunodeficient mice (Fig. 1). This can be complemented by in vitro assays of colony formation, sphere formation, self-renewal ability, invasion, migration, and drug efflux.

Because CSCs are generally more quiescent and display increased DNA repair mechanisms, CSCs often seem resistant to current cancer treatment such as chemotherapy and radiation, which target the cell cycle and/or rapidly dividing cells and cause lethal DNA damage (14). CSCs can also be difficult to reach because they can reside in CSC niches (15, 16). Moreover, CSCs express ABC transporters, enabling them to actively efflux drugs (13, 17). Therefore, chemotherapies and radiotherapy only target transit-amplifying cells and more differentiated cells, causing shrinkage of the tumor. However, as CSCs are not affected, the tumors can relapse (Fig. 2). To efficiently treat cancer, it is important to target the CSCs, ideally in combination therapies that also target the bulk of the tumor (13–15, 17).

**Urothelial stem cells**

With the discovery of urothelial CSCs, it has been suggested that aberrant cells (due to carcinogen exposure) with stem cell/progenitor-like properties create fields of premalignant cells in the urothelium. Resulting multifocal heterogenic tumor development is a consequence of additional accumulation of genetic alterations (i.e., LOH, mutations), epigenetic modifications, and interaction with the tumor-associated stroma.

For example, sarcomatoid carcinoma is an uncommon (0.1%–0.3% of carcinomas), aggressive variant of urothelial carcinoma and displays heterogeneous morphology (both epithelial and mesenchymal differentiation). Analysis of these tumors by either TP53 mutation status or comparative genomic hybridization provided evidence that sarcomatoid carcinoma is derived from a single, urothelial progenitor cell (monoclonal origin). Subsequently, further mutations can be accumulated during tumor progression. In addition, EMT may be responsible for the different phenotypes found in sarcomatoid carcinoma (18).

In urothelial cancer, putative CSCs have been identified using a variety of markers, many of which are also expressed in normal urothelial stem cells (Table 1). The urothelium, the epithelium lining the bladder wall, is a transitional epithelium consisting of basal cells, intermediate cells, and umbrella cells. The basal cells, which are attached to the basement membrane, are thought to comprise the urothelial stem cells that differentiate into transit-amplifying cells of the intermediate urothelial cell layer, which in turn differentiate into umbrella cells. The umbrella cells make-up the upper layer of cells at the bladder lumen, forming a protective barrier for toxic components in urine. The proliferative capacity of the urothelial cells decreases upon differentiation toward the lumen. Corresponding to the differences in function, the cell layers of the urothelium have different cell surface markers, which are depicted in Table 1. It is important to note that is has been proposed that umbrella cells might not be derived from the basal and intermediate cell layer but develop separately. According to this hypothesis, stem/progenitor cells might give rise to 2 cell lineages, the basal/intermediate cells and the umbrella cells (7).
Figure 1. The CSC hypothesis. A, CSCs can arise from either mutated normal tissue stem cells or from more differentiated cells that due to genetic and epigenetic changes acquire stem cell properties, including self-renewal capacity and epithelial plasticity (EMT). CSCs can differentiate into all cell types of the heterogeneous tumor. B, CSCs are often identified functionally by serial xenotransplantation of a small number of cells in immunodeficient mice. CSCs can form a heterogeneous tumor in the recipient mice, whereas transit-amplifying/progenitor cells have limited growth capacity and will eventually regress. Differentiated cells are not able to form tumors and will undergo apoptosis.

In line with the hypothesis of the basal origin of CSCs, a putative urothelial stem cell population of label retaining cells has been identified in rats, residing at the basal layer of the urothelium. When stained with bromodeoxyuridine, 9% of the basal cells retained their label for more than a year, indicating a low proliferation rate and a long lifespan, characteristic for stem cells. The label retaining cells were shown to be small and to have a low granularity, slow cycling capacity, and high colony-forming efficiency, consistent with their putative stem cell role. Furthermore, they were shown to have uniquely high ITGB4 expression, making this a putative urothelial stem cell marker (19). The same group has provided further evidence of a small number of basal cells as putative urothelial stem cells through isolation and characterization of the different urothelial cell types of porcine bladders. All cell types showed clonogenic capacity. Basal cells at the centers of colonies showed stem cell properties. They originally showed a low proliferation rate but had the ability to become highly proliferative upon passage. Moreover, they had a higher clonogenic capacity and showed a lower apoptosis rate than the other cell types (20).

Further evidence supporting the hypothesis that a small portion of the basal cell layer contains putative urothelial stem cells was recently provided by the identification of clonal patches of cells in the human urothelium that are maintained by a single stem cell (21). Furthermore, it appears that urothelial stem cells are not evenly distributed along the rat bladder but are mostly located in the caudal region of the bladder, consisting of the trigone and bladder neck. This would be consistent with the clinical observations in humans that bladder malignancies in this region have a relatively high incidence of carcinoma per–surface area. Because urothelial stem cells already possess self-renewal capacity, they require less mutations than more differentiated cells to transform into malignant cells (22).

