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Review

Understanding the biology of urothelial cancer metastasis

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Abstract Management of unresectable urothelial cancer (UC) has been a clinical challenge for decades. While drug resistance is a key issue, precise understanding of biology of UC metastasis is another challenge for the improvement of treatment outcome of UC patients. Introduction of the cell biology concepts including epithelial-mesenchymal transition (EMT) and cancer stemness seems to explain UC metastasis. Molecular genetics based on gene expression profiling, next generation sequencing, and explosion of non-coding RNA world has opened the door to intrinsic molecular subtyping of UC. Next steps include, based on the recently accumulated understanding, the establishment of novel disease models representing UC metastasis in various experimental platforms, particularly *in vivo* animal systems. Indeed, novel knowledge molecular genetics has not been fully linked to the modeling of UC metastasis. Further understanding of bladder carcinogenesis is needed particularly with regard to cell of origin related to tumor characteristics including driver gene alterations, pathological differentiations, and metastatic ability. Then we will be able to establish better disease models, which will consequently lead us to further understanding of biology and eventually the development of novel therapeutic strategies for UC metastasis.

1. Introduction

Bladder cancer is the sixth most common malignancy excluding non-melanoma skin cancers with an estimation of 330,380 new cases and 123,051 deaths from bladder cancer worldwide in 2012 [1]. Approximately 95% of bladder cancers are histologically classified as urothelial carcinoma (hereafter referred as UC, formerly called transitional cell carcinoma) with the exception of a particular area where squamous cell carcinoma due to chronic infection of *Schistosoma hematobium*is more prevalent. Urothelium, where UC is originated, lines all through the urinary tract

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except for distal urethra. Therefore, UC can arise from renal pelvis, ureter, bladder, and proximal urethra.

As many of other malignancies, bladder UC is a disease of older individuals. Patients are typically in their fifth or greater decades of life. The majority of bladder UC occur in males where there is an approximately 2- to 3-fold greater incidence compared with females. In the United States, Caucasians are at highest risk for bladder UC among all races including African, Asian, and Latin Americans. It is known that smoking and occupational or endemic exposures to certain chemicals predispose us to UC. The underlying mechanisms that link urothelial carcinogenesis to the sexual and racial disparities and risk factors including aging and carcinogens are currently not fully understood.

Treatment and prognosis of UC depend on several key factors including anatomical site, extent (stage), and histological grade of the disease. Non-muscle-invasive bladder UC (NMIBC),with the exception of carcinoma *in situ* (CIS), can be treated by transurethral resection with excellent survival outcomes, whereas muscle-invasive bladder UC (MIBC) and upper urinary tract UC (UTUC) often need radical cystectomy (RC) or nephroureterectomy (RNU). While these treatments are usually indicated with a curative intent, there are currently few curative treatment options for a metastatic or recurrent UC that has progressed outside of the urinary tract. MIBC is associated with higher incidence of distant metastasis compared with NMIBC. Bladder UC often metastasize to lymph nodes, bone, lung, liver, and peritoneum [2]. Systemic chemotherapy, the standard treatment for metastatic UC, can hardly achieve durable disease control. Therefore, treatment outcome of the patients with metastatic UC has been very poor with approximately 15% of overall survival rate [3].

Thus, it is apparently important to understand the underlying biology for metastatic progression of UC. Recently published series of molecular genetics of bladder cancer provided novel information for intrinsic molecular subtyping of UC [4–6], which will potentially lead us to the effective prevention and cure of this currently lethal form of the disease. This article reviews recent key findings that have been accumulated in the research field of UC metastasis, barriers that hamper our research progression, and future perspectives that may potentially overcome them.

2. Cell biology of UC metastasis

2.1. Epithelial-mesenchymal transition (EMT)

Several cellular processes are implicated in metastatic progression of UC. EMT is referred as a complex process that reprograms and transmogrifies epithelial cells to mesenchymal phenotype characterized by loss of cell adhesion and polarity. Although EMT is a phenomenon that physiologically observed during development and wound healing, it has long been implicated in cancer metastasis and treatment resistance. As essential roles of EMT in urothelial cancer metastasis was extensively discussed in an excellent review by McConkey et al. [7] in 2009, this article focuses on relatively recent findings.

