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CRISPR-Cas9 potential for identifying novel therapeutic targets in muscle-invasive bladder

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Abstract

Gene editing technologies help identifying the genetic perturbations driving tumour initiation, growth, metastasis, and resistance to therapeutics. This wealth of information highlights tumour complexity and is driving cancer research towards precision medicine approaches based on an individual's tumour genetics. Bladder cancer is the 10th most common cancer in the UK, with high rates of relapse and low survival rates for patients with muscle-invasive disease. Muscle-invasive bladder cancer (MIBC) is highly heterogenous and encompasses multiple molecular subtypes, each with different responses to therapeutics. This evidence highlights the need to identify innovative therapeutic targets to address the challenges posed by this heterogeneity. CRISPR-Cas9 technologies have been used to advance our understanding of MIBC and determining novel drug targets through the identification of drug resistance mechanisms, targetable cell-cycle regulators as well as novel tumour suppressor and oncogenes. However, the use of these technologies into the clinic remains a substantial challenge and will require careful consideration of dosage, safety, and ethics. clustered regularly interspaced short palindromic repeats-CRISPR-associated protein (CRISPR-Cas9) offers considerable potential for revolutionizing bladder cancer therapies, but substantial validation research is required before these technologies can be used in the clinical setting.

[H1] Introduction

Advances in gene editing technologies provided vast information about the genetic perturbations driving tumour initiation, growth, metastasis, and resistance to therapeutics, highlighting the need for precision medicine approaches based on an individual's tumour genetics. Gene editing technologies enable to study the phenotypic effects of genes at an individual level, up to genome-wide scale. Traditional nuclease-based gene targeting methods, such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), were the main used for carrying out genetic modifications [1]. However, these methods have been replaced by the development of the clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) technologies, which have increased efficiency, reduced costs, and are easier to design[2] . These

CRISPR-Cas technologies are a highly valuable research tool for studying many aspects of cancer research, including genetic drivers of tumorigenesis, invasion, and drug resistance, which can lead to the detection of novel therapeutic targets[3–5].

Bladder cancer is the 11th most common cancer in the UK across males and females but the 8th most common cancer in males in the UK, with over 10,000 diagnoses in the UK every year [6]. In general, non-muscle invasive bladder cancer (NMIBC) is highly treatable with good survival statistics [7]. However, relapse is common, and ~20% of NMIBC instances progress to muscle-invasive disease, which is associated with a 5-year survival rate of only 45% [8], with nodal and metastatic disease considered to be incurable. The current standard of care for MIBC includes chemotherapy, radical cystectomy, or radiotherapy with a radiosensitiser, and in metastatic disease, immunomodulation [9–11]. Targeted therapies currently available for the treatment of bladder cancer are limited. However, some therapies such as PDL1 and FGFR3 inhibitors have been approved as second-line therapies to be provided following failure of first-line treatments [12]. However these second-line therapies have limited therapeutic benefit, as resistance remains a substantial problem [13–15].

Muscle-invasive bladder cancer (MIBC) can be categorised into six molecular subtypes: luminal papillary; luminal non-specified; luminal unstable; stroma-rich; basal/squamous; and neuroendocrine-like [16–18]. These subtypes differ in treatment response and intrinsic therapy resistance, which leads to substantial negative impact in patients. Hence, further advances are needed to identify innovative therapeutic strategies to address the challenges posed by both the intra-tumour and inter-tumour heterogeneity of bladder cancer. CRISPR technologies could give valuable insight in this field.

In this Perspective, we aim to summarise how CRISPR-Cas9 gene editing technologies have been used to advance our understanding of MIBC for novel target discovery, and how these technologies could be further used to improve patient outcomes.

[H1] CRISPR Cas mechanisms and methodologies

Within genetic engineering, CRISPR Cas has become the key technology utilised for the specific and precise targeting of DNA sequences. Among the CRISPR Cas systems, the most commonly used is CRISPR Cas9 due to its simplicity and versatility.

[H2] CRISPR-Cas9

Advances in gene editing technologies enabled researchers to study phenotypes and functional effects of mutations or knock-out of specific genes. One of the main technologies currently used to this aim is CRISPR-Cas9, which enables us to target any gene of interest with high specificity for a variety of purposes. CRISPR-Cas9 is the CRISPR system based on the Cas9 endonuclease from *S. pyogenes* 'SpyCas9', and is the most widely used gene editing system[19]. CRISPR-Cas9 has been adapted from a naturally occurring gene disabling system in bacteria, in which CRISPR-associated protein (Cas) nuclease activity is guided to specifically degrade phages' DNA as a defence mechanism against invading viruses[20]. In mammalian cells, CRISPR-Cas9 has been re-purposed to knock-out or modify target genes [20,21], supporting the identification of genetic drivers of disease progression and therapeutic resistance. This discovery by Emmanuelle Charpentier and Jennifer Doudna was so instrumental in genetics research that it was awarded the Nobel prize in chemistry in 2020.