Evidence is accumulating that urothelial CSCs resemble basal urothelial stem cells. Bladder CSCs have been identified through functional assays and have been further characterized for molecular markers that correspond to basal markers (Table 1). Several studies focused on identification of extracellular cell surface markers for urothelial CSCs, which might be...
useful for diagnostic purposes and could be interesting drug targets. For example, it has been shown that urothelial CSCs are enriched in an MUC1 \(^{-}\) CD44v6\(^{+}\) subpopulation of cells. This conclusion was based on the observation that MUC\(^{-}\) and CD44v6\(^{+}\) cells were only present in the basal layer of normal urothelium, which is thought to comprise urothelial stem cells. Subsequently, MUC\(^{-}\) and CD44v6\(^{+}\) cells were isolated, and a slightly increased clonogenic capacity was observed for these cells compared with unsorted bladder tumor cells (23). CD44v6 expression on CSCs is supported by a study that correlates CD44v6 expression on bladder cancer cell lines with stem cell properties (24). However, studies linking absence of CD44v6 expression to an increased recurrence risk and a worse survival rate seem to contradict these results (25, 26).

CD44 expression, independent of CD44 subtypes, has also been suggested as an urothelial CSC marker. A differential expression pattern was identified between urothelial CSCs and differentiated cancer cells. A CD44\(^{+}\) cell population showed an at least 20-fold higher xenograft-forming capability in immunocompromised mice after subcutaneous inoculation as than in CD44\(^{-}\) cells. The CSCs showed coexpression of KRT5, which in combination with CD44 expression indicates a basal-like phenotype. Furthermore, these cells show upregulation of several oncogenes involved in self-renewal pathways of stem cells such as CTNNB1 (β-catenin), BMI1, STAT3, GLI1, POU5F1 (Oct4), and NANOG (10). A tumor-initiating cell gene signature was identified that was able to predict the progression of non-invasive to invasive tumors. This gene signature contained genes that were previously implicated in bladder cancer progression, such as TWIST, a transcription factor involved in EMT induction and metastasis. This link between CSCs and EMT will be further discussed below. Chan and colleagues paid particular attention to the expression of CD47 on tumor-initiating cells. CD47 binds to SIRPA on macrophages and thereby inhibits phagocytosis. According to Chan and colleagues, inhibition of CD47 might be a promising drug target, enhancing phagocytosis of tumor cells by macrophages (10).

A KRT17\(^{+}\)67LR\(^{+}\)CAECAM\(^{-}\) highly tumorigenic subpopulation was identified in primary urothelial cancers, which follows a similar differentiation pattern to that of normal urothelial basal cells (27). Expression of the basal cell markers KRT17 and 67LR was found at the tumor–stroma interface in xenograft tumors. In the center of these tumors, KRT18 and KRT20 were expressed, which are, respectively, intermediate and umbrella cell markers. This indicates differentiation toward the center of the tumor, much like normal urothelial differentiation. In vitro, no colocalization of KRT17 and 67LR expression, which forms the putative CSC population, was observed, indicating that tumor–stroma interactions influence the urothelial cancer cell differentiation. Furthermore, the tumorigenic cells were shown to have a differential expression signature, in which genes of the WNT signaling cascade and genes involved in chemoresistance were upregulated, suggesting a stem-like phenotype. This expression pattern correlated with urothelial cancer progression and poor survival. These results suggest that the invasive properties of urothelial carcinoma cells could be
determined by the ability of CSCs to interact with the stroma (27).

In addition to cell surface makers, several intracellular proteins associated with self-renewal and drug resistance have been correlated with urothelial CSCs. For example, expression of the embryonic stem cell marker POU5F1 has been shown in bladder cancer samples (28). Because embryonic stem cells share several properties with CSCs, including asymmetric proliferation, motility, invasion, and drug resistance, expression of embryonic stem cell markers might reflect the existence of a bladder CSC population. POU5F1 expression in bladder cancer has been confirmed, and a correlation has been shown between POU5F1 expression and tumor progression. Furthermore, it was shown that POU5F1 expression promotes migration and invasion of bladder cancer cells both in vitro and in vivo. POU5F1 overexpression in a murine bladder cancer cell line resulted in enhanced migration in both a wound-healing and a Boyden chamber migration assay. In line with these observations, POU5F1 overexpression resulted in increased metastasis formation in immunocompetent mice (29).

Recently, it has been shown that a cell population with high aldehyde dehydrogenase activity (ALDHhi), was enriched in bladder CSCs (30). ALDHhi has previously been shown to be involved in stemness and metastasis formation in several other solid tumor types, including breast (31) and prostate cancer (32). The bladder cancer cell lines HTB-2, HTB-4, and HTB-9 have a subpopulation of 6.4% to 8.2% ALDHhi cells, which showed increased clonogenic capacity in vitro and a 100-fold increased heterogeneous tumor formation after subcutaneous inoculation in vivo as compared with ALDH− cells. The ALDHhi cells represent a subpopulation of CD44+/C0 CSC cells. In 26% of human bladder cancer samples, high ALDH expression was observed, which correlated with grades and stages of the tumor and inversely correlated with cancer-specific and overall survival (30).

Bentivegna and colleagues have used a combination of extra- and intracellular markers to identify PROM1+/CD133+ (POU5F1+/NES+ (nestin) cells as putative urothelial CSCs in primary transitional cell carcinoma, with POU5F1 and NES being intracellular markers (33). Urospheres were cultured, a spherical cluster of cells generated by urothelial stem cells in vitro, which were shown to be of clonal origin. A small proportion of these cells showed self-renewal ability in clonogenic assays, but over time, the spheres lost their stem cell phenotype, adhered to the tissue culture plastic, and lost their proliferative capacity. The spheres expressed the stem cell markers PROM1, POU5F1, and the early progenitor marker NES, suggesting initiation of differentiation. A small population (5%–10%) expressed the epithelial markers KRT5, 14, and 8/18. The isolated

Table 1. Markers basal cells, intermediate cells, umbrella cells of the urothelium, and putative bladder CSCs