One of the most particular molecular characteristics of cells undergoing EMT is downregulation of surface CDH-1 (cadherin 1, also known as E-cadherin) and EMT is best characterized by decreased expression of CDH-1 and increased expression of CDH-2 (N-cadherin). Indeed, aberrantly attenuated expression of CDH-1 was reported to be associated with high progression rate of bladder UC [8,9]. Recently Al-Ahmadie et al. [10] observed truncating somatic mutations in the *CDH-1* gene in 84% of plasmacytoid bladder cancers, a highly invasive histological variant of UC. Knock-out of CDH-1 in bladder UC cells enhanced cell migration, suggesting that loss of CDH-1 expression is not just a marker for EMT, but has some central, causal, and functional significance in tumor invasion and progression.

It is well known that numerous signaling pathways involving TGFβ, integrins, Notch, Wnt, and sonic hedgehog (SHH) induce EMT [7,11]. Recent studies addressed molecular mechanisms involved in TGFβ-induced EMT in UC cells. Those studies revealed malat-1 [12,13] and EIF5A2 [14] downstream mediators of TGFβ signaling pathway that induce EMT. Another study showed that PPM1A functions as a negative regulator of EMT by dephosphorylating TGFβ-activated Smad2/3 [15]. Although those reports suggest that TGFβ signaling promotes EMT in UC, another study showed that GDF15, a member of TGFβ superfamily, inhibits EMT through upregulating mammary serine protease inhibitor (MASPIN) and N-myc downstream-regulated family genes (*NDRG1*, *NDRG2*, and *NDRG3*) [16]. Additionally, it is yet to be fully understood what cellular interaction including auto- and para-crine mechanisms induce TGFβ signaling in the microenvironment of UC tumors.

Several recent reports suggested that integrins and associated signaling pathways are implicated in EMT of UC. Integrins mediate cellular adhesion to extracellular matrix (ECM). In the process of EMT, coordinated regulation of the integrin-mediated cell–ECM adhesion and E-cadherin-mediated cell–cell adhesion is required [17]. A report showed that knockdown of αv integrinsled UC cells to a shift towards more epithelial track characterized by increased CDH-1/CDH-2 ratio and downregulation of EMT-associated genes including *SNAI2*, *NANOG*, *BMI1*, *ALDH1* [18]. Importantly, these phenotypic changes were associated with decreased metastatic growth ability of the cells. Integrin-linked kinase (ILK) is highly evolutionally conserved serine/threonine kinase binding to β1 integrin [19]. Like other focal adhesion molecules such as c-Src and FAK, it mediates outside-inside signal transduction from ECM–integrin interaction. It was reported that ILK expression is higher in mesenchymal UC cells compared with epithelial ones [20]. Exogenous expression of ILK led epithelial UC cells to mesenchymal shift through activation of GSK3β–Zeb1 pathway. Importantly, ILK expression is positively correlated with invasive phenotype of human and murine bladder UC. These findings indicate that ECM–integrin adhesion plays a key role for cancer cell plasticity as well as E-cadherin-mediated cell–cell adhesion.

When we consider that EMT occurs physiologically during embryonic development and tissue repair, it is not surprising that EMT in cancer cells are also regulated by the developmental signaling pathways including SHH, Wnt, and Notch pathways. Specifically, recent studies have shed light on the role of SHH signaling in urothelium and UC. Beachy's group found that basal urothelial cells expressing SHH gave a rise of whole urothelial layer [21]. They also

demonstrated that SHH-expressing basal cells were essential for the development of CIS, an early form of mesenchymal UC [22]. However, they observed that SHH expression in CIS was lost as it progresses to invasive bladder UC. Additionally, they reported that inhibition of SHH dramatically accelerated tumor progression and decreased survival time [23]. These findings suggest a tumor-suppressive function of SHH signaling despite another essential role in the maintenance of epithelial stem cell or tumor-initiating cells. It is consistent with a previous report showing more abundant SHH expression in NMIBC compared with MIBC [24]. However, there have been a few reports showing positive correlations between SHH expression and clinicopathological aggressiveness of bladder UC [25,26]. Accordingly, the role of SHH in UC is warranted to be elucidated in the future studies as it can be a potent therapeutic target.