The CRISPR-Cas9 system induces double-strand breaks (DSBs) in DNA using the Cas9 endonuclease bound to a single-guide RNA molecule (sgRNA), which guides the Cas9 to the DNA target site (Box1). Cas9 is the most commonly used CRISPR system, but other CRISPR methodologies are available involving alternative forms of Cas9, such as SauCas9, as well as other Cas proteins including Cas12,

Cas13 and Cas3 [22,23].DSBs are subsequently repaired through non-homologous end joining (NHEJ), which, however, is highly error prone[24], or homology-directed repair (HDR), which is highly precise and can be used to induce mutations of choice within the DNA [25].Other repair pathways exist, such as microhomology-mediated end joining, but in this Perspective, we will focus on the most used NHEJ and HDR [26].

[H2] CRISPR-Cas methodologies

Since the development of CRISPR Cas gene editing, there have been extensive advancements in these technologies. This has meant there is now a wide breadth of options available for studying the genetic landscape of cancer.

[H3] CRISPRa and CRISPRi libraries

CRISPR-Cas9 technology is most commonly used to achieve gene knock-out by inducing a DSB in DNA target genes (Figure 1A). However, in the past 10 years this technology has been further adapted to alter target gene expression using a catalytically inactive form of the Cas9 endonuclease (dCas9), which lacks the ability to cut DNA, but can still bind target DNA sequences guided by a sgRNA. CRISPR-activating (CRISPRa) and CRISPR-inteference (CRISPRi) are two systems in which dCas9 is bound to a transcriptional effector (Fig. 2B) or to a transcriptional repressor, (Fig. 2C), respectively, to alter signalling downstream from a target gene of interest without causing permanent genetic changes [27]. In CRISPRa, dCas9 binding to the promoter region of the gene of interest induces recruitment of transcription activators, such as VP64 and the synergistic activation mediator (SAM), leading to increased gene expression [28,29]. Conversely, in CRISPRi, sgRNA binding to the promoter region prevents transcription-factor binding, leading to reduced mRNA transcription [30]. Using these inducible systems enables to study essential genes, which is not possible during knock-out screening. Furthermore, these systems induce transient changes in gene expression, which can be used, for example, to simulatethe responses of cancer cells to selection pressures such as that induced by drugs, thereby increasing our understanding of these processes [31]

[H3] Base editing and prime editing

Further derivatives of CRISPR-Cas9 techniques include base editing (Fig. 2D) and prime editing (Fig. 2E), which enable to induce point mutations and small changes in the genome without inducing a DSB. These strategies reduce the chance of unwanted addition of indels whilst maintaining high efficiency. Base editing is carried out using CRISPR-Cas9 base editors, which consist of a catalytically inactive form of Cas9 (dCas9) or, more commonly, Cas9 D10A nickase, which only nicks the DNA[32,33]. Deaminases cytidine or adenine deaminase are tethered to this complex, and can direct DNA base substitutions without the need for DSB [34,35]. This technology offers the potential to correct cancer-causing genetic mutations or create desired genetic variations to observe the phenotypic outcome of individual base changes [36]. Similarly, prime editing is a modification of the CRISPR-Cas9 system that also leverages the RNA-guided binding of a Cas9 form that is unable to generate DSBs. In this system, a H820 nickase Cas9 is fused to an engineered reverse transcriptase, which is directed to genomic targets by a prime editing guide RNA (pegRNA). The pegRNA contains an RNA template that can be copied into the nicked genome by the reverse transcriptase [37,38]. This approach enables to achieve precise DNA edits without the need for a DSB or a supply of exogenous DNA.

[H2] Whole-genome knockout CRISPR screening

Whole genome CRISPR-Cas9 knockout screens (WGCS) consists in ablating gene expression on a genome-wide scale and studying the resulting phenotypic alterations to elucidate the relationship between genotype and phenotype In this approach, an sgRNA library (consisting of sgRNA against every gene in the genome, or a subset of genes) is transduced into Cas9-expressing target cells at a moiety of infection low enough to result in each cell receiving one single sgRNA, leading to a

population of cells each with a different gene knock-out [39]. This population of genetically altered cells can then be exposed to different conditions of interest or challenges. The DNA from cells surviving the selective pressure can be extracted and sequenced to determine which guides (genes) are enriched or depleted using the integrated sgRNA as a barcode [40,41]. This approach provides information about the roles of specific genes and how these genes are affected in different conditions. Following the discovery of new technologies such as CRISPRa and CRISPRi, CRISPR screens are also carried out using different Cas9 variants such as dCas9, Cas12 and Cas13, which induce altered gene expression instead of gene knockout [42–44].

[H1] CRISPR-Cas9 gene editing in MIBC research for target discovery

Before the widespread use of CRISPR, siRNA gene knockdown was a popular method for manipulating gene expression. Silencing of the receptor-interacting protein kinase 4 (RIPK4) through siRNA in multiple bladder cancer mouse models including an in-situ bladder tumour mouse model showed reduction in tumour stage[45]. However, this methodology has not successfully moved to use into the clinic[46,47] owing to issues including the lack of longevity of response to siRNA, due to siRNA modifications being transient as well as safety considerations. CRISPR-based technologies have overcome these issues, as gene knock-out or knock-in achieved through CRISPR-Cas are permanent genetic alterations. Furthermore, CRISPR screens using CRISPRa, CRISPRi, prime and base editing enable us to introduce a broader range of alterations and to test a wider range of effects than previously used techniques.

