GALNT1-Mediated Glycosylation and Activation of Sonic Hedgehog Signaling Maintains the Self-Renewal and Tumor-Initiating Capacity of Bladder Cancer Stem Cells

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Abstract

The existence of bladder cancer stem cells (BCSC) has been suggested to underlie bladder tumor initiation and recurrence. Sonic Hedgehog (SHH) signaling has been implicated in promoting cancer stem cell (CSC) self-renewal and is activated in bladder cancer, but its impact on BCSC maintenance is unclear. In this study, we generated a mAb (BCMab1) against CD44 antibody identifying BCSC subpopulation and was required for BCSC self-renewal. Furthermore, the glycotransferase GALNT1 was highly expressed in BCMab1+CD44+ cells and correlated with clinicopathologic features of bladder cancers. Mechanistically, GALNT1 mediated O-linked glycosylation of SHH to promote its activation, which was essential for the self-renewal maintenance of BCSCs and bladder tumorigenesis. Finally, intravesical instillation of GALNT1 siRNA and the SHH inhibitor cyclopamine exerted potent antitumor activity against bladder tumor growth. Taken together, our findings identify a BCSC subpopulation in human bladder tumors that appears to be responsive to the inhibition of GALNT1 and SHH signaling, and thus highlight a potential strategy for preventing the rapid recurrence typical in patients with bladder cancer.

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Introduction

Bladder cancer is the second most common urological malignancy with over 69,000 new cases and an estimated 150,000 deaths worldwide each year (1–3). The big problem of bladder cancer is the extremely easy recurrence after treatment and no recognized second-line therapy (4). Thus, for new therapeutic strategies, it is of great importance to delineate the mechanism of bladder cancer tumorigenesis.

Accumulating evidence from leukemias, germ cell tumors, and many solid tumors support a cancer stem cell (CSC) model in which cancers are initiated by a rare subpopulation of self-renewing and differentiating tumor-initiating cells (TIC; refs. 5–7). The cancer stem cell (CSC) population can self-renew limitlessly and differentiate into heterogeneous cell populations, resulting in phenotypic and functional heterogeneity of tumors (8, 9). Quiescent CSCs may resist therapeutic intervention and confer to recurrence after initial therapy (7). Chan and colleagues identified a subpopulation (Lineage−CD44−CK5−CK20+) from primary human bladder cancer as bladder cancer stem cells (BCSC) (10), which shared similar expression markers to normal bladder basal cells, suggesting the basal-like property of BCSCs. A subpopulation of primary urothelial cancers, coexpressing the 67-kDa laminin receptor (67LR) and the basal cell–specific cytokeratin CK17, possesses more carcinogenic ability (11). These subsets harbor high tumorigenicity, multipotency, and involvement in bladder tumor progression, suggesting that the CSCs exist in bladder tumors.

Bladder cancer arises from the urothelium, a transitional epithelium lining the bladder lumen. This multilayered epithelium is composed of a luminal layer of fully differentiated umbrella cells that overlie intermediate cells, and a basal layer of Sonic Hedgehog (SHH)-expressing cells (12). The SHH-expressing basal cells, capable of regenerating all cell types of the urothelium, have been defined as urothelial stem cells. The same group reported that muscle-invasive bladder cancers originate exclusively from the SHH-expressing basal stem cells of the basal urothelium in procarcinogen N-butyl-N-4-hydroxybutylyl nitrosamine (BBN)-induced mouse bladder cancer (13). These SHH-expressing basal stem cells act as BCSCs to initiate bladder oncogenesis. Aberrant activation of the Hedgehog signaling is implicated in many malignancies (14, 15). Emerging data support that Hh signaling plays a pivotal role in the self-renewal and differentiation of CSCs.
Activation of Hh pathway causes CSC self-renewal and expansion, while the SMO antagonist cyclopamine or the ligand-neutralizing antibody induces terminal differentiation and loss of clonogenic growth capacity (16, 18). In this study, we defined that Hh signaling is essential for the self-renewal of BCSCs. GALNT1-mediated glycosylation is required for SHH activation in BCSCs.