It is well known that Wnt/β-catenin signaling pathway also regulates EMT in various cancers [27]. β-catenin interacts with E-cadherin or triggers the activation of EMT-inducing transcription factors including SNAIL (Snail 1/2/3), TWIST (Twist 1/2), and ZEB (Zeb 1/2). It was reported that homeodomain-interacting protein kinase-2 (Hipk2) negatively regulated EMT and subsequent invasion by inhibiting Wnt/β-catenin signaling pathway in UC cells [28]. Several other reports also demonstrated that Wnt/β-catenin pathway activation induced EMT in UC cells [13,29].

Recent studies have shed light on several different aspects of the biology of Notch in bladder cancer including EMT. A report revealed that Notch acted as a tumor suppressor by inhibiting ERK pathway [30]. Another report showed that disruption of *Rbpj* and *Psen*, important mediators of Notch signaling, promoted bladder squamous cell carcinoma (SCC) with mesenchymal features in *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine (BBN)-induced mouse bladder carcinogenesis model [31]. Another group, inspired by genomic gain of Notch2 in The Cancer Genome Atlas (-TCGA) dataset, has shown that overexpression of Notch2-intracellular domain (N2ICD) promoted growth and EMT of UC cells [32]. These findings indicate that Notch1/2/3 have distinct and context-dependent functions in various aspects of biological processes in UC. Further understanding is needed for specific targeting of Notch pathways in the management of UC patients.

Finally, some carcinogenic chemical substances have been reported to induce EMT in UC cells. Benzidine is known as a strong urothelial carcinogen. A previous report showed that benzidine induced EMT of normal bladder urothelial cells through activation of ERK1/2 pathway [33]. Chronic exposure to arsenic acid causes UC as well as skin and lung cancers [34]. It was reported that arsenic acid induced EMT in lung and prostate epithelial cells [35—37]. However, precise mechanisms of arsenic acid-induced UC carcinogenesis or progression have not been elucidated. Aristolochic acid, a compound found in Chinese herbs, is known to induce progressive tubulointerstitial fibrosis, chronic renal insufficiency, and upper urinary tract UC [38]. Aristolochic acid was reported to induce EMT in human proximal tubule epithelial cells [39], while affected urothelial cells frequently undergo T→A transversion in codon 139 of exon 5 of *p53* gene [40]. Mechanistic association between the two observations is not fully understood and subject to the future studies. These agents have been implicated in carcinogenesis based on their potential to induce EMT on normal epithelial cells. As described above and elsewhere [7], however, EMT is strongly associated with local invasion and metastatic progression of cancer cells as well. Indeed, it was reported that bladder cancers associated with arsenic acid exposure was more aggressive than those in unexposed patients [41].

2.2. Cancer stem cell or tumor-initiating cell

Several investigators reported that cancer stem cells or tumor initiating cells of UC were successfully isolated based on the expression of cell surface markers or some functional molecules [42—45]. In addition to markers commonly proposed for tumor-initiating cells of solid cancer including CD24, CD44, CD133, and ALDH1A1, several unique markers such as CK14 have been identified as UC stem cell markers [45]. Intriguingly, tumor-initiating cells displayed distinct marker profiles according to stage and grade of original tumor, suggesting that phenotype of tumor-initiating cells defines biological and clinical aggressiveness of bladder UC [46].