CRISPR gene editing techniques have widespread utility in cancer research, facilitating the identification of genetic drivers involved in tumour progression and drug resistance. The ultimate goal of these approaches is to uncover novel drug targets to improve the efficacy of cancer treatments. The heterogeneity and wide range of molecular subtypes in bladder cancer makes CRISPR-based screening approaches particularly relevant and led to rapid advances in understanding how to improve patient outcomes (Fig.2).

[H2] CRISPR-Cas to identify mechanisms of chemotherapy resistance

Chemotherapy resistance is prevalent in individuals with MIBC, and is mainly attributed to acquired resistance mechanisms within the tumour, including overexpression of drug efflux pumps, activation of anti-apoptotic signalling pathways and increased DNA repair[48–51]. However, the exact understanding of these mechanisms remains limited, leading to treatment failure and disease progression. CRISPR screening methods have been used to help identify the genetic factors contributing to these acquired resistance mechanisms.

In MIBC, WGCSs have been used to identify multiple genes controlling chemotherapy sensitivity and resistance. Cisplatin stands as a primary option among chemotherapeutic agents for the treatment of MIBC[9], underscoring the importance of comprehending both resistance mechanisms and the genetic factors influencing chemotherapy sensitivity in tumours. Results from a WGCS in MIBC cells showed that Schlafen 11 (*SLFN11*) knockout caused chemotherapy resistance[52]. This result was validated both in vivo in animal models and in patient tumour samples[52]. Following cisplatin-induced DNA damage, SLFN11 acts as a regulator of cell-cycle progression by interacting with the Mediator of DNA damage checkpoint 1 protein (MDC1) [52]. Results from this WCGS suggest that high SLFN11 expression in patients could be an indicator that neoadjuvant chemotherapy (NAC) would have high efficacy [53]. Considering this chemo-sensitizing effect of SLFN11 overexpression across multiple cancer types [54], epigenetic modification of *SLFN11* to increase expression has been hypothesized to be an effective novel therapeutic strategy for re-sensitisation of tumours to cisplatin-based chemotherapy [55]. Multiple inhibitors of epigenetic modulators targeting DNA methyl transferases, histone lysine methyltransferases, or histone lysine acetyltransferases have been explored in clinical trials and

showed promise in combination with current standard of care treatments including chemotherapy and immunotherapy in a range of tumour types, indicating future potential for [56]novel therapeutics.

In another WGCS study in MIBC, *MSH2* knockout was shown to confer resistance to cisplatin-based chemotherapy through downregulation of DNA-damage response mechanisms [57]. This reduced DNA-damage response is probably mediated by MSH2-mediated recruitment of ERCC1, a known marker of cisplatin resistance, during DNA mismatch repair [58]. Moreover, low MSH2 expression in tumour samples from patient with MIBC positively correlated with reduced overall survival and poor prognosis[59], indicating that MSH2 could act as a predictive biomarker for cisplatin sensitivity. Other mechanisms of cisplatin resistance include onset of apoptosis orchestrated by the circular RNA circLIFR which interacts with MSH2 [60].

In another study, the heterogenous nuclear ribonucleoprotein U (*HNRNPU*) gene has been identified by WGCS as an important gene for cisplatin resistance in T24 MIBC cells, as *HNRNPU* knockout inhibited cell proliferation and migration and increased apoptosis in response to cisplatin. This effect was shown to be mediated by the modulation of the HNRNPU downstream target neurofibromin 1 gene (*NF1*), a tumour suppressor gene with a role in chemosensitivity . HNRNPU CRISPR Cas9 knockout induced upregulation of NF1 in T24 cells, inducing chemosensitivity to cisplatin[61]. The increased chemosensitivity observed in HNRNPU knockout cells was reversed by the knockout of *NF1*, highlighting that the effect of HNRNPU knockout was mediated by NF1 upregulation [61]. Furthermore, analysis of samples in The Cancer Genome Atlas (TCGA) showed that high HNRNPU expression is positively correlated with low survival rates in patients with MIBC [61]. Thus, inhibition of HNRNPU has been identified as a potential target for re-sensitisation of tumours to cisplatin-based therapy [62].

Multiple drivers of chemotherapy resistance have been identified using CRISPR technologies, but further studies are needed to validate these targets in vivo in order to move towards a clinical trial setting. Additionally, to date, only cisplatin resistance and sensitivity have been studied, although multiple other chemotherapies are used in bladder cancer treatment. Thus, further CRISPR screening approaches could be used to identify genetic drivers of response to different chemotherapy agents..

[H2] CRISPR-Cas to identify mechanisms of radiotherapy resistance

Radiotherapy has a widespread use in the treatment of bladder cancer, but very few studies have been carried out using CRISPR to determine the genetic drivers of radioresistance mechanisms. Hypoxia is a crucial driver of therapeutic resistance in bladder cancer to radiotherapy, as well as to other therapeutics, but the mechanisms underlying this resistance are poorly understood. Radiotherapy is given in combination with the radiosensitiser carbogen and nicotinamide (CON) as a standard of care recommended by the National Institute for Health and Care Excellence (NICE) in the UK. CON is used to alleviate tumour hypoxia to re-sensitise the tumour to the radiotherapy [63] and has been shown to increase overall survival by 14 months [64], highlighting the therapeutic benefit of targeting hypoxia. However, CON only alleviates the low oxygen tension but does not eradicate the hypoxic radiotherapy-resistant phenotype that has evolved. Thus, further research using CRISPR-Cas9 screens to identify novel drivers of hypoxia-induced treatment resistance is of paramount importance in bladder cancer research. This approach could also be used to identify drivers of pseudohypoxia or any oxygen-independent HIF activation.

