Blockade of integrin signaling reduces chemotherapy-induced premature senescence in collagen cultured bladder cancer cells

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Abstract

Background
A diminished sensitivity towards chemotherapy remains the major impediment to the clinical treatment of bladder cancer. However, the critical elements in control of chemotherapy resistance remain obscure.

Methods
We adopted improved collagen gels and performed cytotoxicity analysis of doxorubicin (DOX) and mitomycin C (MMC) of bladder cancer cells in 3D culture system. We then detected the expression of multidrug resistant gene ABCB1, dormancy-associated functional protein chicken ovalbumin upstream-transcription factor 1 (COUPTF1), cell proliferation marker Ki-67, and cellular senescence marker senescence-associated β-galactosidase (SA-β-Gal) in these cells. We further tested the effects of integrin blockade or AKT inhibitor on the senescent state of bladder cancer. Also, we examined the tumor growth and survival time of bladder cancer mouse models given the combination treatment of chemotherapeutic agents and integrin α2β1 ligand peptide TFA (TFA).

Results
Collagen gels played a repressive role in bladder cancer cell apoptosis induced by doxorubicin (DOX) and mitomycin C (MMC). In mechanism, collagen activated integrin β1/protein kinase B (AKT) cascade to drive bladder cancer cells into a premature senescence state via the p21/p53 pathway, thus attenuating chemotherapy-induced apoptosis. What’s more, TFA had the ability to mediate the
switch from senescence to apoptosis of bladder cancer cells in xenograft mice.

Meanwhile, TFA combined with chemotherapeutic drugs produced a substantial suppression of tumor growth as well as an extension of survival time \textit{in vivo}.

\textbf{Conclusions}

Based on our finding that, integrin β1/AKT acted primarily to impart premature senescence to bladder cancer cells cultured in collagen gel, suggesting that integrin β1 might be a feasible target for bladder cancer eradication.

\textbf{Key words}: premature senescence; integrins; bladder cancer; chemotherapy; collagen
Introduction

Bladder cancer is among the major sources of cancer-related morbidity, accounting for approximately 200,000 deaths worldwide in 2018.\(^1\) Cytotoxic chemotherapy has been the systemic management of bladder cancer for decades, but the efficacy remains limited.\(^2\) The lack of response to chemotherapy is eyed as a major culprit for treatment failure in bladder cancer patients, leading to disease relapse and poor overall survival.\(^3\) Therefore, it would be desirable to unravel the underlying mechanisms of drug resistance aiming to devise potential treatment strategies.

Cancer cells could seek refuge from cytotoxic effects in a wide range of ways, among which the most straightforward method is to restrict drugs reaching the site of action.\(^4\) One such mechanism is through the upregulation of the adenosine triphosphate (ATP)-binding cassette transporters, which are able to promote drug expulsion.\(^5\) DNA damage repair is also considered as a key player in modifying the response to chemotherapy, which may be attributed to the induction of cell cycle arrest.\(^6\) What’s more, accumulating evidence suggested that the tumor microenvironment (TME) provided shelter for cancer cells from cytotoxic stress, leading to the development of acquired resistance.\(^7\) The TME is mainly made up of stromal cells and extracellular matrix (ECM) components. It has been demonstrated that collagen, the most abundant ECM protein, exerts modulatory functions in chemotherapeutic sensitivity, which may be partially dependent on cellular adhesion.\(^8,9\) Integrins, a large family of heterodimeric receptors, serve as cell surface adhesion molecules that connect cells to the ECM. Ligand binding to integrins modulates multiple downstream signals,
including the phosphoinositide 3-kinase (PI3K)-AKT, extracellular-signal regulated kinase (ERK) and NF-κB pathways, culminating in the formation of a resistant genotype.\textsuperscript{10} A recent study indicated that the interaction of integrin β\textsubscript{1} with collagen I transmits breast cancer cells into chemoresistance, and ITGB1 inhibition sensitized the cells to drugs.\textsuperscript{11} Nevertheless, the role of integrins in chemoresistance development and underlying mechanism remain a matter of further elucidation.