<table>
<thead>
<tr>
<th>Marker</th>
<th>Basal cells</th>
<th>Intermediate cells</th>
<th>Umbrella cells</th>
<th>Putative CSCs</th>
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<td>ND</td>
<td>+</td>
<td>(6, 27)</td>
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<td>(6, 10)</td>
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<tr>
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<td>−</td>
<td>+</td>
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<td>(7)</td>
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<td>−</td>
<td>+</td>
<td>ND</td>
<td>(5, 7)</td>
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</table>

Abbreviations: +, positive; +/-, intermediate; −, negative; ND, not determined.
PROM1⁺, POU5F1⁺, and NES⁺ cells were not able to form xenografts when injected into CD1 nude mice, which are deficient for T cells. The authors explain the loss of stem cell characteristic and inability to form xenografts by plasticity between stem cells and progenitors (33). The hypothesis of plasticity between CSC and non-CSC was previously proposed by Gupta and colleagues (34) who stated that EMT indicates dedifferentiation of cells to form CSCs. This concept will be described in more detail in the section on EMT.

Although many markers have been proven useful for identification, isolation, and characterization of urothelial CSCs, there is no consensus on these markers. Therefore, functional assays including xenotransplantation are required in addition to molecular markers to confirm the CSC phenotype of isolated cell populations.

The growth of spherical colonies is considered to be indicative of self-renewal and seems a valid approach for the identification of tumor-initiating cells in vitro (35).

One of the functional assays that have been used for urothelial CSC isolation makes use of the multidrug resistance of CSCs. Because CSCs express ABC transporters, or multidrug resistance pumps, they are able to pump out the DNA-binding dyes Hoechst 33342 and DyeCycle violet. Efflux of these dyes identifies a side population of cells, which has been shown to be enriched for CSCs. Multiple groups have used this approach to identify and isolate urothelial CSCs. She and colleagues showed that side population cells isolated from bladder cancer cell lines have self-renewing ability, clonogenic capacity, and can initiate tumor formation when subcutaneously injected in low numbers (1 × 10³) into immunocompromised mice (36). Furthermore, expression of the ABC transporters ABCG2 and ABCB1 (also known as MDR1) was confirmed, and side population cells express the stem cell makers POU5F1 and BMI1, implying a stem-like phenotype of side population cells (36). In an independent study, side population cells isolated from the T24 bladder cancer cell line showed a more quiescent cell state, asymmetric cell division, rapid cell growth, and colony formation and chemoresistance, providing further evidence for CSC enrichment (37). Moreover, side population cells isolated from patient samples have increased clonogenic and proliferative capacity, a higher percentage of quiescent cells, and long-term self-renewal. These cells showed increased expression of the stem cell markers CDKN1B, PAX2, and SHH (38). Thus, side population cells isolated from cell lines or patient samples both show enrichment of CSCs.

Epithelial Plasticity

Epithelial plasticity is defined as the ability of cells to dynamically switch between different phenotypic cellular states. EMT is the transition from a sessile, epithelial cell to a motile cell with a mesenchymal phenotype. It has been shown that induction of EMT can induce stem cell properties (39, 40). Therefore, in addition to CSCs differentiating into transit-amplifying progenitor cells, the reverse process (dedifferentiation into CSCs) may also occur under certain conditions at various stages of cancer progression. Epithelial plasticity enables cancer cells to adapt to changes in the tumor (micro)environment.

In cancer, EMT contributes to progression to metastatic disease and therapeutic resistance, enabling cancer cells to become invasive, disseminate, resist apoptosis, stimulate angiogenesis, and acquire stem/progenitor cell properties (41–43). EMT is categorized in 3 different subtypes (41), according to its function and pathways involved. Type I is involved in embryonic development. Type II is required for tissue repair, can cause organ fibrosis, and is triggered by inflammatory signals such as TGF-β. Type III or oncogenic EMT is associated with cancer and can be induced by multiple growth factors, for example, hepatocyte growth factor (HGF), EGF, platelet-derived growth factor (PDGF), and TGF-β, through regulation of the expression of EMT-inducing transcription factors (44).

It is important to note that EMT in cancer is usually not a complete transition but a rather transient and reversible process.

EMT and mesenchymal-to-epithelial transition in metastasis formation

EMT and the reverse process of mesenchymal-to-epithelial transition (MET) play important roles in different steps of metastasis formation. For successful metastasis, cancer cells have to undergo a multistep process including invasion of the surrounding tissue, intravasation, survival in the bloodstream, extravasation, and colonization of distant sites (45). For successful dissemination, epithelial tumor cells often switch to a mesenchymal phenotype, as motility, invasion, and survival are required (46). At distant sites, a more epithelial phenotype might be favorable because cells have to adhere to their surroundings. This can be induced through MET under influence of the microenvironment at distant sites through mechanisms that remain largely elusive. The occurrence of MET at distant sites would also explain the similarities in pathology of primary tumors and metastases. Taken together, epithelial plasticity, or switching between epithelial and mesenchymal phenotypes, appears to be essential for successful metastasis formation (44).

An important characteristic of EMT is decreased CDH1 (E-cadherin) expression followed by increased CDH2 and/or CDH3 expression (respectively, N- and P-cadherin, a process called cadherin switching; ref. 47). CDH1 is responsible for cell–cell adhesion in the epithelium through homophilic interactions between CDH1 proteins on adjacent cells, occurring in adherens junctions (48). Therefore, down-regulation of CDH1 enables cells to become more motile and migrate.