[H2] Immunotherapy

Bladder cancer therapy has been enhanced throught the development of immunotherapy aiding in tumour treatment in both the first line and second line treatment settings. These therapies have

been found to be effective in the treatment of MIBC, however there is large scope for more use of these therapies [65]. CRISPR Cas based research is an essential tool in understanding and uncovering novel immunotherapy targets.

[H3] CRISPR screening to uncover gene targets for immunotherapy

A number of immunotherapies are being investigated for clinical use to treat MIBC. Inhibitors of programmed cell death protein-1 (PD1), programmed death ligand 1 (PDL1), and cytotoxic T-lymphocyte antigen 4 (CTLA4) have been particularly studied. Specifically, in a trial including patients with MIBC, combination therapy with the PD1 inhibitor nivolumab plus gemcitabine and cisplatin chemotherapies increased clinical complete response reducing the need for radical cystectomy [66]. Inhibition of these proteins prevents the suppression of T cell-driven tumour killing. CRISPR gene editing could help understand the mechanisms underlying this phenomenon. However, this approach has not yet been well-exploited in bladder cancer research.

Understanding the role of peripheral circulating blood lymphocytes and immune checkpoint-related protein expression on tumours is of high interest. A study was carried out on peripheral blood mononuclear cells from patients with bladder cancer and healthy donors to determine the differences in the expression levels of the immune checkpoint molecules PD1 and CTLA4. Expression levels of both PD1 and CTLA4 were shown to be elevated in patients with MIBC compared with healthy donors[67] . Additionally, CTLA4 was silenced on CTLs using CRISPR-Cas9 in MIBC cells co-cultured with control cytotoxic T lymphocytes (CTL). *CTLA4* KO was shown to enhance anti-tumour activity of CTL through increasing apoptosis in co-cultured MIBC cells, highlighting the role of CTLA4 in bladder cancer immune system evasion [67] . *CTLA4* KO-mediated increased cytotoxicity was also confirmed in in vivo mouse models of bladder cancer [67]. These results indicate that high CTLA4 expression is a marker of CTL exhaustion, and *CTLA4* KO counteracts this phenotype. Thus, immune checkpoint modification of CTLA could be an effective combination treatment with first-line therapies, chemotherapy and radiotherapy, to reduce T cell exhaustion and cancer immune evasion [67].

[H3] CRISPR as a tool to uncover mechanisms of response to immunotherapies

Tumours might become resistant to checkpoint inhibitors. Thus, CRISPR is being used to identify mechanisms of resistance associated with immune checkpoint inhibitors, specifically PD1. In a study in bladder cancer cells, dual CRISPR-Cas9 knockout of the checkpoint blockade *PD1* and the long non-coding RNA urothelial carcinoma associated 1 (UCA1) enhanced PD1-induced anti-tumour activity both in vitro and in vivo mouse model, reducing tumour growth and increasing survival [68]. In humanized SCID mice xenografted with a human bladder cancer cell line (5637) knockout for PD1 and UCA1, a shift in the tumour microenvironment— from immunosuppressive to stimulatory —was observed, and occurred through enhanced expression of Th1-associated immune-stimulating genes [69]. These results highlight how the efficacy of PD1 immunotherapies could be increased using a combination approach. However, this dual targeting of PD1 and UCA1 has yet to be tested in a clinical setting. Furthermore, lncRNAs based therapies are still within early stages due to issues such as the variability of lncRNA expression patterns between different patients[70]

[H2] Synthetic lethality studies using CRISPR-Cas9

When looking for novel cancer therapies, targeting only one gene might be insufficient, as, often, more than one gene mutation can be responsible for a phenotypic change. Thus, high interest has been growing around identifying synthetic lethal partners driving tumour progression. The gene pair of the CREB binding protein (*CBP*) and *p300* — which are histone acetyltransferases acting as

transcriptional coactivators— have been identified as potential therapeutic targets in other cancer types(such as breast and prostate cancer) [71]. The potential of these gene as therapeutic targets in bladder cancer has not been studied. However, CRISPR interference technology using the dCas9 system was used to achieve dual inhibition of these genes in bladder cancer cells. Fusion of the Kruppel-associated box1 (KRAB) is commonly known to increase effectiveness of gene silencing of dCas9 [31]. To make this inhibition bladder cancer cell-specific, the dCas9-KRAB system was combined with the hUPII and hTERT promoters, which are cancer- and bladder-specific promoters, respectively [72]. This strategy successfully prevented dual gene silencing in normal bladder urothelial cells. Dual suppression of *CBP* and *p300* in MIBC cells inhibited cell growth and increased apoptosis through a reduction of c-MYC expression [73]. This bladder cancer cell-specific approach enables to selective killing bladder cancer cells without causing damage to normal urothelial cells which, if successfully transferred into the in vivo setting, could provide a novel therapeutic approach in the treatment of bladder cancer. To date, this work has not been validated in in vivo models. Thus, translation of this targeting strategy in the clinic remains highly speculative.