Materials and Methods

Reagents and mice

Mouse mAb BCMab1 was purified through protein A-Sepharose from ascites. Corresponding species-specific FITC-conjugated secondary antibodies were purchased from Sigma. The commercial primary antibodies were phycoerythrin (PE)-conjugated anti-CD44 (BD Pharmingen; 550989), goat anti-human C-terminus of SHH (Santa Cruz Biotechnology, sc-33942), goat anti-human N-terminus of SHH (Santa Cruz Biotechnology; sc-1194), rabbit anti-Gli1 (Santa Cruz Biotechnology; sc-20687), goat anti-GALNT1 (Santa Cruz Biotechnology, sc-68491), and mouse anti-β-actin antibody (Sigma, A1978). NOD/SCID mice were obtained from Vital River Laboratory Animal Technology Co. Ltd. GALNT1+/− mice were obtained from Jackson Laboratories. BBN, benzyl-N-acetyl-D-galactosaminide (BG), and cyclopamine were from Sigma. GALNT1 siRNA (human) and GALNT1 siRNA (mouse) were from Santa Cruz Biotechnology.

Xenograft establishment

Human bladder cancer specimens were obtained with patient's consent, as approved by the Research Ethics Board at The Second Affiliated Hospital of Kunming Medical University (Kunming, China). For the generation of xenografts, cells were injected with Matrigel subcutaneously into the backs of NOD/SCID mice. Animal work was carried out in compliance with the ethical regulations approved by the Animal Care Committee, Institute of Biophysics, Chinese Academy of Sciences (Beijing, China).

Flow cytometry sorting

Primary tumor tissues were digested with 200U type I collagenase, 20U type IV collagenase (Sigma, C-0130, C-5138), and DNase at 37°C for 2 to 6 hours. Then, the cancer cells were stained with FITC-conjugated BCMab1 and PE-conjugated anti-CD44 antibody or the same isotype FITC/PE–conjugated antibody for 30 minutes on ice. Labeled cells were analyzed and sorted by flow cytometry (BD FACSAria III).

DNA methylation analysis

Methylation was assayed by using the procedure as described previously (19). Genomic DNA was treated with sodium bisulfate using the EZ DNA methylation direct kit (Zymo Research). PCR products were analyzed with the MassArray Epityper system (Invitrogen).

Quantitative real-time PCR

Total RNA was isolated from primary sorted cells, human bladder cancer cell lines, and mouse tissues using the RNA Isolation Kit (Tiangen Biotech). RNA was then subjected to cDNA synthesis using M-MLV Reverse Transcriptase (Promega). The cDNA was then processed for an amplification step with the SYBR Green reaction system (Tiangen Biotech) running on an ABI 7300 (Applied Biosystems). Primer oligonucleotides for qPCR are listed in the Supplementary Table S1.

In vitro glycosylation assay

Recombinant human wild-type (WT) SHH and T282A-SHH mutant proteins were expressed in E. coli. WT SHH or T282A-SHH mutant was incubated with GALNT1 (R&D Systems) plus BCMab1 “CD44” cell lysates at 37°C for 4 hours. Reaction products were analyzed by immunoblotting.

Immunohistochemical staining

Tumor specimens were fixed in 10% buffered formalin and embedded in paraffin as described (20). The immunosignals were detected using the ABC kit.

Intravesical administration of siRNA/liposomes and cyclopamine for treatment

Female 6-week-old NOD/SCID mice with a body weight of approximately 15 g were used and kept under specific pathogen-free conditions. BCMab1 “CD44” cells were labeled with luciferase and transplanted into the murine bladder cavity via 24-gauge angiocatheters (21). The mice were grouped (24 mice per group) and intravesically applied with 6 μmol/L siCtrl/liposomes or siGALNT1/liposomes with or without 100 μmol/L cyclopamine next day.