Another way in which downregulation of CDH1 might contribute to tumor progression is through release of CTNNB1 (β-catenin). The cytoplasmic domain of CDH1 is anchored to the actin cytoskeleton by CTNNA1 and CTNNB1, providing mechanical stability to the adherens junctions. Upon downregulation of CDH1, CTNNB1 is released and could migrate to the nucleus and activate WNT
target genes, resulting in EMT and increased metastasis formation. However, CTNNB1 can be ubiquitinated and subsequently degraded by a destruction complex consisting of several proteins including APC and GSK3β. By degrading CTNNB1 that is released from CDH1, this complex prevents CTNNB1 to migrate to the nucleus and exert its transcriptional activity. Therefore, loss of CDH1 only results in nuclear localization of CTNNB1 and thereby WNT pathway activity, when the destruction complex is impaired (49).

In contrast to CDH1, CDH2 drives tumor progression and invasion. CDH2 is thought to promote tumor invasion through interaction with FGFR1, which activates the mitogen-activated protein kinase (MAPK)/extracellular signal–regulated kinase (ERK) pathway and leads to matrix metalloprotease-9 (MMP9) expression (50). MMP9 is an extracellular matrix (ECM)-degrading protein, which facilitates invasion. Furthermore, CDH2 might enable migration and survival in distant tissues through homophilic interactions of CDH2 expressed on tumor cells and CDH2-expressing tissue, such as stroma and endothelium (51). In line with this, CDH2 has been shown to be an independent prognostic marker for progression of superficial bladder tumors (51).

Although the term cadherin-switching suggests mutually exclusive expression of CDH1, 2, and 3, cells can coexpress the cadherins. This illustrates the epithelial plasticity and could be an intermediate state between an epithelial and mesenchymal phenotype. When CDH1 and CDH2 are coexpressed, CDH2 function is dominant over the tumor-suppressive function of CDH1. This has originally been shown for breast cancer, in which forced expression of CDH2 in the CDH1-positive breast cancer cell line MCF-7 resulted in increased motility and invasiveness, without downregulating CDH1 expression (52). In human bladder cancer, coexpression of CDH1, 2, and 3 has also been observed (53), and different cadherin expression patterns in bladder cancer cell lines were correlated with their invasive capacity. Cells expressing only CDH1, coexpressing CDH1 and CDH2, or expressing only CDH2 show low, intermediate, and high invasive capacity, respectively. Moreover, the invasive capacity of bladder cancer cells expressing only CDH1 could be increased by transfection of these cells with CDH2. Furthermore, invasive capacity could be decreased by both CDH2 antibodies and inhibitors of the PI3K pathway, suggesting that CDH2 regulates invasiveness through the PI3K/Akt pathway, rather than the MAPK/ERK pathway (54).

The role of CDH3 appears to be context-dependent, showing opposite roles in different tumor types (55). Even in the same tissues, contradicting results have been obtained for the role of CDH3 expression. For example, CDH3 expression has been associated with a good prognosis of breast cancer in some studies (56), whereas other studies associated CDH3 expression with a poor prognosis (57). In bladder cancer, it appears that CDH3 favors tumor progression and metastasis, showing a correlation of CDH3 expression with a poor prognosis (49, 58, 59). Interestingly, CDH3 is only expressed in the basal layer of normal urothelium, which is thought to comprise urothelial stem cells. It has been suggested that the less adherent CDH3 may allow for migration of basal cells into the more superficial layers of the urothelium (58). Thus, also implying a role for CDH3 in stemness and migration of bladder cancer cells. Indeed, forced CDH3 expression has been shown to increase the migratory capacity of the bladder cancer cell lines EJ and UM-UC-3 in vitro (58). This role was confirmed by knockdown of CDH3 in these cell lines and the RT-112 cell line, which resulted in decreased migratory and invasive capacity (57, 58).

The roles of epithelial plasticity at the different stages of metastasis formation are illustrated by differences in metastatic potential of phenotypically epithelial or mesenchymal bladder cancer cells at different inoculation sites. At the orthotopic inoculation site, representative for growth at the primary site and the multistep metastatic process, a mesenchymal phenotype is favorable for metastasis formation. However, after intracardiac or intratibial inoculation, circumventing the first steps of metastasis formation, an epithelial phenotype is favorable for metastasis formation. These epithelial phenotype cells, however, retained expression of some mesenchymal markers, including VIM (vimentin) and MMPs (60). Because the shift toward an epithelial phenotype was not complete, this might have enabled survival in the circulation of the phenotypically more epithelial cells. True epithelial cells would have undergone apoptosis as a result of loss of adherence to the ECM (anoikis; ref. 46).

An alternative model for the metastatic spread of cancer cells is the cooperation model, which postulates that phenotypically epithelial and mesenchymal cancer cells cooperate to form metastasis. This model was based on a series of experiments, in which athymic mice were subcutaneously or intravenously inoculated with hamster cheek pouch carcinoma 1 cells. These cells have an epithelial phenotype (non-EMT cells), and EMT was induced by forced expression of CDK2AP1, resulting in phenotypically mesenchymal cells (EMT cells). Subcutaneously inoculated EMT cells showed invasiveness into the bloodstream, but no metastatic spread, thus no colonization of distant sites. In contrast, the non-EMT cells were not able to invade when injected subcutaneously but did show metastatic spread after intravenous inoculation. Subcutaneous inoculation of a combination of epithelium- and mesenchyme-like cells resulted in metastatic spread (61). These observations lead Tsujii and colleagues to postulate that EMT-cells are required for invasion through degradation of the ECM, leading the way for non-EMT cells to enter the bloodstream, which are in turn responsible for metastasis formation (62). However, this model appears less likely than the sequential EMT–MET model, as it fails to explain therapy resistance, tumor dormancy, and disease recurrence (47).