[74][H2] Using CRISPR-Cas to achieve cell-cycle regulation

Cyclin dependent kinases (CDKs) have a central role in cell-cycle regulation and, therefore, have become attractive targets for cancer therapy. CDK inhibitors have been developed to specifically block the abnormal activation of CDK-cyclin complexes in cancer cells, causing cell-cycle arrest and inducing apoptosis. Some CDK inhibitors have been approved for the treatment of certain types of cancer, such as breast cancer [75]. However, none of these inhibitors have yet been approved for use in bladder cancer owing to lack of response in clinical trials [76]. A whole-genome CRISPR-dCas9 activation screen was carried out in MIBC cells to identify resistance mechanisms of bladder cancer to CDK4 and CDK6 inhibitors [77]. Enrichment of sgRNAs indicated genes that mediated CDK4 and CDK6 inhibitor resistance. Nearly 1000 targets were identified in this screen, which were then analysed to determine signalling pathways commonly associated with CDK4 and CDK6 resistance[77]. The vast number of enriched guides highlights the complexity of CDK4 and CDK6 inhibitor resistance. Multiple signalling pathways were identified as contributors to this resistance mechanism, such as receptor tyrosine kinase and PI3K—AKT signalling pathways[77]. These findings suggest that a panel of predictive resistance markers could be created to stratify patients, and combination therapy involving CDK4 and CDK6 inhibitors along with inhibitors of the identified resistance markers could be used, potentially improving treatment outcomes [78].

[H2] CRISPR-Cas to study tumour suppressors and oncogenes

CRISPR-Cas9 can also be used to study the role of tumour suppressors and oncogenes. The Kruppel like factor 4 (KLF4) is a transcription factor with varying roles in different tumour sites. Low expression of KLF4 is implicated in bladder cancer progression and promotion of epithelial to mesenchymal transition (EMT)[79]. Conversely, KLF4 overexpression is associated with reduced tumour progression in advanced bladder cancer [80]. CRISPR-mediated KLF4 activation was used to determine the anti-tumour mechanism of KLF4 overexpression in MIBC cells (T24). KLF4 overexpression was shown to suppress T24 cell proliferation through the regulation of AKT—p21 signalling, which induced cell-cycle arrest in the G1 phase. Furthermore, KLF4 activation reduced T24 cell invasion, migration and EMT. The authors from this study concluded that this CRISPR activation system, if translatable to the clinical setting, could act as a novel therapeutic strategy in MIBC [81].

Mounting evidence suggests that epigenetic regulatory proteins have a crucial role as tumour suppressors in diverse types of cancers. *UTX* and its paralog *UTY*, located on the X and Y chromosome, respectively, are histone demethylases that frequently harbour copy number

variations and mutations in bladder cancer[82]. These genes have been implicated in tumorigenesis in multiple cancer types through the regulation of proliferation, cell differentiation and metastasis [83,84]. In bladder cancer cell lines, CRISPR-Cas9-mediated knock-out was use to silence these epigenetic regulators, either singularly or in combination. This silencing resulted in a substantial increase in cell proliferation, with the most noteworthy effect observed in the double knock-out cells[85]. These results were consistent with results in patient samples from patients undergoing therapy for muscle invasive or high grade bladder cancer, with many patients showing reduced UTX and or UTY copy number and truncating mutations, suggesting a tumour suppressor role of these genes[85]. Moreover, results from this study emphasize the crucial role of X-Y gene pairs in carcinogenesis, particularly in tumour types such as bladder cancer, in which a higher prevalence is reported in male than in female patients [86]. Following this study, research focused on assessing whether this loss of function can be targeted therapeutically. *UTX* has a role in antagonizing the enhancer of zeste homolog 2 (EZH2), a methyltransferase involved in histone methylation [87]. Thus, *UTX* knockout has similar effects to EZH2 overexpression[87], EZH2 inhibition has been shown to delay tumour onset and suppress tumour growth in xenograft mouse models derived from UTX knockout cell lines as well as patient- derived tumours [87] . These findings suggest that EZH2 inhibition could potentially serve as a novel therapeutic approach for patients with bladder cancer harbouring mutations in *UTX* [88]. However, UTX is a component of the COMPASS family of histone H3K4 methylases. Thus, research into other components of this complex, such as the commonly mutated MLL3 and MLL4 proteins, and their interactions should also be explored, as the role of the COMPASS family in tumorigenesis is not fully understood[89].

Other CRISPR systems can be used in different ways. For example, Cas13, an RNA-targeting system, was used to target and degrade the oncogene MYC mRNA, rather than introducing alterations in the gene. Consequently, a decrease in both MYC mRNA and protein expression was achieved, leading to reduced bladder cancer cell proliferation and increased apoptosis [74]. This reduction in protein expression without the need to introduce DNA mutations is advantageous as Cas13 directly targets cytoplasmic mRNA sequences increasing the rate of downregulation of gene expression[90]. These findings introduce a potential targeted therapeutic approach for MIBC. However, these results must be validated in vivo to determine whether this strategy is effective in an organism wide setting.

