Transgenic Animal Models of Bladder Cancer

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Abstract: Traditional animal models for cancer research represent an excellent experimental tool in bridging the gap between pre-clinical and clinical studies. The advances in gene transfer technologies allow the generation of transgenic animal models through the integration of the gene of interest into the animal models through gene transfer methods. Overcoming the limitations of traditional animal models, transgenic animal models are beneficial for studying carcinogenesis with respect to the genes. Besides, the utilization of transgenic animal models is also necessary for the development of novel targeted therapy. Bladder cancer (BC) is the most common cancer type worldwide and effective therapies targeting BC are urgently needed. Thus, with the delineation of molecular signatures of BC pathogenesis, the research involving transgenic animal models of BC will facilitate the development of clinical medicine, thereby improving the clinical outcome of BC patients in the future.

Keywords: Bladder cancer, Transgenic animal models, Gene transfer

1. Introduction

Bladder cancer (BC) is the most common malignant tumor of urinary system and one of the most common tumors around the world. About 81,400 new cases and 17,980 deaths of BC were reported in 2020 worldwide[1]. It is more common in western countries than in developing countries and more prevalent in males than in females. However, the reason is still not clear. Since the effects of conventional radiotherapy and chemotherapy on BC remain unsatisfactory, there is an urgent need to re-evaluate the mechanisms of BC pathogenesis for designing more effective therapy[2]. An estimated 90% of BC cases arise from the urothelium, so it is also known as urothelial carcinomas. The remaining 10% of cases comprise adenocarcinoma, primary squamous cell carcinoma, small cell carcinoma, etc.[3,4]. Non-muscle invasive BC is a common (estimated 70%) subgroup which is usually associated with a favorable prognosis. While muscle-invasive BC (estimated 30%) is less prevalent but usually associated with a poor prognosis and contributes to most of morbidity and mortality[5,6].

The molecular basis that underlies BC is gaining clarity, but BC is still a major public health issue. Since conventional radiotherapy and chemotherapy have limited effect on BC, it is necessary to develop more effective tumor therapy either for improving survival or chemoprevention. Animal models are instrumental in research for getting...
an understanding of the development and progression of BC and screening or developing novel therapies. The utilization of animal models not only allows us to study the behavior of tumor cells but also the complexity of human malignancy and immune contexture within the tumor microenvironment.

There are three principal types of animal models: engraftment models, chemical carcinogenesis models, and transgenic models. Engraftment models which have the cancer cells engrafted into the mouse bladder were first mentioned in 1975, and now this type of model is widely used. Before the engraftment, the gene expression of the cultured cell can be manipulated to attain the desired effect. Thus, the engraftment model is useful to evaluate the effects of a mutation on tumor growth. Since the engrafted tumors are non-autochthonous, the model itself cannot simulate the evolution of tumor. Oppositely, the tumor in chemical carcinogenesis models is induced by chemical carcinogens, simulating the actual events that cause BC in humans. However, the disadvantage of the chemical carcinogenesis model is that it is difficult to rule out another potential carcinogenic factor, making it difficult to model specific tumor subtypes. Conventionally, these animal models for cancer research were mainly established through xenograft transplantation and induction with carcinogen. Along with the advantages of low cost and widespread availability, these traditional animal models provide insights of basic mechanisms in the progression, metastasis, and recurrence of cancer. Besides, they can also be used in the drug screening process that predicts the therapeutic response as well as monitor the growth of tumor.

On the other hand, transgenic animal models, which are defined by the integration of the gene of interest into the animal models through gene transfer means, are becoming more common due to the development of genetic engineering technologies. Transgenic models usually refer to the transgenic or gene-targeted models characterized by the over-expression of oncogenes or the silencing of tumor suppressor genes, which can be applied for detecting whether genetic changes are related to bladder carcinogenesis and how the development of tumor correlates with the genetic signatures. Apart from that, the pleiotropic effects of the constitutive or conditional expression of oncogenes and silencing of tumor-suppressor genes directed by the transgene will be delineated in-depth. In clinical medicine, this helps refine and develop more effective and robust targeted therapy to improve the clinical outcome of cancer patients. Transgenic models are beneficial for studying the mechanism of tumorigenesis and tumor immune evasion compared to engraftment models, and modeling specific tumor subtypes compared to chemical carcinogenesis models.

In this review, we will provide an overview of transgenic animal models used in BC research. Furthermore, the methods for the generation of transgenic animal models with their contribution to BC development and enhancement in drug discovery are discussed.