**Integrated concept of EMT and CSCs**

The CSC and EMT concepts were integrated by Brabletz and colleagues who proposed a subdivision of 2 types of...
EMT-inducing transcription factors

EMT-inducing factors, such as cytokines or hypoxia, stimulate the expression of EMT activators, transcription factors of the ZEB and SNAIL family, which directly or indirectly repress CDH1 expression. ZEB and SNAIL family members not only induce phenotype changes enabling migration and invasion but also affect stemness, growth arrest, and cellular survival (reviewed in ref. 63).

The zinc-finger transcription factors SNAI1 (also known as SNAIL1), SNAI2, ZEB1, ZEB2, TCF3 (E47), and KLF8 have been shown to bind directly to E-boxes of the CDH1 promoter, thereby repressing CDH1 expression. Whereas, the helix-loop-helix transcription factors TWIST, GSC (Goosecoid), TCF4 (also known as E2-2), and FOXC2 show indirect repression of CDH1 (60). Expression of these transcription factors can be induced by the growth factors HGF, EGF, PDGF, and TGF-β, which are secreted by the tumor stroma (44).

In bladder cancer, several of these transcription factors are differentially expressed, showing increased SNAI2, TWIST, and ZEB1 expression and decreased SNAI1 expression in human bladder carcinoma tissue (64, 65). TWIST has been correlated with reduced CDH1 expression and increasing bladder tumor stage and grade in cell lines and bladder carcinoma tissue and has been shown to be significantly upregulated in metastases compared with primary bladder tumors, suggesting an important role for TWIST in bladder cancer progression and metastasis formation (66, 67). Furthermore, it has been shown that TWIST expression is associated with smoking, which is an important risk factor for bladder carcinoma (66).

In addition, SNAI2 expression also seemed to be involved in tumor progression and metastasis formation. Upon knockdown of SNAI2 in the bladder carcinoma cell line T24 in vitro proliferation, survival, invasion, and angiogenesis induction were reduced, which was associated with increased CDH1 expression and decreased MMP2 expression. MMP2 was shown to be required for angiogenesis, as illustrated by a regained capability to induce angiogenesis upon forced MMP2 expression in the SNAI2 knockdown cells. Moreover, knockdown of SNAI2 resulted in a 60% reduction of tumor growth and inhibition of metastasis formation after implantation in immunodeficient mice (64, 68).

The role of SNAI1, ZEB1, and ZEB2 in bladder cancer cells is less clear. Contradictory results have been obtained for SNAI expression. Yu and colleagues showed a reduction of SNAI1 expression in bladder carcinoma compared with normal bladder tissue (64), whereas Bruyere and colleagues suggested SNAI1 expression as a predictive marker for tumor recurrence of superficial bladder tumors (69). Similarly, inconclusive results have been obtained for ZEB1 expression. Although ZEB1 expression has been associated with reduced CDH1 expression and increased migration and invasion in bladder cancer in vitro, no correlation has been detected with clinical variables, including tumor stage, grade, node (N) stage, metastasis (M) stage, and survival (69, 70). Furthermore, ZEB1 and ZEB2 mRNA and protein expression showed contradictory results in bladder cancer cell lines and tumor samples. ZEB1 expression was detected in 3 tested mesenchymal bladder cancer cell lines and was shown to inversely correlate with CDH1 expression. However, ZEB1 was rarely expressed in tumor tissues. In contrast, ZEB2 was rarely expressed in bladder cancer cell lines but was detected in bladder tumor samples, of which 31% were positive for ZEB2. ZEB2 expression in tumor samples was shown to inversely correlate with CDH1 expression and was associated with a worse 5-year survival rate than ZEB2-negative cells (71).

miRNA regulation

Members of the ZEB family of EMT activators are connected in a reciprocal double-negative feedback loop to the miR-200 family of miRNA and miR-205. Mir-200 family members have been shown to repress ZEB1 and ZEB2 expression, thereby maintaining CDH1 expression and an epithelial phenotype (72). To illustrate the role of the miRNAs, a TGF-β–induced EMT mouse model was used, in which EMT is induced in normal murine mammary epithelial cells (NMuMG), resulting in cells with a more mesenchymal morphology, CDH1 downregulation, and CDH2 upregulation. Overexpression of each of the 5 different miRNAs of the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) inhibited EMT in this model, maintaining high levels of CDH1 expression. Using a reporter assay, it was shown that these miRNAs directly suppress ZEB1 and ZEB2 (73). Furthermore, it has been shown that ZEB1 can inhibit miR-200 expression, which has lead to the hypothesis of a ZEB1/miR-200 feedback loop that regulates cellular plasticity through regulation of EMT (72, 74).

These miRNAs have also been implicated in the regulation of EMT in bladder cancer (74). miRNAs of the miR-200 family have been shown to be silenced in several bladder cancer cell lines (e.g., UM-UC-3 and KU7) with a mesenchymal phenotype. Upon forced expression of miR-200c in the UM-UC-3 cell line, these cells shifted toward a more epithelial phenotype (MET) as a result of reduced ZEB1 and ZEB2 expression, as illustrated by increased CDH1.
expression, morphologic changes, and decreased migratory capacity (68). Furthermore, the miR-200 and miR-205 loci were shown to be silenced through hypermethylation of the promoters in both primary bladder tumors samples and bladder cancer cell lines (73). Taken together, miR-200 family and miR-205 seem to play pivotal roles in maintaining an epithelial phenotype in bladder cells, thereby preventing tumor formation and/or progression.