Statistical analysis

The relationship between the expression levels of GALNT1 and clinicopathologic features was analyzed using the χ² or the Kruskal–Wallis test. Kaplan–Meier analysis was used to estimate the cumulative cause-specific survival rates, and the log-rank test was used to correlate differences in patient survival with staining intensity of GALNT1. The influence of GALNT1 on the growth of bladder cancers was analyzed by the Student t test.

Results

A BCMab1 “CD44” subpopulation of cancerous cells acts as BCSCs

To identify unique biomarkers of BCSCs, we isolated CD44+ BCSCs from human bladder cancer resection specimens (Fig. 1A), which were used as immunogen to immunize mice to generate mAbs. About 458 hybridomas were screened to bind BCSCs. Among these hybridomas, we identified several antibodies, including BCMab1 and BCMab37, which were determined to recognize the BCSC surface antigens, aberrantly glycosylated integrin α3β1 and CD47, respectively. We previously showed that the BCMab1 antibody was also generated by T24 cell immunization, recognizing the aberrantly glycosylated integrin α3β1 on bladder cancer cells (20). The aberrantly glycosylated integrin α3β1 is highly expressed in bladder cancer tissues. CD47 was also confirmed to be a surface marker of BCSCs (11).

By using BCMab1 and anti-CD44 antibodies, we distinguished a BCMab1 “CD44” subset from the tumor cells of human bladder tumor specimens (Fig. 1A), comprising 3% to 5% of the total cell population. BCMab1 “CD44” cells were further confirmed by immunofluorescence staining (Fig. 1C). We next determined whether BCMab1 “CD44” cells were more tumorigenic than their BCMab1 “CD44” counterparts. BCMab1 “CD44” cells freshly isolated from bladder cancer specimens were inoculated subcutaneously into NOD/SCID mice. As few as 10 BCMab1 “CD44” cells could form xenograft tumors in vivo (Fig. 1D and E), whereas at least 1 × 10⁵ BCMab1 “CD44” cells were necessary to establish xenografts. The xenograft tumors formed with BCMab1 “CD44”
Figure 1.

A BCMAb1\(^+\) CD44\(^+\) subpopulation of cancerous cells is defined as BCSCs. A, representative cystoscope of a typical bladder cancer. B, a subset of BCMAb1\(^+\) CD44\(^+\) cells was fractionated in one human bladder cancer specimen. Single tumor cells were stained with BCMAb1 (B) and anti-CD44 (C) antibodies followed by flow cytometry. C, immunofluorescence staining of BCMAb1\(^+\) CD44\(^+\) (left) and BCMAb1\(^+\) CD44\(^-\) (right) cells. D, tumor formation frequency of BCSCs from unsorted, BCMAb1\(^+\) CD44\(^+\) and BCMAb1\(^+\) CD44\(^-\) subpopulations derived from bladder cancer patient specimens. CI, confidence interval. Six bladder cancer samples got similar results. E, representative photographs demonstrating xenografted tumors (arrowheads). Bladder tumor cell subpopulations (BCMAb1\(^+\) CD44\(^+\) or BCMAb1\(^+\) CD44\(^-\)) with limiting dilutions were injected subcutaneously into NOD/SCID mice with the indicated numbers of cells. F, FACS analysis of BCMAb1 and CD44 expression in xenograft tumors generated from \(1 \times 10^6\) BCMAb1\(^+\) CD44\(^+\) or BCMAb1\(^+\) CD44\(^-\) cells. G, injection of 10, 100, 1,000, 10,000, and 100,000 freshly isolated BCMAb1\(^+\) CD44\(^+\) (left) or BCMAb1\(^+\) CD44\(^-\) (right) cells from the primary graft tumors. Data represent means \(\pm\) SD; \(n = 6\). H, serial tumor formation assay. Tumor volumes were measured at the indicated time points and calculated as means \(\pm\) SD in BCMAb1\(^+\) CD44\(^+\) (left) or BCMAb1\(^+\) CD44\(^-\) (right) cell transplantations; \(n = 6\). I, oncospheres were counted in five separate fields after two weeks and are shown as means \(\pm\) SD.
cells and BCMab1−CD44− cells in Fig. 1E were used for further analysis. Intriguingly, the tumors induced by BCMab1−CD44+ cells were able to reconstitute a mixture of BCMab1+CD44+ and differentiated BCMab1+CD44− tumor cells (Fig. 1F), but the tumors grafted by BCMab1−CD44− cells could not reconstitute the BCMab1+CD44+ cell subpopulation.