2. Methods for establishing transgenic animal models

2.1. Microinjection

Microinjection was first introduced in the 1980s. Since then, thousands of transgenic mouse strains have been produced. Many new approaches using zinc fingers or CRISPR/Cas9 for manipulating genomes have also been developed, but pronuclear microinjection remains the essential technique for delivering exogenous DNA. In the establishment of transgenic mouse models by microinjection, an DNA fragment is injected into one of the pronuclei of fertilized ova, and then the embryos were transferred into the oviducts of pseudopregnant surrogates. There are many factors that could affect the efficiency of the establishment of transgenic models by microinjection; one of the most prominent factors being the transgenic rate of double-pronuclear microinjection (2.08%), which is significantly higher than single-pronuclear microinjection (1.20%). Microinjection technique is simple and efficient, but it does have some limitations. First, the level of transgene expression is uncertain because the copy numbers of inserted genes and their insertion sites are uncontrollable. Second, the percentage of successful cases of transgenic offspring generation is relatively low, which could be overcome by increasing the number of embryos in rodents, but the procedures would become more complicated and high costs are required if larger animal species are involved. In general, transgenic mouse model established by microinjection has provided information on virtually every aspect of gene control and function. To facilitate future research, it is necessary to establish an efficient microinjection platform.

2.2. Retrovirus-mediated gene transfer

Retroviruses are a group of RNA viruses that go through a reverse transcription process from RNA to DNA during replication. There are many species of retrovirus, such as human immunodeficiency virus (HIV), Rous sarcoma virus (RSV), and avian leukemia virus (ALV). Retrovirus-mediated gene transfer is a method that utilizes the infection ability of retroviruses to transfer foreign genes into the host genome. Gene transfer using the retrovirus vector system can date back to 1987. Retrovirus-mediated gene transfer is appropriate for vertebrates, avian, and mammalian species. Retrovirus-mediated gene transfer has the advantages of high infection efficiency and wide host range. However, the disadvantages of this technique are that the retrovirus vectors can only carry the DNA fragment of <10 kb, and the viral nucleic acids may activate oncogenes, thereby increasing the risk for carcinogenesis. Thus, the practical application of retrovirus-mediated gene transfer method is still limited.
2.3. Embryonic stem (ES) cell-mediated gene transfer

It was first demonstrated in 1989 that a predetermined genetic modification could be transferred into an animal using the mouse embryonic stem (ES) cells. This experiment corrected the HPRT deletion mutation in the mouse ES cell by gene targeting and transferred the ES cell into the mouse blastocysts, achieving the ES cell-mediated gene transfer. The exogenous genes are usually transferred into ES cells by electroporation or virus infection. The advantage of ES cell-mediated gene transfer is that the special genotype can be selected in vitro before the embryo is implanted, and it is conducive to the selection of positive ES cells. However, so far, only stable ES cell lines in mice and humans are available. In other large animals, such as pigs, cattle, and horses, stable ES cell lines are difficult to be obtained, which restricts the application of this method.

2.4. Somatic cell nuclear transfer

In 1999, the first transgenic goat was produced by somatic cell nuclear transfer technology. Oocytes at the arrested metaphase II stage or telophase II stage were enucleated and electrofused with donor's somatic cells to produce recombinant in vitro. Then, the recombinant embryo was transplanted into surrogate animals. Theoretically, the offspring born are all transgenic animals, obviating the need for further selection since the expression of foreign gene is usually stable. About 2.6% of embryos modified by nuclear transfer and 0.5–3% of embryos by microinjection give rise to transgenic offspring, suggesting that the efficiency of somatic cell nuclear transfer is comparable to that of microinjection. The nucleases developed in recent years for gene targeting such as ZfC, TALENs, or CRISPR/Cas9 have greatly improved the methods of somatic cell nuclear transfer.

3. Different types of transgenic animal model of bladder cancer

At present, transgenic animal models are widely used in many BC researches, including analyses of tumor genesis, modeling of tumor subtypes, exploration of the mechanism of candidate genes and signaling pathways, and preclinical study of therapeutic agents. Some methods that can be used to establish the transgenic animal model of BC include UPII-derived urothelium-specific gene expression and Cre/loxP system-derived transgene in urothelium.