CRISPR-Cas9 can also be used to determine the unknown function of suspected oncogenes or tumour suppressors. Long noncoding RNAs (lncRNAs) have no protein coding ability, but have been implicated in a wide range of cellular functions including pre- and post-transcriptional regulation, epigenetic regulation, and chromosome stability [91]. Interest in the involvement of lncRNAs in cancer is growing, and CRISPR-Cas9 techniques can be used to further elucidate the phenotypic effect of lncRNA manipulations. The lncRNA *SNHG3* has been shown to be implicated in bladder cancer progression, is overexpressed in bladder cancer tumour tissues and is correlated with poor prognosis [92].CRISPR-Cas9 Knockout of SNHG3 in two muscle-invasive bladder cancer cell lines resulted in reduced cell proliferation, migration, invasion, and angiogenesis [92]. SNHG3 was also shown to stabilise B lymphoma Mo-MLV insertion region 1 (BMI1)—through MYC binding—, which inhibits cell senescence and, therefore, is strongly associated with poor prognosis in multiple cancer types [92]. These results indicated that targeting the SNHG3—MYC—BMI1 axis could emerge as a promising novel therapeutic target to regulate tumour growth and metastasis in MIBC [92].

A crucial component of CRISPR screens is a functional assay to enrich and separate different cell population behaviours. In many instances, this assay can be based on a relatively simple living or death response of cells after a drug or another challenge has been applied. Functional screens can also exploit CRISPR and HDR to create reporter systems, such as knock-in of genes encoding

fluorescent proteins into relevant endogenous loci. This approach creates systems in which cell populations can be separated according to fluorescence intensity that correlates with gene activity. For example, in a 2023 study, knock-in of eGFP into the peroxisome proliferator-activated receptor gamma (*PPARγ*) gene was achieved through CRISPR-Cas9[93]. PPARγ is a transcription factor driving tumour growth, and is known to be upregulated in luminal bladder cancer tumours [93]. In this study[93], following a whole-genome CRISPR screen, cell populations could be sorted according to PPARγ high and low expression, which enabled to identify genes regulating PPARγ expression[93]. Multiple genes were identified and functionally validated as putative positive regulators of PPARγ expression including *GATA3*, *SPT6*, *SMC1A* and *RAD21* [93]. These genes were speculated to be druggable targets for novel luminal bladder cancer therapy [94]. These targets were validated using mRNA expression data from TCGA; however, mRNA expression might not accurately reflect the protein expression and activity of these genes. Additionally, these results were not confirmed using in vivo models, adding further doubt about the possibility to translate these targets into the clinic.

[H1] CRISPR-Cas technology use for gene therapy

CRISPR gene editing shows high promise in the future treatment of cancers, but many factors still prevent the use of these technologies as a direct therapeutic through a gene therapy approach, The main use of CRISPR-Cas gene editing for disease therapy consists in the treatment of monogenic disorders such as sickle cell disease using haematopoietic stem cell transplantation, which have shown high rates of success [95]. In this approach, stem cells are eliminated through chemotherapy before gene therapy, and CRISPR-Cas9 modified stem cells with the mutated gene restored can then be introduced to reconstruct both the blood and immune systems[96]. Cancer derives from multiple genetic mutations that varies between patients, and is far more complex than monogenic disorders. However, CRISPR has been used to improve existing cancer therapies. One example is the chimeric antigen receptor (CAR)-T cell therapy. CAR-T cell therapy has showed efficacy in treating acute lymphoblastic leukaemia, but the need of using patients' own CAR-T cells can lead to treatment delays. To overcome this obstacle, CRISPR-Cas9 was used to modify CAR-T cells targeting CD19 and CD22. This modification involved disrupting the TRAC region and CD52 gene to prevent immune rejection. The resulting edited CAR-T cells were subsequently introduced into patients, resulting in favourable anti-cancer responses and minimal adverse effects. [97].

These CRISPR-mediated therapies have shown success in haematological malignancies, but the translation of these therapies to solid tumours, such as bladder cancer, has been a challenging task. However, promising advances have been obtained in pre-clinical studies targeting solid tumours. An emerging therapeutic strategy based on T cells targeting solid tumours is the T-cell receptor (TCR) therapy. TCR therapy involves the use of genetically modified T cells engineered to recognise tumour-specific markers [98]. This strategy should provide therapeutic benefit without causing damage to normal cells. The efficacy of TCR therapy was initially shown in xenograft tumours derived from patients, prompting clinical trials to evaluate the potential of this innovative treatment approach in a variety of tumours including lung cancer, renal cell carcinoma and gastrointestinal cancers with varying levels of success [99, 100]. In a 2023 study, patient-specific TCR therapy was generated using patient's peripheral blood mononuclear cells (PBMCs) from patients with a variety of solid tumours [101]. This approach enabled to compare germline and tumour DNA to identify each patient's specific tumour mutations. Subsequently, CRISPR-Cas9 gene editing was used to create genetically modified T cells with neoantigens designed to target the tumour[102]. This result was achieved through knockout of the endogenous *TRAC* and *TRBC* genes and introduction of engineered neoantigen-specific TCRs [102]. These sequences were cloned into DNA plasmids designed for homologous recombination with the endogenous TRAC site on the DNA, prompting the neoantigen's expression on the T cell surface.