Cancer stem cell hypothesis is an important concept for the understanding of cancer biology including the multistep process of tumor metastasis. Provided that cancer stem cell theory is closely associated with the process of EMT [45,47,48], it is not surprising that tumor-initiating cells seem to play an important role in UC metastasis, while it is also reported to be responsible for chemo-resistance of UC [45,49]. Indeed, cancer stemness and EMT were mutually linked by common markers including *OCT4*, *NANOG*, *SNAI1/2*, *ZEB1/2* and *TWIST* [42,45]. Indeed, expressions of various stem cell markers including *OCT4* [50,51], *ALDH1A1* [52], *ZEB1/2* [53], and *TWIST1* [54] were reported to be associated with bladder UC metastasis (extensively reviewed by van der Horst et al. [45]). However, another study on stem cell markers and EMT signature questioned the strict correlation between cancer stemness and EMT in bladder UC cells [55].

Apart from EMT, Overdevest et al. [56] reported that bladder UC cells expressing stem cell marker CD24 had higher potential of lung metastasis. CD24-expressing cells showed higher early retention to the lungs after tail vein injection, suggesting that CD24 expression promoted lung colonization. However, another group reported that CD24 expression did not alter tumor-initiating ability in the subcutaneous xenograft model [57].

Importantly, matched pair immunohistochemical analysis using primary bladder UC and lung metastasis tumors demonstrated that lung metastatic lesion expressed higher CD24 compared with corresponding primary tumors. Additionally, another study showed that CD24 expression was associated with inferior post-RC survival in bladder UC patients [57].

CD24 is considered as a luminal marker as it is expressed in superficial umbrella cells of normal urothelium [57] and luminal subtypes of UC [5]. It is notable that a luminal marker is associated with higher metastasis ability and poor prognosis of cancer. In this regard, these traits of CD24 as a molecular marker for poor prognosis and luminal subtypes have been also reported in breast cancer [58], suggesting

that CD24 has a common function correlated with metastatic ability and luminal characteristics. Further studies are warranted for the elucidation of CD24 function in cancer in the future.

2.3. Role of tumor microenvironment

It is obvious that above-mentioned cellular signaling pathways (TGFβ, integrins, Notch, Wnt, and SHH) regulating EMT and tumor stemness are closely associate with tumor microenvironment, which has been increasingly studied in recent years as exemplified by the Beachy's reports showing epithelial-mesenchymal interaction mediated by SHH and Wnt in urothelial regeneration [21] and UC development and progression [22,23]. Another report showed that, in the tumor microenvironment, UC cells and recruited mast cells cooperatively enhanced EMT and metastasis by modulating ERβ/CCL2/CCR2 signaling pathway [59]. Another group investigated infiltration of tumor-associated macrophage (TAM) by assessing CD163 expression [60]. CD163 expression was positively correlated with local expression of IL-6 and IL-10. Interestingly, CD163 expression was observed not only on TAMs but also on tumor cells in some cases. Moreover, CD163 expression on tumor cells was significantly associated with more advanced disease stage, higher histological grade, and inferior clinical outcomes. More recently, some investigators focused on exosome that promotes EMT in an autocrine manner [61]. Indeed, exosomes extracted from some mesenchymal bladder UC cells contained EMT-related proteins and reduced expression of CDH-1 and β-catenin in urothelial cells [62,63].

Like other solid tumors [47,64,65], it is believed that UC requires tumor-associated stroma to maintain tumor-initiating cells [43,45,47]. Thus, epithelial-mesenchymal interaction in tumor microenvironment is increasingly recognized as important biological processes that are essential to induce EMT or maintain tumor-initiating cells of UC. This should be subject to further investigation in the future studies.