In addition, the self-renewal capacity of BCMab1+CD44+ cells freshly isolated from human bladder cancer specimens exhibited significantly higher oncosphere-forming efficiency compared with their respective BCMab1−CD44− counterparts (Fig. 1I). Taken together, the BCMab1−CD44− subpopulation is highly tumorigenic, with self-renewal and differentiation properties. Thus, we defined this BCMab1+CD44+ subset as the BCSC.

Hedgehog signaling is essential for the self-renewal of BCSCs. SHH-expressing basal stem cells, acting as BCSCs, initiate tumorigenesis in BBN-induced mouse bladder cancer (15). These results suggest that Hh signaling may be implicated in the stemness of BCSCs. To determine the Hh pathway of BCMab1+CD44+ cells, we performed transcriptome microarray analysis of BCMab1+CD44+ and BCMab1−CD44− cells isolated from human bladder tumor specimens. Microarray analysis revealed that several Hh signaling molecules, including SHH, Smo, Gli1, and Gli2, were highly expressed in BCMab1+CD44+ cells compared with their respective BCMab1−CD44− counterparts (Fig. 2A). High Gli1 mRNA levels in BCMab1+CD44+ cells were significantly inhibited by cyclopamine (Fig. 2F and G).
CD44+ cells were verified in BCMab1+CD44+ cells derived from each specimen of 72 human bladder cancer samples (Fig. 2B). Bladder cancer tissues displayed high expression of SHH and Gli1 by immunohistochemical staining (Fig. 2C). In addition, Gli1 was highly expressed in the nuclei of BCMab1+CD44+ cells, but undetectable in BCMab1+CD44− cells (Fig. 2D). More importantly, SHH was highly expressed in BCMab1+CD44+ cells and apparently hydrolyzed to form its catalytic band (Fig. 2E), but undetectable in BCMab1+CD44− cells. These data suggest that the Hh signaling pathway is activated in BCSCs.

To further confirm the involvement of Hh signaling in BCSCs, we used the SMO antagonist cyclopamine to treat BCMab1+CD44+ cells for an in vitro sphere formation assay. We observed that cyclopamine treatment dramatically impaired oncosphere formation (Fig. 2F). In addition, using a soft agar colony formation assay, cyclopamine treatment also significantly repressed anchorage-independent tumor growth (Fig. 2G). Overall, the Hh signaling pathway is essential for the maintenance of self-renewal of BCSCs.

GALNT1 is highly expressed in BCSCs

We performed transcriptome microarray of BCMab1+CD44+ cells versus BCMab1+CD44− cells derived from bladder cancer samples. Microarray analysis displayed that the glycotransferase GALNT1 mRNA level in BCMab1+CD44+ was over 7-fold higher than that in BCMab1+CD44− cells (Fig. 3A). However, other glycotransferases (GALNT4, 7, 8, 9, and 12) that the microarray detected had no significant changes in BCMab1+CD44+ cells compared with BCMab1+CD44− cells. As the GALNT family has 12 members, we then examined other undetected glycotransferase members in the microarray, including GALNT2, 3, 5, 6, 10, and 11, in the BCMab1+CD44+ cells. We noticed that these undetected glycotransferases were not expressed in the BCMab1+CD44+ cells (data not shown). These results were verified in BCMab1+CD44+ cells isolated from 72 bladder cancer specimens by using qPCR (Fig. 3B and C). Moreover, high expression of GALNT1 in pooled BCMab1+CD44+ cells from ten bladder cancer specimens was further validated by immunoblotting (Fig. 3D). High expression of GALNT1 in bladder cancer tissues was confirmed by immunohistochemical staining (Fig. 3E). In addition, GALNT1-positive staining in bladder cancer tissues was related to BCMab1 and CD44 staining.