These genetically altered T cells were then reintroduced into the patient [102]. Notably, these cells successfully localized to the tumour sites. Encouragingly, some patients showed disease stabilization or even a reduction in tumour size 28 days after the T cell infusion [102]. This outcome is relevant, as these patients had refractory cancers and had already undergone six rounds of previous therapies. Results from this study indicated the safety of infusing transgenic T cells into patients and suggested the potential therapeutic value of these modified cells in diminishing tumour size in a wide range of solid tumours. CAR T cell therapy shows high potency and rapid tumour killing within shorter timeframes than TCR, but an inverse relationship is observed between CAR T cell proliferation and increased target antigen expression[103], which diminishes the effectiveness of tumour targeting. Conversely, TCR cell therapy showed high proliferation also in high antigen environments, sustaining efficient rates of tumour clearance [103]. This evidence suggests that TCR cell therapy in combination with CRISPR-Cas9-based gene modifications might held good performance across a broader spectrum of tumour antigen presentations than CAR T [104].

A crucial issue in drug efficacy is the infiltration of the extracellular matrix (ECM) to target tumour cells. In one study in 5A2-SC8 liver cells, this issue was overcome using liquid nanoparticles to co-deliver siRNA targeting focal adhesion kinases to break down the ECM alongside Cas9 mRNA and a sgRNA targeting PDL1 [105]. The introduction of siRNA targeting focal adhesion kinases decreased the ECM, resulting in two important outcomes: a tenfold enhancement in CRISPR-mediated gene editing within tumour in in vitro spheroid models; and increased infiltration of immune cells, contributing to decreased tumour growth during in vivo mouse experiments[105]. These results are encouraging and show potential for the development of innovative CRISPR-Cas9-based therapies for solid tumours, with potential applicability also in bladder cancer.

Multiple clinical trials using CRISPR-based therapeutics are ongoing. For example, there is a phase I/II trial currently recruiting patients with metastatic gastrointestinal epithelial cancer for treatment with neoantigen specific tumour infiltrating lymphocytes CRISPR Cas9 genetically edited to inhibit the intracellular immune checkpoint CISH [106]. Another ongoing phase I/II trial is investigating the safety and efficacy of CRISPR-Cas9 edited CAR T cells in patients with refractory solid tumours [107]. However, these trials are still in very early stages, with a high focus on dosage and safety of treatments. Additional research is required before these treatments are available for widespread use in the clinic. However, advances in the translation of these therapies in solid tumours provides hope about the possibility of using gene editing-based therapies to treat bladder tumours. The design of TCR therapies based on individual tumour neoantigens could be of high importance in bladder cancer, in which the high tumour heterogeneity remains a substantial problem. Bladder tumours have high tumour mutational burden (TMB) and, therefore, are ideal candidates for TCR therapies, as higher the TMB the more neo-antigens are available for targeting, leading to increased therapeutic success [108].

[H1] Limitations and ethical considerations

An important limitation of CRISPR screening in cancer research is that most current studies have been completed in vitro but not in vivo. CRISPR-Cas9 screens have shown to be an invaluable resource in uncovering potential therapeutic targets, but translating these findings into the clinic is crucial. Thus, advancements in generation and ease of access of in vivo-based CRISPR-Cas9 methodologies are required to validate the findings of CRISPR screens and accelerate the future use of CRISPR-Cas9-based therapies into the clinic. These in vivo models could include animal models and tumour xenografts, as well as the development of conditional tissue-specific knockouts to study gene functions at a tissue-specific level.

With regards to the development of CRISPR-based therapeutics, the safe delivery of CRISPR into patients remains a substantial challenge. These therapies need to target tumour cells specifically, without inducing death of normal cells and off-target effects, which is a major aspect to consider for the translation of CRISPR into the clinic[109]. Off-target effects could have catastrophic consequences including induction of novel oncogenic mutations. Results from current pre-clinical and clinical trials in which CRISPR-Cas9 based therapeutics have been used have shown that off-target effects are rare, indicating that CRISPR-Cas9-mediated gene editing is highly targeted, and holds promise in large-scale clinical trials of these therapies [110]. The identification of off target effects has been enhanced through the development of GUIDE-seq. GUIDE-seq identifies where DSBs have occurred and through the detection of double-stranded oligodeoxynucleotides, which enables us to conduct genome-wide profiling of CRISPR-induced off-target effects [111].

For example, the use of these technologies in the treatment of Sickle-cell disease is relatively simple, as delivery of CRISPR components occurs ex vivo as HPSCs are extracted, edited and reintroduced into the patient[95]. A crucial issue in the delivery of gene editing in situ is the targetability of the desired tissue or organ. The liver, for example, has proven to be a targetable organ via lipid nanoparticle-based delivery systems for the treatment of transthyretin amyloidosis[112]. For bladder cancer, the challenge remains how to target cells in situ [113,114]. Only when methods to facilitate the delivery of CRISPR-Cas based therapeutics into the bladder cancer cells avoiding off-target effects will be developed, rapid advancements in the use of CRISPR-based strategies in MIBC treatment will be possible.

Ethical considerations are also associated with the use of CRISPR-Cas gene editing in clinical therapies. Considerations range from somatic cell gene editing as well as use of exogenous transfer of CRISPR-edited cells into patients .As these technologies are still in their infancy there is limited long term effect data limiting the justification for the widespread use of these CRISPR-Cas based therapeutics. [115]. Ethical considerations are reduced considering the use of an individual's genetically altered cells, as only the patient would be affected, whereas germline gene editing could also affect future generations [116]. Lastly, the long-term implications of CRISPR-Cas9 editing in humans are not fully understood. Thus, the use of these technologies must be tightly regulated to ensure patient safety.