3. Metastasis promoters and suppressors of UC

Recent efforts in UC research identified a number of metastasis-related genes in UC. Our group identified RalGAP complex as the GTPase-activating protein (GAP) of small G-protein Ral [66], which has been implicated in bladder cancer metastasis and progression [67—69]. Indeed, activity of Ral in bladder UC cells was inversely correlated with of RalGAP expression. The expression RalGAP is downregulated in high grade bladder UC and exogenous expression of RalGAP attenuated cellular metastatic potentials. Additionally, RalGAPa2 knock-out mice developed more invasive bladder cancers compared with wild-type animals after administration of bladder carcinogen BBN [70]. Our results suggested that RalGAP functions as a tumor metastasis suppressor through regulating the Ral pathway in bladder UC.

p63, a member of p53 tumor suppressor family, is a basal cell marker and implicated in maintenance of normal urothelial stem cells. Similar to breast cancer, p63 is categorized as a basal epithelial marker in UC [5,71]. There are several isoforms for p63 harboring (Tp63) or lacking (ΔNp63) *N*-terminal transactivation domain [72]. It was reported that Tp63 was abundantly expressed in non-invasive bladder UC [73]. Additionally, the same group and others reported that loss of p63 expression was associated with muscle-invasive progression or aggressive pathological phenotypes of UC [72,74—76]. Indeed, a number of reports showed metastasis suppressor function of p63 in various cancers [77,78]. These works collectively suggest a tumor metastasis suppressor function of Tp63 in UC. According to previous reports studying ΔNp63 variants specifically, however, a subset of muscle-invasive bladder cancer expressed ΔNp63 and maintenance of ΔNp63 expression is significantly associated with UC-specific mortality [72,73]. Collectively, p63 isoforms seem to have distinct functional significance that is also context-dependent. Future studies should better dissociate expression profiles and molecular function of p63 isoforms at various biological steps, which would enable us to understand the role of this interesting molecule for the UC pathogenesis.

Several receptor tyrosine kinases including epithelial growth factor receptor [79,80], FGFR (fibroblast growth factor receptor) [81] and c-Met [80,82] have been implicated in UC metastasis (also extensively reviewed by McConkey et al. [7]). However, it is well known that dependency on FGFR signals is considered as one of the characteristics of epithelial UC. Consistently, it was reported that mesenchymal UC cells are more likely to show resistance to an FGFR receptor tyrosine kinase inhibitor [83]. As for transcription factors, FOXQ1 was reported to promote EMT of UC cells upon stimulation of TGFβ as in other malignancies [84]. KLF4 was reported to be downregulated in UC and act as a metastasis suppressor inhibiting EMT [85]. Androgen receptor (AR) was reported to promote UC metastasis by affecting CD24 [86—88]. Several investigators reported that infiltrating neutrophils [89] and B lymphocytes [90] modulated AR transactivation leading to elevated MMPs expression and cell invasion. Interestingly, these reports and others [91] showed that AR in UC cells activated in ligand-independent manners.

c-Src is one of the oldest proto-oncogene that encodes non-receptor tyrosin kinase. c-Src has been recognized to promote metastasis of cancers by modifying focal adhesion and cell—cell junction mediated by integrins and cadherins [92,93]. However, Theodorescu's group [94] recently reported that c-Src acted as metastasis suppressor in bladder UC thorough phosphorylating RhoGDI2. Their reports showed that expression of c-Src was inversely correlated with tumor stage of bladder UC [94,95]. Considering that a number of Src inhibitors such as dasatinib have been developed for cancer treatment, the bilateral roles of c-Src for cancer metastasis should be further elucidated in the future.

Recently microRNAs (miRNA) have been reported to regulate various biological processes including tumor invasion and metastasis. To date, miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429) have been most extensively studied [96—100], while many others such as miR-205 [101], miR-429 [102], miR-218 [103], miR-23b [80,104], miR-34a [105], miR-451 [106], miR-145 [107], and miR-433 [108] have been reported to act as a metastasis promoter or suppressor. More recent studies have shed light on long non-coding RNAs (lncRNA) including lncRNA-UCA1 [109], lncRNA-HOTAIR [110], and MALAT-1 [13] as

metastasis promoters. It is intriguing that these lncRNAs were reported to induce EMT through upregulating EMT-related genes such as *ZEB1/2*. Accordingly, lncRNAs seem to be a promising research subject for potential therapeutic targets and clinically useful urine markers.