To further verify the high expression of GALNT1 in BCMab1+CD44+ cells, we examined the 5′ CpG sites at the GALNT1 promoter of genomic DNAs by bisulfite genomic sequencing analysis. Each group displayed ten independent sequencing clones. The GALNT1 promoter region was over 90% unmethylated in BCMab1+CD44+ cells, whereas the same cytosine residues were about 60% methylated in BCMab1+CD44− cells (Fig. 3F). Similar results were obtained with other three bladder cancer specimens. In sum, these results suggest that the GALNT1 is indeed actively transcribed in BCMab1+CD44+ cells.

GALNT1 is required for the maintenance of stemness for BCSCs

We next wanted to determine whether GALNT1 takes part in the regulation of BCSCs. We silenced GALNT1 in BCMab1+CD44+ cells derived from one human bladder cancer specimen and established GALNT1-depleted cell lines (Fig. 4A). We found that GALNT1 knockdown significantly declined oncosphere formation and anchorage-independent colony formation (Fig. 4B and C). BG is a competitive substrate inhibitor for all glycotransferases (22). BG treatment obtained similar results to GALNT1 depletion (Fig. 4B and C). We detected mRNA levels of Gli1 and GALNT1 in BCMab1+CD44+ cells derived from 72 bladder cancer samples by qPCR. Interestingly, Gli1 displayed a good correlation with GALNT1 expression (Pearson correlation coefficient, r = 0.945; Fig. 4D).
Importantly, we observed that GALNT1 knockdown or BG treatment dramatically reduced Gli1 expression in BCMab1⁺CD44⁺ cells (Fig. 4E). These results were verified by immunofluorescence staining (Fig. 4F). We next overexpressed Gli1 and Gli2 in BCMab1⁺CD44⁺ cells followed by oncosphere formation assays. We observed that Gli1 but not Gli2 overexpression could promote oncosphere formation (Supplementary Fig. S1A), suggesting that Gli1 was activated in BCSCs. In addition, GALNT1 knockdown or BG treatment significantly declined active SHH-N protein levels in BCMab1⁺CD44⁺ cells (Fig. 4G), suggesting that GALNT1 depletion impaired SHH processing. Consequently, GALNT1-silenced BCMab1⁺CD44⁺ cells formed smaller tumor burden and displayed much lower xenograft tumor formation rates compared with shCtrl-treated cells (Fig. 4H and I).

We next rescued SHH and/or GALNT1 in GALNT1-silenced BCMab1⁺CD44⁺ cells. We found that rescue of both SHH and GALNT1 was able to process SHH and induce Gli1 expression (Supplementary Fig. S1B). In contrast, rescue of SHH in GALNT1-silenced BCMab1⁺CD44⁺ cells neither hydrolyzed SHH nor triggered Gli1 expression (Supplementary Fig. S1B). These data