3.1. UPII-derived transgenic animal model

3.1.1. UPII-SV40T transgenic mice

In 1999, Zhang et al. first reported the application of transgenic mice carrying the transitional cell carcinoma of the urinary tract. The investigators found that the promoter of Uroplakin II gene could induce urothelium-specific expression of oncogene in the mice (Table 1). The promoter was combined with SV40T gene encoding for large T antigen to form UPII-SV40T chimeric gene. The UPII-SV40T chimeric gene was microinjected into the pronuclei of fertilized eggs of the mice to establish the transgenic animal model of BC that can be used to study the role of SV40T in BC invasion and metastasis. Four out of 30 experimental mice were incorporated with the chimeric gene, and three out of the four transgenic mice passed the transgene to their progenies. Urothelial UPII expression is limited to the superficially located terminally differentiated umbrella cells in human, but the gene is expressed in the suprabasal layer of the mouse. Pathological reports revealed that the phenotypes and transformation patterns of transitional cell carcinoma in mice were highly similar to human transitional cell carcinoma. The tumor in transgenic model bearing a low copy number of SV40T transgene developed carcinoma in situ (CIS) in 5–8 months, whereas those bearing a high copy number of SV40T developed CIS as well as an invasive and metastatic tumor in 3–5 months. Further studies found that the promoter of Uroplakin II gene could give rise to the occurrence, further invasion and metastasis of bladder carcinoma in situ through the inactivation of p53 and pRb genes.

The establishment of UPII-SV40T transgenic mouse model is helpful not only in studying the molecular mechanism underlying the occurrence of different BC phenotypes but also in preclinical research. In the preclinical experiment, this type of model allows the design and optimization of effective therapies that targeting various signaling pathways for preventing BC. Recent literature reported that UPII-SV40T transgenic mice were used as an experimental tool for studying chemoprevention through the regulation of apoptotic pathways. In this experiment, the UPII-SV40T mouse model and WT mouse were fed diets containing p53-stabilizing agent, CP-31398, or control diet for 34 weeks (Table 1). The findings of this experiment include the inhibition of invasive papillary transitional cell carcinoma (TCC), a decrease in bladder tumor weights, an increase in the level of apoptosis markers (p53, p21, and Bax), and a decrease in vascular endothelial growth factor in the mice model. These results suggest that p53-stabilizing is a potential chemopreventive agent for BC.

In another study, the anti-tumor effect of the chemophylaxis of a dual cyclooxygenase (COX)-lipoxygenase (LOX) inhibitor, licofelone, was examined in UPII-SV40T transgenic mice (Table 1). There was a significant, dose-dependent inhibition of tumor growth in UPII-SV40T transgenic mice, suggesting that licofelone can serve as a potential chemopreventive agent against BC. The screening of chemotherapeutic drugs on these transgenic animals showed that the metastatic behavior and recurrence rate of urothelial carcinoma were reduced. These studies illustrate the significance of preclinical
animal model in BC research, thus offering the prospect of novel therapeutic drug development.

3.1.2. UPII/Ha-Ras transgenic mice

In a study, murine UPII/Ha-ras fusion gene was injected into the pronuclei of FVB/N inbred mice embryos to generate the UPII/Ha-ras mice\textsuperscript{[40]}. Most of the transgenic mice developed urothelial hyperplasia and superficial papillary non-invasive bladder tumors, while none of the control mice developed any urothelial abnormalities (Table 1). This result shows that the activation of Ha-ras gene leads to urothelial hyperplasia and superficial papillary non-invasive bladder tumor\textsuperscript{[39]}. In contrast, the urothelial expression of SV40T antigen-induced invasive and metastatic bladder tumors, as described before\textsuperscript{[50]}. In general, these findings suggest that different BC phenotypes are attributed to different genetic defects\textsuperscript{[39]}.

UPII mutant Ha-ras transgenic mice that have low-grade papillary urothelial cell carcinoma have been used to determine the effect of metformin on superficial BC\textsuperscript{[40]} (Table 1). UPII/Ha-ras\textsuperscript{+/+} mice were generated by breeding of UPII/Ha-ras\textsuperscript{+/−} mice. Then, they were randomly divided into three groups, given normal drinking water or water with metformin (0.5 mg/kg or 1.0 mg/kg). The UPII/Ha-ras\textsuperscript{+/+} mice that were given water with metformin had a higher survival rate and their mean bladder weights were significantly reduced. These results show that intake of metformin significantly increased the survival rate of UPII/Ha-ras\textsuperscript{+/+} mice. Meanwhile, the concentrations of the drug that got into contact with the urothelium through urine following oral administration were higher than those in the circulation as measured by an external calibration curve. These findings provide a strong rationale of using oral metformin in the treatment of superficial BC for clinical trials.