4. Therapeutic opportunities

Recent efforts showed some promising findings that potentially lead us to effective prevention or treatment of UC metastasis. Several Cox-2 inhibitors was shown to inhibit UC cell growth as well as reversal of EMT characterized by induction of CDH-1 and reduction of SNAIL expression [111]. A micro-tubule-targeting agent vinflunine was also reported to reverse EMT [112]. Vinflunine increased CDH-1 by stabilizing it through inhibiting Hakai, E3-ubiquitin ligase of CDH-1. Silibinin, a natural flavonoid, was reported to inhibit GSK3β/β-catenin signaling, decrease *ZEB1* expression, and subsequently suppress metastasis through blocking MET and stemness of UC cells [113]. A report showed the possibility that reactivation of p53 through RNA activation using small activating RNA may inhibit cell growth and EMT of UC cells [114]. A plant alkaloid tetrandrine was reported to act as GLI-1 inhibitor blocking Hedgehog signaling and subsequently suppress EMT and metastasis of UC cells [115]. Finally, Theodorescu's group [116] discovered novel small molecules RBC8 and BQU57 that inhibited Ral activity. They showed promising effects of these drugs on UC cell growth, however it is also anticipated that these drugs may also suppress metastasis of UC since Ral pathways have been implicated in metastasis of various cancers including UC [68—70] and others [117—120].

5. Experimental models of UC metastasis

It is essential to have useful models that recapitulate pathogenesis, biology, and treatment response for the development of novel treatments of human disease. Currently a variety of experimental systems are available for the research on UC metastasis.

5.1. Cell culture experimental systems

A panel of UC cell lines is currently available with the information for their origin and genetic profile [121]. Cell culture-based experiments related to metastasis research include wound healing ("scratch") assay, Boyden chamber transwell assays. Wound healing assay is an easy, well-developed and inexpensive method that has been employed for years to study cell motility [122]. Boyden chamber transwell assay utilizes chemotaxis and leads cells to go across a polycarbonate membrane with micropores (3—15 μm in diameter). If the membrane is coated with ECM such as matrigel or collagens, one can see the cellular potential to invade across basement membrane. Transwell assays with different ECM substances may provide information for key molecules that facilitate invasion such as matrix metalloproteinases (MMPs) [123]. They are useful assays to dissociate intrinsic cellular motility or migratory potential. Indeed, abilities of migration and invasion are not necessarily parallel [124]. Additionally, higher motility or invasiveness in these assays does not always mean high metastatic potential of the cells *in vivo*.

5.2. Cell line-based *in vivo* experimental systems (Table 1)

Various approaches currently available to model bladder cancer *in vivo* are mostly utilizing mouse [125]. Cell-based xenograft models are widely used with their potential advantages. Preparation of the cells is easy including genetic manipulation for functional assays or reporter expression for *in vivo* imaging. However, it inevitably requires immunocompromised animals. Experimental systems based on

Table 1 Urothelial carcinoma cell lines for metastasis researches.

Cell line	Source	Application
253J[a]	MIBC	Orthotopic intramural injection model (LN met.) [137] (Liver met.) [130]
		Orthotopic intravesical injection model (LN and lung mets.) [135,136]
		Caudal vein injection model (lung met.) [130]
J82	MIBC	Intraperitoneal injection model (intraperitoneal spread) [153]
		Subcutaneous injection model (liver met.) [128]
		Caudal vein injection model (liver and lung mets.) [128]
UM-UC-3[a]	Met.	Subcutaneous injection model (lung met.) [129]
		Caudal vein injection model (lung met.) [56,95,132]
		Orthotopic intramural injection model (liver and lung mets.) [82]
UM-UC-14	MIBC	Orthotopic intramural injection model (LN mets.) [138]
T24	MIBC	Caudal vein injection model (lung met.) [131]
TSU-Pr1[a,b]	MIBC	Orthotopic intramural injection model (lung met.) [134]
		Intracardiac (left ventricle) injection (lung, liver, brain, LN, bone mets.) [133,134]
		Intratibial injection (mimicking tumor growth at bone metastatic lesion) [134]
EJ	MIBC	Caudal vein injection model (lung met.) [114]

LN, lymph node; MIBC, muscle-invasive bladder carcinoma; Met., metastatic bladder carcinoma.
[a] Including derivative sublines.
[b] Derivative of T24 [154].

immunocompromised host animals limit the investigation of role of immune systems in UC metastasis. Additionally, the results from these systems should be carefully interpreted provided the known importance of the immune system for cancer metastasis [126]. Use of syngeneic system with mouse bladder cancer cells established from carcinogen-induced tumors seems to be a good solution in the future.