Figure 4.
GALNT1 is required for maintenance of stemness of BCSCs. A, GALNT1 was silenced in BCMab1⁺CD44⁺ cells derived from one human bladder cancer specimen. A scrambled sequence (shCtrl) was used as a control. B and C, GALNT1 silencing or BG treatment declines oncosphere formation (B) and anchorage-independent tumor growth (C) of BCMab1⁺CD44⁺ cells. Oncospheres or colonies were counted in five separate fields after two weeks. **, P < 0.01. D, mRNA levels of GALNT1 and Gli1 were detected by RT-PCR from 72 patient samples. P = 0.001; r = 0.945, Pearson correlation. E, real-time PCR analysis of Gli1 mRNA levels in GALNT1-silenced or shCtrl BCMab1⁺CD44⁺ cells. BG-treated BCMab1⁺CD44⁺ cells were also analyzed. Data were normalized to β-actin and are shown as means ± SD. n = 12; **, P < 0.01. F, immunofluorescence staining of GALNT1 and Gli1 in shCtrl or GALNT1-silenced BCMab1⁺CD44⁺ cells. GALNT1 was labeled with FITC, and Gli1 was labeled with PE. G, ELISA analysis of SHH-N levels shCtrl or GALNT1-depleted BCMab1⁺CD44⁺ cells. BG-treated cells were also analyzed; **, P < 0.01. H, tumor volumes were measured at the indicated time points and calculated as means ± SD (left). Representative tumors at week 8 are shown on the right; **, P < 0.01. n = 12. I, xenograft BCSCs sorted by FACS and infected with retrovirus expressing either scramble vector (shCtrl) or shGALNT1, followed by transplantation into mice 24 hours after infection (n = 10 for each group).
validate that GALNT1-mediated SHH processing can induce Gli1 expression. Finally, we examined expression levels of SHH and its receptor PTCH1 in BCSCs from bladder cancer samples. We noticed that PTCH1 was expressed in the BCMab1⁺CD44⁺ cells (Supplementary Fig. S1C), and SHH was processed in these BCSCs. These results suggest that SHH processing in the BCSCs is directly related to its own Hh signaling in the same type of cell. Thus, we conclude that SHH is an autocrine factor in the BCMab1⁺CD44⁺ cells. Altogether, these data suggest that GALNT1 contributes to the activation of Hh signaling and tumorigenesis of bladder cancer.

GALNT1-mediated glycosylation is required for SHH activation in BCSCs

To further test whether GALNT1 influences SHH activation, we detected the SHH processing status in GALNT1-silenced or BG-treated BCMab1⁺CD44⁺ cells. Interestingly, we found that GALNT1 knockdown significantly impaired SHH hydrolysis in BCMab1⁺CD44⁺ cells derived from bladder cancer specimens (Fig. 5A), whereas shCtrl-treated BCMab1⁺CD44⁺ cells exhibited apparent SHH processing. Moreover, the full-length SHH displayed a smaller size on the gel blot in GALNT1-depleted BCMab1⁺CD44⁺ cells, suggesting that SHH might not be glycosylated after depletion of GALNT1. BG treatment showed similar results (Fig. 5A). Peanut agglutinin (PNA) lectin can specifically bind to the terminal galactose residues of O-glycans (23). To examine whether SHH undergoes O-linked glycosylation, we used PNA to probe SHH immunoprecipitates derived from GALNT1-silenced or shCtrl-treated BCMab1⁺CD44⁺ cells. Interestingly, we found that PNA could bind to SHH and its hydrolyzed SHH-C fragment (Fig. 5A), suggesting the SHH underwent O-linked glycosylation in BCMab1⁺CD44⁺ cells. To further confirm O-linked glycans, we used peptide N-glycosidase F and O-glycosidase to treat BCMab1⁺CD44⁺ cell lysates, respectively. PNA still bound to the SHH from N-glycosidase – treated cell extracts, but not the SHH from O-glycosidase – treated cell extracts (data not shown). These results verify that the O-linked glycosylation takes place in the BCMab1⁺CD44⁺ cells, but not in GALNT1-silenced or BG-treated BCMab1⁺CD44⁺ cells (Fig. 5B). These data indicate that GALNT1 participates in the processing activation of SHH.

We next predicted O-linked glycosylation sites of SHH by using the NetOGlyc 3.1 Server tool. Three potential candidate O-linked
glycosylation sites of SHH protein, including Thr282, Ser288, and Ser289, were selected (Supplementary Fig. S2). To identify the exact glycosylation sites, we generated three mutation construct vectors of SHH (T282A-SHH, S288A-SHH, and S289A-SHH). We cotransfected GALNT1 and SHH (WT) or its three respective vectors of SHH (T282A-SHH, S288A-SHH, and S289A-SHH). We exact glycosylation sites, we generated three mutation construct

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GALNT1-mediated activation of SHH is indispensable for the maintenance of self-renewal of BCSCs.

together, GALNT1-mediated activation of SHH is indispensable for the maintenance of self-renewal of BCSCs.