3.2. Cre/loxP system derived transgenic animal model

Cre is a recombinase, while loxP is a DNA fragment with a length of 38 bp. Cre can specifically recognize loxP site, cleave insertion sequence between the sites, and recombine the ends. The selective marker gene in a transgenic animal can be knocked out by Cre/loxP system through site-specific recombination\textsuperscript{[41]}. For instance, to study the role of estrogen receptor alpha (ER\textalpha) in the occurrence and development of BC, Hsu et al. used Cre/loxP \textit{in vivo} gene knockout strategy to generate CMV-ER\textalpha knockout (KO) mice by cross-breeding female mice bearing CMV-Cre with male mice bearing loxP-ER\textalpha. Female CMV-Cre/ER\textalpha\textsuperscript{−/−} mice were then mated with male ER\textalpha\textsuperscript{−/−} mice to generate wild-type and ER\textalpha KO mice. Genetic analysis showed that CMV-ER\textalpha KO mice carried both Cre and ER\textalpha KO alleles\textsuperscript{[42]} (Table 1). The mice developed mainly transitional cell carcinomas, including muscle invasive and non-muscle invasive, after 12 weeks of induction with N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN). The results show that ER\textalpha KO mice have a higher bladder weight, which is an indicator of higher bladder cellularity and tumor mass compared with wild-type mice with ER\textalpha gene. Mechanistically, immunohistochemical staining assay showed that ER\textalpha reduced the activity of protein kinase B (AKT) by controlling expression of INPP4B, thereby slowing down the growth of BC cells. This study shows that ER\textalpha plays a protective role in BC initiation and growth using Cre/loxP system-derived transgenic animal model and confirms the role of ER\textalpha in suppressing BC development since ER\textalpha KO mice had earlier occurrence and higher incidence rate of BC. Taken together, Cre/loxP system-derived transgenic animal model provides an important tool to identify the function of a specific gene in the tumorigenesis of BC.

3.3. UPII and Cre/loxP derived transgenic animal model

In another research, an activated form of \textbeta-catenin was specifically expressed in the urothelium of the transgenic mouse model to study the function of Wnt signaling pathway in urothelial cell carcinoma (UCC) with PTEN deficiency\textsuperscript{[43]} (Table 1). Uroplakin II Cre mice were bred with mice bearing \textbetacatenin\textsuperscript{ exon3/−} and PTEN\textsuperscript{loxP/loxP}. The UroIICRE/\textbetacatenin\textsuperscript{ exon3/−} mice developed areas of urothelial hyperplasia but not UCC, while the UroIICRE/\textbetacatenin\textsuperscript{ exon3/−} PTEN\textsuperscript{loxP/loxP} mice rapidly developed papillary carcinomas without metastasis. There was a marked upregulation of nuclear \textbeta-catenin and a downregulation of the PTEN protein in bladders. Those lesions in UroIICRE/\textbetacatenin\textsuperscript{ exon3/−} mice led to an increase of the level of the tumor suppressor protein PTEN, while papillary UCC arose in the UroIICRE/\textbetacatenin\textsuperscript{ exon3/−} PTEN\textsuperscript{loxP/loxP} mice model along with the activation of the PI3K-Akt signaling pathway. Along with a dramatic increase in \textit{mTOR}, the number of proliferating cells identified by Ki-67 and BrdU immunohistochemistry in the tumors was higher than that in the UroIICRE/\textbetacatenin\textsuperscript{ exon3/−} mice, suggesting that tumorigenesis is dependent on \textit{mTOR}. These results show that Wnt signaling plays an important role in driving the growth and development of UCC, and \textit{mTOR} inhibition may be a significant therapeutic approach against the tumor that manifests high levels of Wnt.