Our previous study reported that novel 3D collagen I gel model could confer tumorigenic potential to bladder cancer cells and enable them to progress to cancer stem cells (CSCs).\textsuperscript{12} Herein, we further focused on the impact of collagen on chemotherapeutic sensitivity and found that collagen culture rendered bladder cancer cells into a senescent state to evade apoptosis induced by cytotoxic reagents. In mechanism, collagen regulated integrin β1/AKT axis to trigger premature senescence, which was dependent on the p53/p21 pathway. Additionally, we confirmed that interruption of integrin signals had an inhibitory effect on chemotherapy-induced senescence \textit{in vivo}, resulting in enhanced antitumor efficacy. Taken together, our study firstly pointed to the importance of cell senescence during integrin-induced chemoresistance development, which may aid in the elaboration of therapeutic strategies for eliminating bladder cancer.
Methods

Ethical approval

The clinical experiments were carried out according to the guidance of the Declaration of Helsinki and approved by the Ethics Committee of West China hospital, Sichuan University. All patients were informed with written consent and agreed to participate in the study. The animal studies were conducted in accordance with the Public Health Service Policy and complied with the World Health Organization (WHO) guidelines for the humane use and care of animals. All animal protocols were monitored by the Ethics Committee of West China hospital, Sichuan University and conducted in accordance with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

Cell culture and reagents

Human bladder cancer cell line T24 was purchased from the American Type Culture Collection and maintained in RPMI 1640 complete medium (Gibco, MA, USA) supplemented with 10% fetal calf serum (Gibco, MA, USA), at 37°C in a 5% CO₂ atmosphere. 3D collagen culture of cancer cells was performed as previously described. Briefly, collagen I was diluted to 0.6 mg/ml with RPMI 1640 complete medium supplemented with 10% fetal calf serum. 1 × 10⁴ cancer cells were added into the 295 μl collagen solution containing 25 μl 10 × PBS and 20 μl 1N NaOH. After 2 hours of 37°C incubation, the solid clotty collagen (containing tumor cells) was added to RPMI 1640 complete medium supplemented with 10% fetal calf serum.
for further use. Collagen I and type I collagenase were purchased from Sigma (USA). Integrin α2β1 ligand peptide TFA (TFA) and AKT inhibitor Miransertib (Mir) were purchased from MedChemExpress (USA). Chemotherapeutic DOX and MMC were purchased from Sangon (China).

**Primary tumor cells culture and patients’ information**

Human bladder tumor tissues were collected from the West China hospital, Sichuan University. 20 tumor tissues (paraffin sections) were divided into recurrent (R) and non-recurrent (NR) groups according to follow-up visit after chemotherapy. For establishment of primary bladder cancer cell lines, 15 primary tumor tissues (BP 1~15) were collected, and digested to be seeded in 3D Matrigel containing growth factors (#354234, Coring, USA). 2 samples (BP5 and BP7) succeeded to form colonies, which were collected and digested to seed in Matrigel again. After cultured in Matrigel for 2 weeks, BP5 and BP7 were digested and cultured in dish for further analysis. Both BP5 and BP7 survived and displayed proliferative characteristics in dish for 4 months.

**Cell apoptosis analysis**

Cell apoptosis was determined using the FITC-Annexin V and PE-PI apoptosis detection kit (Becton, Dickinson and Company, USA). Briefly, pre-treated tumor cells were harvested and stained with FITC-Annexin V and PE-PI viability staining solution for 15 min at room temperature. Subsequently, apoptosis was analyzed by flow cytometry on a C6 flow cytometer Becton, Dickinson and Company, USA).
**SA-β-Gal assay**

Cells were stained for β-Gal activity according to guidance of SA-β-Gal assay Kit (Biyuntian, China). Briefly, tumor cells were digested and washed in phosphate-buffered saline (PBS), then fixed for 10 minutes in 4% formaldehyde. After that, tumor cells were incubated at 37 °C (containing no CO₂) with staining solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside). After 12 hours, quantification of SA-β-Gal positive cells was performed under photon microscope (Leica, Germany).

**Cell cycle assay**

Cell cycle analysis was performed using the PE-PI cell cycle analysis kit (Becton, Dickinson and Company, USA). In brief, pre-treated cancer cells were harvested and fixed with ice ethanol. Subsequently, samples were incubated with propidium iodide (PI) staining solution for 10 minutes according to the manufacturer’s protocol. The samples were detected by flow cytometry (Becton, Dickinson and Company, USA).