Another double-negative feedback loop important for epithelial plasticity consists of the miR-34 family and the SNAI family members (75). In bladder carcinoma, members of the miR-34 family are frequently inactivated (76).

Importantly, TP53 activates the expression of both miR-200 family members and miR-34 family members (77–79). These data indicate that TP53 is an important regulator of epithelial plasticity. However, the abundance of mutations in TP53 in several solid tumors suggests that mutations in TP53 alone cannot be sufficient for a mesenchymal CSCs phenotype.

**The Tumor Microenvironment**

It is becoming increasingly clear that the tumor-surrounding microenvironment (or tumor stroma) is pivotal for regulation of tumor behavior, including EMT and CSC properties. The importance of the tumor stroma in EMT and CSC maintenance is illustrated by the presence of EMT and CSC markers specifically at the tumor edge, where tumor cells interact with the stroma. It has been proposed that in the absence of EMT-inducing signals derived from the tumor stroma, cancer cells will undergo MET as a default mechanism, enabling cells to colonize distant sites and providing an explanation for the epithelial differentiation observed in distant metastasis (12, 14, 43, 61).

The influence of the microenvironment can be either unfavorable or beneficial for tumor progression (80). However, cancer cells can alter their microenvironment (and vice versa) to become supportive for tumor growth and progression by activation of local stromal cells, such as fibroblasts, smooth muscle cells, and adipocytes, and by recruitment of endothelial and mesenchymal progenitors and inflammatory cells (60). This is achieved through secretion of several stroma-modulating growth factors, including basic FGF2, VEGF, PDGF, EGF receptor (EGFR) ligands, colony-stimulating factors, and TGF-β (81). As a result, the tumor microenvironment acquires properties that resemble wound-healing (82) and therefore stimulate cell proliferation, motility, invasion, and angiogenesis.

The stromal cells can contribute to tumor progression through secretion of chemotactractors, growth factors, and ECM-degrading proteins, such as MMPs. Degradation of the ECM is not only required for invasion but also releases growth factors, including EGF and TGF-β, and exposes RGD sites that can be recognized by integrins expressed on the cancer cell surface. Binding of these growth factors to their respective receptors and binding of the RGD site to integrins activates RAS-induced pathways, such as the MAPK/ERK and the PI3K/Akt pathway, that provide signals for tumor cell proliferation, motility, and survival (81, 83). Furthermore, specific MMPs, secreted by either cancer or stromal cells, have been shown to induce EMT, possibly through reactive oxygen species–induced upregulation of SNAI (84).

In addition to alteration of the microenvironment induced by the cancer cells, the cancer cells themselves may also acquire stromal support by epithelial plasticity to produce the necessary stromal cell types. For example, cancer cells that have undergone EMT may well take up the tasks of cancer-associated fibroblasts (reviewed in (85)).

In bladder cancer, a stimulatory, supportive effect of the microenvironment has been suggested on the basis of discrepancies between in vitro and in vivo invasive behavior of bladder cancer cell lines. In vitro, only cells lines lacking CDH1 expression (T24 and J82) showed high invasive capacity, whereas in vivo invasive behavior of cell lines appeared to be less dependent on CDH1 expression, showing invasive behavior in the CDH1-positive SD cell line after subcutaneous inoculation. CDH1 expression in vivo was shown to be downregulated in this cell line. This loss of CDH1 expression was observed particularly in the invasive regions of these tumors, indicating a role for the microenvironment in CDH1 downregulation (86).

Alternatively, tumor cell invasion can be explained by collective migration of cells as sheets, strands, or clusters rather than as individual cells. The main difference is that the cells retain the cell–cell junction both at the leading edge as well as inside the moving cell cluster (87). Although the specific mechanisms of EMT and CSC maintenance of the tumor stroma in bladder cancer remain to be elucidated, several stromal cell types have been shown to be involved in bladder cancer progression. One of these cell types is the mesenchymal stem cell, which can be recruited by tissue damage or inflammation and are involved in tissue repair through differentiation into several cell types, including fibroblasts and endothelial cells. In a rabbit bladder cancer model, engrafted mesenchymal stem cells were shown to differentiate into endothelial cells, thereby initiating or contributing to angiogenesis, and tumor growth (88).

Furthermore, tumor-associated fibroblasts (TAFs; also known as cancer-associated fibroblasts or myofibroblasts) have been shown to be upregulated in muscle-invasive T2 and T3 bladder tumors, compared with non–muscle-invasive Ta and T1 tumors, indicating a role for these cells in invasion and metastasis formation (89). Indeed, fibroblasts have been shown to increase bladder cancer cell motility and invasiveness in vitro through secretion of HGF. This was illustrated by a more invasive phenotype of bladder cancer cell lines in the presence of TIG-1 human diploid embryonic lung fibroblasts, which could be inhibited by treatment with anti-HGF. In line with these observations, increased serum HGF levels were detected in patients with muscle-invasive disease compared with healthy controls. Moreover, conditioned medium of bladder cancer cell lines increased both HGF expression and the enhancement of invasiveness caused by the TIG-1 fibroblasts. This conditioned medium
LOX family oxidases interact with SNAI transcription factor prostate carcinoma (95). In line with these data, LOX family oxidases are important for covalent modification of ECM components. Integrins are heterodimeric receptors, consisting of α- and β-subunits. Eighteen different α-subunits and 8 different β-subunits have been identified, which can form at least 25 distinct heterodimers of different combinations, each specific for a unique set of ligands. Integrins are special in that—in addition to "outside-in" signaling—they can also facilitate motility by modulating their affinity for ECM ligands in response to intracellular signals, a process called "inside-out" signaling. By changing their affinity, associations with ECM ligands can be broken and new ones can be formed, enabling cells to move along the ECM. Furthermore, through inside-out signaling, integrins can modulate the cytoskeleton, regulate the expression of matrix-degrading enzymes, and provide antiapoptotic signals. Through these processes, integrins are involved in migration, invasion, and survival of cancer cells during metastasis formation (93). Furthermore, integrins expressed on endothelial cells play an important role in the regulation of pathologic angiogenesis (94).