GALNT1 deficiency inhibits bladder tumor growth

We induced bladder cancer in GALNT1-deficient mice or littermate control mice (WT) by feeding BBN. At 4 months of BBN exposure, 23 WT out of 24 mice displayed robust tumors in bladders, with bigger tumor sizes (Fig. 6A and B). In contrast, only 5 GALNT1-deficient mice of 24 GALNT1−/− mice showed bladder tumors with smaller sizes. Tumors in WT or GALNT1-deficient mice were confirmed by histology staining (Fig. 6C). We prepared single tumor cells from WT or GALNT1−/− mice for an in vitro sphere formation assay. We observed that GALNT1−/− tumor cells formed much fewer oncospheres than WT tumor cells (Fig. 6D). As expected, mRNA and protein levels of Gli1 were undetectable in GALNT1−/− tumors (Fig. 6E and F), whereas Gli1 was highly expressed in WT tumors. In sum, GALNT1 deletion in mice suppresses BBN-induced bladder tumors.

GALNT1 silencing or blockade of Hh signaling displays antitumor efficacy of bladder cancer in vivo

The implanted mice were grouped (24 mice/group) and intravesically administrated GALNT1 siRNA/liposomes, or GALNT1 siRNA/liposomes plus cyclopamine the next day. Intriguingly, both GALNT1 siRNA and cyclopamine could significantly suppress tumor growth (Fig. 7A and B). Importantly, GALNT1 siRNA plus cyclopamine were able to abrogate tumor formation. To detect the delivery efficiency of siRNA with liposome, we analyzed

Figure 6.

GALNT1 deficiency inhibits BBN-induced bladder tumor growth. A and B, GALNT1−/− mice show a dramatic decrease in tumor formation rates (A) and sizes (B) of BBN-induced bladder cancer. Tumor formation rates of BBN-induced bladder cancer in mice were calculated after four months with BBN exposure. Then, the mice were sacrificed for measurement of tumor volumes. (n = 24); **P < 0.01. C, hematoxylin and eosin counterstaining for GALNT1−/− and GALNT1−/− mice treated with BBN. D, bladder tumor. D, tumors induced from GALNT1−/− mice suppress oncosphere formation. Sphere formation assays were performed for tumor cells induced from GALNT1−/− and GALNT1−/− mice analyzed by PCR (E) and immunoblotting (F).
GALNT1-Mediated SHH Activation in BCSCs

GALNT1 expression in bladder cancer tissues after 5 weeks of intravesical instillation of GALNT1 siRNA. GALNT1 siRNA was mixed with liposome for intravesical administration as previously described (24). GALNT1 was almost undetectable in GALNT1 siRNA/liposome-treated bladder cancer tissues (Fig. 7C), whereas GALNT1 was abundantly expressed in siCtrl-treated control mice. These data suggest that intravesical instillation of GALNT1 siRNA/liposome could efficiently deliver into bladder tissues and successfully deplete GALNT1 expression. Notably, both GALNT1 siRNA and cycloamine treatment were able to prolong survival rates of treated mice compared with siCtrl-treated mice. However, over 60% mice treated with GALNT1 siRNA plus cycloamine lived as long as WT mice (Fig. 7D). Therefore, the therapeutic efficacy is due to the elimination of BCSCs.

We next examined whether GALNT1 affects metastasis of bladder cancer. BCMab1 CD44+ cells, which were sorted from human bladder cancer samples and injected via tail vein injection for 5 weeks, were implanted with the indicated human bladder cancer cells via tail vein injection for 5 weeks. These cells were injected into the indicated agents. B, photon counts of each mouse are indicated by pseudo-color scales. Growth curves of orthotopical tumors were measured by IVIS (n = 24 per group). **, P < 0.01. C, GALNT1 expression levels were analyzed by immunoblotting. D, comparison of survival rates with the indicated treatment. n = 24 per group. E, representative bioluminescence images showing bladder cancer metastasis. Mice were implanted with the indicated human bladder cancer cells via tail vein injection for 5 weeks. F, imaging by stroboscope (middle) and hematoxylin and eosin (H&E) counterstaining (bottom) at 12 weeks after intravesical administration of GALNT1 siRNA with BBN exposure (top). G, tumor formation rates of GALNT1 siRNA or siCtrl-treated mice with exposure of BBN.