Subcutaneous injection model is one of the most widely used cell-based xenograft experimental systems. It is technically simple and allows easy access to the tumor. Renal subcapsular engraftment was also reported [127]. It seems to yield higher take rate due to more abundant blood flow but requires more sophisticated experimental skills. Although there are numerous previous reports studying tumor growth at engraftment site, there are the limited number of studies that addressed metastatic ability of the UC cells using these ectopic engraftment models [128,129].

Direct injection of tumor cells into circulation is more widely used for metastasis researches. In the intravenous injection, caudal vein injection in most cases, tumor cells enter the right ventricular (pulmonary) circulatory system and subsequently form lung metastasis [56,70,95,114,128,130—132]. On the other hand, in the left ventricular intracardiac injection, tumor cells enter the left ventricular (systemic) circulatory system and subsequently metastasize to bone, liver and brain [133,134]. These experimental systems allow us to focus biological behavior of the tumor cells after intravasation into the circulation.

Orthotopic engraft models can be stratified into intravesical injection (instillation into bladder lumen) [135,136] and intramural injection (inoculation into bladder wall) [82,130,137,138]. The former primarily mimics the seeding implantation of superficial bladder cancer and the latter submucosal progression of infiltrating bladder cancer. Both methods can be used to investigate metastasis once after successful tumor engraftment and progression. These models have an advantage of representing tumor microenvironment at the primary site and reflecting multistep metastatic process more faithfully. However, the rate of metastasis formation are usually much lower compared with intravascular injection models. The primary tumor at the engraftment site often grows rapidly and the host animal has to be sacrificed before the development of metastatic lesions [139]. Additionally, the intramural inoculation used to require bladder exposure via lower abdominal incision and very sophisticated skills for precise injection. A recent report has shown a modification with less invasive and more precise procedure using ultrasonography scan [140].

5.3. Patient-derived tumor graft

Patient-derived xenograft (PDX) is a classical experimental system that has been revisited and growingly become important [141,142]. Almost all PDX lines are subcutaneously engrafted and passaged. This seems to be the main reason for no prior report on the application of PDX lines for metastasis researches although only one previous report showed highly invasive characteristics of the xenograft [143]. In this regard, orthotopic engraftment of PDX tumor or dissociated tumor cells seems to be a resolution although it will be technically challenging.

5.4. Chemical-induced bladder cancer

Although a number of chemical carcinogens are known to induce bladder cancer in rodents, BBN is currently most widely used for bladder cancer research. Mice administered with BBN in the drinking water develop CIS and then muscle invasive bladder cancer eventually. A problem is the model is high prevalence of squamous cell carcinoma [144,145], which accounts for less than 10% of human invasive bladder cancer [146]. Only a few studies reported the metastatic progression in this model [86,144]. BBN-induced bladder carcinogenesis model has a potential to be combined with genetically engineered mice and can be used for functional association of metastasis-related genes. Additionally, syngeneic bladder tumors developed in immunocompetent animals are suitable for immune-oncological investigations that are becoming increasingly crucial for the era [147].

5.5. Genetically-engineered mouse (GEM)

As well as the BBN-induced model, GEM models can represent *de novo* carcinogenesis in immunocompetent hosts. Therefore, given relevant genetic alterations in the right population of the urothelial cells, GEM may recapitulate the pathogenesis and tumor microenvironment of human disease more faithfully. However, bladder cancer is still underrepresented by GEM models compared with other types of malignancies although there are an increasing number of GEM models of bladder cancer being reported [125]. Most models develop non-muscle-invasive disease and metastatic progression is rare even in invasive bladder cancer models with a few exceptions.