LOX family oxidases, which are important for covalent cross-linking and stabilization of ECM components elastin and collagen, have first been shown to function as tumor suppressors (reviewed in ref. 95). In bladder carcinoma, the expression of LOXL1 and LOXL4 is epigenetically silenced (96). However, recently, LOX peroxidases seem highly expressed in invasive tumors and are associated with poor outcome in patients in several solid tumors such as breast and prostate carcinoma (95). In line with these data, LOX family oxidases are also actively implicated in the process of EMT. LOX family oxidases interact with SNAI transcription factor and protect SNAI from GSK3B-dependent phosphorylation and degradation. As a result, the cells undergo mesenchymal-like morphologic changes and display enhanced cell motility. TGF-β, one of the major EMT activators, induces the expression and secretion of LOX family oxidases. Inactivating LOX activity impaired TGF-β1–mediated EMT and invasion in breast cancer cells (97). Furthermore, hypoxia induced the expression of LOX and LOXL2, resulting in repression of E-cadherin, possibly through stabilization of SNAI (95). LOX family oxidases are, therefore, important for processes such as tumorigenesis and metastasis, that is, EMT, and remodeling of the tumor microenvironment. This has been confirmed by data showing that addition of pharmacologic inhibitors of LOX family oxidases inhibited tumor progression and metastasis in breast and lung carcinoma (95).

Inflammation can contribute to tumorigenesis and tumor growth of many different carcinomas by providing growth factors that support proliferation, survival, angiogenesis, invasion, and metastasis (98–100). For example, inflammatory cells that accumulate at the tumor edge can produce ECM-degrading enzymes and other factors that enable invasive growth (100, 101). Inflammation has been shown at early stages of tumor progression and reactive oxygen species generated by the inflammatory cells result in the development of additional mutations in the cancer cells (99, 100).

As observed in many cancer types, the role of immune cells in bladder cancer appears to be contradictory, showing an antitumor response of the adaptive immune system and a protumor response of the innate immune system. For example, tumor-infiltrating lymphocytes have been associated with a favorable prognosis in bladder cancer, whereas infiltration with tumor-associated macrophages has been shown to be required for hypoxia-induced angiogenesis, thereby contributing to cancer progression (102). Moreover, chronic inflammation is a well-known cause for bladder cancer (103).

Recently, it has been shown that tumor-infiltrating immune cells can also activate the EMT program. The EMT activator TGF-β has been shown to promote invasion and metastasis by acting on both the tumor cells and the immune cells in the tumor microenvironment. TGF-β induces a shift in macrophages from a M1 (antitumor) to a M2 protumorigenic phenotype. In addition, neutrophils shift from an N1 to an N2 phenotype in reaction to TGF-β. Furthermore, TGF-β promotes the differentiation of regulatory T cells. This leads to increased matrix remodeling, angiogenesis, and EMT (reviewed in ref. 104).

Implications for Therapy

For patients with metastatic bladder cancer, systemic cisplatin (CDDP)-based combination chemotherapy is the first-line choice of treatment. Although up to 70% of patients with advanced bladder cancer initially show good tumor response to this form of combination chemotherapy, more than 90% of good responders relapse and eventually die of the disease. It is now widely thought that treatment resistance involves cells that have a mesenchymal stem cell
like phenotype. Indeed, bladder CD44<sup>+</sup> CSCs have been isolated that are more resistant to CDDP than their CD44<sup>low</sup> counterparts (105). The acquired knowledge about the roles of CSCs, EMT, and the tumor microenvironment has provided researchers with many potential drug targets for bladder cancer treatment. As described above, it is important for effective tumor therapies to target both differentiated cancer cells and CSCs, thereby removing not only the bulk of the tumor but also the source of the cancer (Fig. 2). Unfortunately, CSCs have often been shown to be resistant to current cancer therapies because of their quiescent state, drug efflux, and their localization in the protective CSC niche (15–17, 19, 20). Several approaches have been explored to overcome therapy resistance.