[H1] Conclusions

CRISPR-Cas9 gene editing techniques have provided a vast amount of knowledge in MIBC research, from the identification of drug resistance mechanisms to novel drug targets. However, the use of these technologies into the clinic as a gene-therapy approach remains a substantial challenge. The current use of CRISPR-Cas9 gene editing in the treatment of monogenic disorders is encouraging for future perspectives in cancer treatments. Currently, the area of CRISPR-based therapies showing most promise in the treatment of solid tumours in clinical trials is the use of genetically engineered patient-derived T cells to recognise tumour neoantigens. This area of research highlights how CRISPR technologies could advance the field of immunotherapies and personalised medicine in cancer research.

However, the use of CRISPR technologies for the treatment of baldder cancer into clinical settings will require careful evaluation of dosage and safety. The greatest challenges in this area concern off-target effects, ethical implications of somatic cell gene editing, and the unknown long-term outcomes of CRISPR-Cas9-related therapeutics.

CRISPR-Cas9 offers considerable potential for revolutionizing bladder cancer therapies, but substantial research into validation of these technologies is required before achieving wide use in the clinical setting. The intricate genetic landscape of MIBC shows the need for a multidisciplinary treatment approach, and continued research will be required to use the full potential of CRISPR-based technologies in the development of novel precision therapeutics.

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The authors declare no competing interests.

Author contributions

D.J.S. researched data for the article. D.J.S. contributed substantially to discussion of the content. D.J.S. wrote the article. S.L., A.A., S.N., T.S.,K.R., P.H. and A.C. reviewed and/or edited the manuscript before submission.

Box1

CRISPR-Cas9 system

The CRISPR-Cas9 system induces double-strand breaks (DSBs) in DNA using the Cas9 endonuclease bound to a single-guide RNA molecule (sgRNA), which guides the Cas9 to the DNA target site. The sgRNA molecule consists of two parts, a trans-activating CRISPR RNA (tracrRNA) and a user-defined CRISPR RNA (crRNA), which is complementary to the target region of the genome. A pre-requisite for DNA targeting is a protospacer adjacent motif (PAM) site, a 2–6 nucleotide DNA sequence that must be located adjacent to the target sequence, and is necessary for Cas9 to bind the cleavage site[117]. Even one-nucleotide change within the PAM site has been shown to prevent Cas9 binding [118]. Upon sgRNA binding to the target DNA following PAM site recognition, the DNA unwinds, and a DNA:RNA hybrid R-loop structure is formed between sgRNA and the target DNA strand, whereas the non-target DNA strand is displaced [119]. If the DNA and RNA sequences match, the target DNA will continue to unwind until the whole crRNA has successfully bound. This binding causes a conformational change of Cas9 to the active state that can recognise and bind DNA. Cas9 has two nuclease domains, HNH and RuvC, each responsible for the cleavage of opposite DNA strands [120,121]. The formation of the R-loop enables the Cas9 nuclease domains to access and cleave the individual strands, inducing a double-strand break (DSB). Following DSB, DNA-repair pathways are activated. The most commonly used repair mechanism is non-homologous end joining (NHEJ), which, however, is highly error prone, because an intact homologous DNA strand is not used as a template, and often results in insertions or deletions in the genome. In coding regions of the genome, these modifications can result into frameshift mutations and premature stop codons, leading to amino acid changes[24]. The alternative and less common mechanism of DNA repair following a Cas9-induced DSB is homology-directed repair (HDR), which requires a homologous DNA sequence as a template to repair the original DNA [122]. This homologous sequence could be supplied exogenously or could be from a sister chromatid [123]. This mechanism is highly precise and can be used to induce mutations of choice within the DNA by including these mutations into the homologous template DNA [25].

Figure 1. CRISPR-Cas9 mechanism and alternative methodologies

a) Single-guide RNA (sgRNA) complexed with Cas9 binds to the DNA inducing a double-strand break (DSB), which is subsequently repaired through different DNA-repair mechanisms, including non-homologous end joining (NHEJ) and homology directed repair (HDR). NHEJ is error prone, resulting in

indels in the DNA sequence, whereas HDR accurately repairs the DNA, as a homologous DNA repair strand is required. **b)** CRISPR activation (CRISPRa). A catalytically dead Cas9 (dCas9) bound to a transcriptional activator and sgRNA induces transcriptional activation of target genes. **c)** CRISPR interference (CRISPRi). dCas9 bound to a transcriptional repressor and sgRNA inhibits transcription of target genes. **d)** Base editor. dCas9 bound to deaminase and sgRNA removes an amino acid from a nucleoside inducing a single-nucleotide polymorphism. **e)** Prime editor. A Cas9 nickase is bound to a prime editing guide RNA (pegRNA) and a reverse transcriptase (RT) to induce small base edits in the DNA.

Figure 2. CRISPR-Cas gene editing in muscle-invasive bladder cancer research.

Short summary

This perspective details the current research using CRISPR-Cas gene editing technologies in the discovery of novel targets in muscle invasive bladder cancer. Furthermore, the future prospectives of these technologies have been discussed along with the current limitations and ethical considerations.