In addition, Ras pathway plays a synergistic role with Wnt\textsuperscript{44}. Urothelial expression of Ras \textit{mutation} (H-Ras\textsuperscript{Q61L}, or K-Ras\textsuperscript{G12D}) with an activating \textbeta-catenin mutation in the mouse bladder was constructed using Cre/loxP technology. UroplakinII Cre mice were intercrossed with mice bearing \textbetacatenin\textsuperscript{ exon3/−}, K-Ras\textsuperscript{G12D}, H-Ras\textsuperscript{Q61L}, and p21\textsuperscript{−/−} in combinations (Table 1). UPIICRE/H-Ras\textsuperscript{Q61L} mice carrying Ras \textit{mutation} developed global hyperplasia but not tumor. UPIICRE/\textbeta-catenin\textsuperscript{ exon3/−}H-Ras\textsuperscript{Q61L} mice carrying both Ras \textit{mutation} and activating \textbeta-catenin \textit{mutation} rapidly developed non-invasive papillary carcinomas without
<table>
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<td>UPII-SV40T was microinjected into the pronuclei of fertilized eggs of the mice</td>
<td>UPII-SV40T (low copy number)</td>
<td>4 out of 30 mice were integrated with the chimeric gene; 3 out of 4 transgenic mice passed the transgene to their progenies</td>
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<td>The murine UPII/Ha-ras-M fusion gene was injected into the pronuclei of mice embryos</td>
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<td>UPII/Ha-ras+/− mice were generated by the breeding of UPII/Ha-ras+/− mice</td>
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<td>Uroplakin II Cre mice were bred with mice bearing β-cateninloxPloxP and PTENloxPloxP</td>
<td>UroIICRE/β-cateninloxPloxP</td>
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metastasis. This mouse model manifested high levels of p21 and PTEN, suggesting that these tumor suppressors play an important role in the progression of these lesions. The UPIICRE/β-catenin<sup>exon3/exon3</sup>p21<sup>−/−</sup> mice rapidly developed urothelial tumors while UPIICRE/p21<sup>−/−</sup> mice did not develop tumors. This result suggests that p21 has a role in suppressing β-catenin-induced UCC. Finally, to detect whether this cooperative effect was restricted to H-Ras mutation, UPIICRE/β-catenin<sup>exon3/exon3</sup>K-Ras<sup>G12D</sup> mice were generated, which also rapidly developed symptoms of bladder tumorigenesis. All of these results show that Ras pathway activation works with Wnt signaling pathway to drive the progression of UCC in vivo.

4. Conclusions

The application of transgenic animal model in studying human diseases enables us to study multiple aspects of BC from the perspective of molecular biology. This provides an ideal experimental system for studies on pathogenesis, drug screening, and clinical medicine of BC. In addition, transgenic animal models can be combined with traditional animal models, such as BNN-induced tumorigenesis transgenic mouse[42], providing a greater range of animal models for BC research. However, the animal models, including the transgenic animal model, are unable to reflect the complexities of human BC due to the intricacies of gene expression and signaling pathways[45,46]. Thus, more studies are warranted to better translate the findings from transgenic animals to clinical studies.

Some advantages of applying transgenic animal model of BC include the following: (i) the induced tumor in the transgenic BC animal models is conserved and heritable; (ii) the interactions between drugs and tumor cells can be delineated through the insertion of marker gene to indicate migration pathway and organ selectivity during metastasis[17,47]; (iii) the transgenic animals are well-suited for studying the interaction between an environmental factor and genetic background due to its aberrant gene regulation; and (iv) compared to immunodeficient mice, the transgenic animal model is more superior for studying the mechanisms of BC occurrence and immune escape mechanisms of cancer cell, thus giving a more sophisticated understanding of the interactions between tumor cells and other cells in tumor microenvironment[48].

On the other hand, the shortcomings in the use of transgenic animal models in studying BC include the following: (i) since the gene regulations in animal models are complex and do not resemble the human’s completely, it is difficult to translate relevant findings into clinical studies[49]; (ii) transgenic animal model cannot fully mirror the complex dysregulation of signaling pathways that contributes to tumorigenesis in human[49,50]; (iii) the gene expression, after manipulation, is unspecific in region and not restricted to only the bladder of the transgenic animal model (for instance, the widely used promoter
such as UPII not only specifically leads to gene expression in the bladder, but also in some other tissues such as hypothalamus[51]; (iv) some technical difficulties in the generation of transgenic BC animal models exist due to the variable transfection efficiency and heterogeneity in animal models[52]; (v) due to the pleiotropic effects of gene regulation, the introduction of an oncogene for studying its functions may activate other oncogenes, leading to the growth of undesirable tumors[45]; and (vi) the establishment of transgenic animal model involves long cycle, high cost of animal model generation and maintenance, and the difficulty in technical operations.

The use of transgenic BC animal models in cancer research has overcome the limitations that commonly occur in traditional animal models, allowing the researchers to decipher the molecular mechanisms of BC and discover novel targeted therapy with the aim of improving and overcoming the limitations in the conventional chemotherapy. Taken together, the transgenic BC animal models offer broad prospects in translating the pre-clinical findings into clinical studies, ultimately benefiting the clinical and therapeutic aspects of BC.

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Conflicts of interest

The authors declare no potential conflicts of interest.

Author contributions

C.L. and Z.Y. conceived the idea of the review. L.W. and F.Z. wrote the paper. C.L. and C.Y. revised the paper.

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