First, the capacity of the cells to redifferentiate offers a promising strategy for resensitization for conventional chemotherapy. Differentiation therapies aim to induce MET, transforming phenotypically mesenchymal CSCs to more epithelial cells, thereby losing self-renewal properties and becoming more vulnerable for therapies targeting proliferating cells (106). The differentiation agent retinoic acid (RA or vitamin A) is currently used in treatment of acute promyelocytic leukemia and has shown to be very effective, curing patients with a more than 70% success rate when administered in combination with chemotherapy. Another promising approach, which is being explored for glioblastoma, is differentiation induced by bone morphogenetic proteins (BMP). BMP4 has been shown to induce differentiation of glioblastoma cell lines in vitro, decreased clonogenicity, and a reduction of the PROM1<sup>+</sup> glioma stem cell population. Moreover, BMP4 treatment resulted in inhibition of tumor growth and mortality in a glioblastoma mouse model (107). In addition, treatment with BMP2, BMP7, and particularly the heterodimeric BMP2/7 strongly reduced the size of the ALDH<sup>hi</sup>/CD44<sup>hi</sup>/CD24<sup>−/low</sup> breast cancer CSC subpopulation (108). In addition, pretreatment of breast cancer cells with BMPs for 72 hours before systemic inoculation of the cancer cells inhibited the formation of bone metastases (108). A possible role of differentiation therapies in bladder cancer is restoring EGFR dependency, thereby restoring sensitivity to EGFR inhibitors. EGFR has been used as a drug target in several solid cancers, as it promotes migration and invasion, and overexpression of EGFR is associated with a poor prognosis. Indeed, several EGFR inhibitors have been shown to have clinical benefits in, for example, breast and colorectal carcinomas. However, only phenotypically, epithelial cancers appear to respond to EGFR inhibitors. This was confirmed in bladder cancer in which the anti-EGFR antibody cetuximab has been shown to be effective only in bladder cancer cell lines with an epithelial phenotype, expressing CDH1. Interestingly, responsiveness to cetuximab could be decreased in sensitive cell lines by silencing CDH1 expression (109). Moreover, it has been shown that forced expression of the miR-200 family can induce MET in mesenchymal bladder cancer cell lines, thereby restoring EGFR inhibitor sensitivity in several phenotypically mesenchymal bladder cancer cell lines (88). Therefore, a potential strategy could be to pretreat patients with drugs increasing the expression of the miR-200 family members before applying conventional chemotherapy.

In addition to CSC differentiation strategies, direct inhibition of the EMT program can potentially reduce migration, invasion, and survival of cancer cells (110). For example, induction of MET through SNAI2 inhibition might be a promising treatment strategy. However, direct targeting of transcription factors is difficult, therefore therapies are being developed that inhibit pathways that are required for EMT induction, including inhibitors and/or antibodies directed against the receptors for EGF, PDGF, and TGF-β (67). An example in bladder cancer is the green tea component (-)-epigallocatechin-3-gallate (EGCG), which has been shown to reduce clonogenicity, migration, and xenograft growth of bladder cancer cell line through inactivation of the PI3K/Akt pathway, coinciding with reduced CDH2 levels (111).

Nevertheless, when considering novel therapies, one should take the intrinsic epithelial plasticity of the carcinoma cells into consideration as well as the notion that the MET process might be required for the establishment of metastasis at distant sites. It is possible that anti-EMT drugs could be applied to patients diagnosed at early stages of the disease to prevent invasion and dissemination, whereas anti-MET drugs could be potentially efficient in patients with established metastasis.

Other CSC properties, including self-renewal and modulation of chemoresistance, are targeted by elimination therapies (15, 112). For example, studies have been conducted combining a range of cytotoxic drugs and ABC transporter inhibitors trying to prevent drug efflux. So far, these therapies have been unsuccessful possibly because they inhibit the wrong ABC transporter or the use of chemotherapeutics that are unable to target CSCs (14, 106). Alternatively, drug-detoxifying enzymes that are upregulated in CSCs, such as ALDH, can be targeted.

Chemical screening programs for drugs that selectively kill cells with EMT-CSC–like phenotype have identified salinomycin, which specifically acts on breast CSCs (113). In addition, in triple-negative breast cancer, it has been shown that dasatinib, an inhibitor of SRC and ABL kinases, can inhibit breast cancer cells with an EMT/stem cell–like phenotype (114).

Furthermore, CSC markers are potential targets for CSC-eliminating therapies. For example, treatment with oncolytic viruses that specifically replicate in cells expressing particular markers has been shown to be effective in preclinical metastatic bladder cancer models, using POU5F1-responsive oncolytic adenoviruses to target POU5F1-expressing bladder cancer cells (115). Although CSC-related proteins or pathways provide attractive targets for therapy, caution must be applied as many markers are shared by normal stem cells (Table 1).

Another promising approach is targeting of the tumor microenvironment, which has the advantage that stromal cells are less likely to develop therapy resistance than cancer cells, as they are more genetically stable. Therapies are being...
developed that target a wide range of tumor stroma components, including signaling molecules, ECM-degrading proteases, inflammatory cells, endothelial cells, and fibroblasts (112). Inhibiting integrin signaling may provide an additional strategy. The αv subunit of the vitronectin receptor (ITGAV), for instance, has been shown to be upregulated in both carcinoma cells and activated endothelial cells of the tumor stroma (116). Inhibition of ITGAV has been shown to reduce angiogenesis, tumor growth, and metastasis in several solid tumor types in which ITGAV is upregulated, including breast (117) and prostate cancer (118).

In conclusion, combination therapies are necessary, combining conventional therapy targeting the bulk of the tumor and targeting of the mesenchymal CSC progenitor–like cells, which on its own might not be sufficient, as non-CSCs can be converted to CSCs by activating the EMT program.

Conclusion
To develop more efficient therapies for prevention and/or treatment of metastatic cancers, including bladder cancer, it is important to further unravel the mechanisms that cause current therapies to fail. In this review, we discussed the importance of CSCs, EMT and the tumor microenvironment in tumorigenesis, tumor progression, metastasis formation, and drug resistance. The secret for effective cancer therapies might therefore lie in a combination of therapies that target not only the bulk of the tumor but also the CSCs subpopulation and, last but not the least creating an inhospitable microenvironment for tumor cells at primary and distant sites.

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No potential conflicts of interests were disclosed.

Authors’ Contributions
Conception and design: G. van der Horst, L. Bos, G. van der Pluijm
Development of methodology: G. van der Pluijm
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): G. van der Pluijm
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References


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Epithelial Plasticity, Cancer Stem Cells, and the Tumor-Supportive Stroma in Bladder Carcinoma

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