One of these exceptions is SV40T-based transgenic mouse models. Wu's group [148] first established a GEM model harboring Simian Virus 40 T antigen (SV40T) transgene driven by *uroplakin II* promoter. Although mice having low copy number of SV40T transgenes developed only CIS, those having high copy number developed invasive UC and some of them showed metastatic progression to lymph nodes and liver. Another group reported a similar GEM model based on SV40T transgene driven by *cytokeratin 19* (*Krt19*) promoter [149]. This model was characterized by lung metastasis that was observed in 25% of the mice. Unfortunately, non-specific activation of Krt19 led the mice to develop tumor lesions in other organs including adrenal glands, prostate and mesothelium. In these GEM models, however, metastasis is observed in a subset of affected mice and other investigators reported that they found no metastasis in similar models [150,151]. These findings suggest that these gene alterations are not sufficient and that the accumulation of other mutations or tumor suppressor inactivation are required metastatic progression.

Another problem in using SV40T-based model is the low relevancy of the gene alteration that drives mouse bladder carcinogenesis and progression. The role of SV40T in human UC is very limited, although inactivation of p53 and Rb, major targets of SV40T, is among the most frequently observed genetic events in human MIBC [4] and molecular profiles of SV40T-based mouse bladder cancers were reported to be conserved with those of human disease [151].

Another GEM model for MIBC metastasis is based on inactivation of *Trp53* and *Pten* in urothelium [127]. This model is characterized by stochastic gene recombination in urothelium induced by Adeno virus expressing Cre recombinase that is directly injected into bladder lumen. It was reported that about 60% of affected mice developed macroscopically visible metastatic spread. Metastatic loci were observed most frequently in regional lymph nodes, while distant metastatic lesions were detected in spleen, liver, and diaphragm.

However, UC metastasis has not been studied extensively using these autochthonous mouse models. There seem to be several aspects of difficulties that hamper UC metastasis research using autochthonous mouse models. A primary tumor often becomes lethal causing obstructive renal insufficiency before the tumor cells metastasize to distant organs. Bladder tumor of 1 cm in diameter can kill the host mouse, while a tumor of that size is hardly identified with concomitant distant metastasis. Higher induction rate (whatever chemical exposure or gene recombination) or rapid growth can be an advantage for studies on primary tumor, but those characteristics may sometimes make metastasis research difficult. In this regard, use of lentivirus that usually yields lower infection efficiency compared with that of Adeno virus may benefit as succeeded in a lung cancer research [152]. Another issue is that anatomies of human and mouse urinary tracts are not exactly same, with particular regard to blood supply and lymphatic drainage. Mouse bladder is not enveloped with fat tissue and not insulated from peritoneal cavity by dense peritoneum like human bladder. While recent efforts have provided relatively detailed information about bladder cancer genetics [4,6,42,71], molecular correlation between subtypes of human UC and GEM models are still under investigation. Since the cell of origin for MIBC is still inconclusive [22,145], there are still limited options to induce gene alterations specifically to the urothelium. We need to overcome these barriers in order to establish more relevant and efficient GEM models for UC metastasis research in the future.

6. Future directions

The biology of UC metastasis is not fully understood. The lack of experimental models that accurately recapitulate the human disease has been one of the main barriers that has precluded our precise understanding of UC metastasis. Although several concepts in cell biology such as EMT or cancer stem cell appear to be attractive to explain biology of UC metastasis, it does not seem to be fully linked to *in vivo* findings in animal models. There is an urgent need for the establishment of novel animal models that recapitulate molecular, genetic, and clinical characteristics of human disease. It is anticipated that precise modeling of the disease will lead us to more profound understanding and consequently the development of novel therapeutic strategy to overcome metastasis of UC.

Conflicts of interest

The authors declare no conflict of interest.

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