Discussion

BCSCs were considered as TICs (25), which could be responsible for bladder cancer initiation and recurrence. In this study, we identified a BCMab1 CD44+ cell population as BCSCs. The BCMab1 antibody, generating against CD44+ cells from human bladder cancer specimens, was defined to recognize aberrantly glycosylated integrin α3β1 in our previous study (20), which is a novel biomarker for BCSCs. We demonstrate that BCMab1 CD44+ cells have increased tumorigenicity and self-renewal property in vivo and in vitro. We defined that the GALNT1-mediated Hh signaling activation is essential for the maintenance of self-renewal of BCSCs and bladder tumorigenesis.

The mammalian secreted Hh ligands include SHH, Indian Hedgehog, and Desert Hedgehog, which initiate a signaling cascade via engaging with their common receptor Patched1 (PTCH1; ref. 17). Upon Hh engagement, PTCH1 releases its inhibitory role in the positive regulatory transmembrane protein SMO, resulting in activation of Gli family transcription factors (Gli1, Gli2, and Gli3; refs. 26, 27). The Hh pathway is largely inactive in the adult, except for its action in tissue repair and maintenance (14, 28). The role of Hh signaling in the tumorigenesis of bladder cancer remains controversial. Inconsistently, loss-of-function mutations of PTCH1 were rarely identified in bladder cancer (29, 30). Recently, Robbins and his colleagues showed that the Hh signaling is highly expressed in bladder cancer tissues and implicated in the tumorigenesis of bladder...
cancer (31, 32). However, the role of Hh signaling pathway in BCSCs is largely unknown.

Newly synthesized Hh proteins require a series of posttranslational processing reactions to generate their respective active forms for signal transduction. Many properties of Hh autoprocessing were defined from studies of the Drosophila protein (33). It is widely considered that the Hh mature processing procedure is highly conserved in various species. It has been revealed that the secreted Hh signaling domain is covalently modified by cholesterol and palmitate (26). The N-terminal cysteine is catalyzed by the acyl-transferase Skinny hedgehog to form the amide linkage of cholesterol and palmitate (26). Hydrophobic modifications confer membrane affinity such that the signaling domain is successfully associated with its membrane receptors, leading to a signaling cascade (36, 37). Here we defined a novel glycosylation modification for SHH in BCSCs, which is uniquely mediated by GALNT1.

Superficial tumors are usually treated by surgical resection and intravesical chemotherapy and/or immunotherapy. The difficulty in managing bladder cancer is unable to predict which tumors will recur or progress (38). A previous report showed that intravesical instillation of polo-like kinase (PLK-1) siRNA can effectively prevent bladder cancer growth (24). siRNA-mediated RNAi is sequence-specific gene silencing and widely used to silence gene expression (39, 40). Here we showed that intravesical administration of GALNT1 siRNA can significantly suppress bladder tumor growth in orthotopic bladder cancer mouse models and BBN-induced bladder tumors. The SMO antagonist cyclopamine and its derivatives have been reported to be effective agents against several tumors (18, 41). One of cyclopamine derivative GDC-449 has been approved to treat advanced unresectable basal cell carcinoma as a first-line therapy. Of note, intravesical administration of GALNT1 siRNA and cyclopamine can eradicate implanted tumor formation without overt adverse effects. Finally, therapeutic use of GALNT1 siRNA, the SMO inhibitor, and the BCMAb1 antibody will provide a potent antitumor strategy against bladder cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Concept and design: C. Li, J. Wang, Z. Fan
Development of methodology: C. Li, Z. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Li, Y. Du, Z. Yang, L. He, Y. Wang, R. Yan, J. Wang, Z. Fan
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Li, Y. Du, L. Hao, Z. Fan
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