**Real-time quantified polymerase chain reaction (PCR)**

The quantification of mRNA levels was conducted by real-time PCR using SYBR green dye (Thermo, MA, USA). GAPDH was used for normalization. The primers used are listed as follows: human GAPDH forward primer 5'-GGAGCGAGATCCCTCCAAAAT-3', reverse primer 5'-GGCTGTTGTACTTCTCATGG-3'; human COL1A1 forward primer 5'-GAGGGCAAAGACGAAGACATC-3' and reverse primer
5’-CAGATCACGTCATCGCACAAC-3’; human COL1A2 forward primer
5’-GTTGCTGCTTGCAAGTAACCTT-3’ and reverse primer
5’-AGGGCCAAGTCCAACCTCCTT-3’; human ABCB1 forward primer
5’-TTGGCTGATGTTTGTGGGAAG-3’ and reverse primer
5’-CCAAAAATGAGTAGCAGCCT-3’;

Western blotting

Cells were harvested and homogenized in lysis buffer (Sigma, USA) containing phosphatase and proteinase inhibitors (Sigma, USA). Protein samples were then quantified using the Pierce BCA Protein Assay (Thermo Fisher, USA). Protein samples (25 μg) were separated by SDS-PAGE, transferred to PVDF membranes, and incubated with primary antibodies for integrin β1 (ITGB1) (1:1000; Abcam, B179471, UK), p21 (1:1000; Abcam, ab109199, UK), p53 (1:1000; Abcam, ab32389, UK), total AKT (1:1000; Abcam, ab8805, UK), p-AKT (1:500; Abcam, ab38449, UK) or β-actin (1:1000; Abcam, ab8226, UK) at 4°C overnight. Bound antibodies were detected by horseradish peroxidase–linked secondary antibodies (Thermo Fisher, USA) and processed with Pierce ECL Western Blotting Substrate (Thermo Fisher, USA).

Immunofluorescence and immunohistochemistry

Tumor tissues were fixed in 10% formalin solution for at least 48 hours. Subsequently, samples were processed, embedded in paraffin, and sectioned at 5 μm for immunofluorescence and immunohistochemical staining. Sections were then dewaxed, rehydrated, quenched of endogenous peroxidase, blocked, and incubated with the primary antibody anti-collagen I (1:100; Abcam, ab270993, UK) at 4°C overnight,
followed by signal amplification using a horseradish peroxidase–linked secondary antibodies (Thermo Fisher, USA). The intensity of collagen I expression was determined by Image-Pro Plus 6.0 software (USA).

**In vivo experiments**

Nude mice (6–8 weeks old) were purchased from Huafukang (Beijing, China) and housed in a specific pathogen-free facility. For cell senescence and apoptosis analysis in vivo, $5 \times 10^6$ T24 cells were subcutaneously injected into nude mice. On day 20, mice were treated with PBS, MMC (5 mg/kg) or DOX (5 mg/kg) by tail vein injection (n=3 in each group). On day 23, mice were sacrificed for tumor tissues collection. P21 and p53 expression were determined by western blotting. Part of tumor tissues were digested for cell apoptosis and senescence analysis. For tumor volume and survival analysis, $5 \times 10^6$ T24 cells were subcutaneously injected into nude mice. On day 20, mice were treated with PBS, MMC (5 mg/kg), TFA (2.5 mg/kg) or combination twice a week by tail vein injection (n=6 in each group). Tumor volume and survival were recorded every day. The calculation formula of tumor volume is: tumor volume = length $\times$ width$^2$/2.

**Statistical analysis**

All data were presented as the mean ± SEM. Statistical significance was analyzed using GraphPad 6.0 software (La Jolla, California, USA). Statistical significance between groups was calculated by Student’s t test for two groups or by one-way ANOVA for more than two groups. Bonferroni analysis were further used for the post hoc test. The survival rates were determined by Kaplan–Meier survival analysis. *p <
Results

Collagen reduced chemotherapy-induced cell apoptosis and correlated with tumor recurrence in patients

Our previous study has demonstrated that collagen I served as the major element in ECM, tightly correlated with the tumorigenic potential in tumor initiating cells (TICs). Stemming from this evidence, we next sought to conduct more clinically relevance of the role of collagen in regulating chemoresistance during bladder cancer progression. To do this, 15 tumor tissues were collected from bladder cancer patients and tumor cells were digested, seeded in 3D matrix gels (containing growth factors) for primary cancer cells culture. Two primary bladder cancer cell lines (bladder patient #5, BP5 and bladder patient #7, BP7) succeeded to survive in dish culture and revealed proliferative characteristics for 4 months (Fig. 1A). Subsequently, bladder cancer cell line T24 and primary BP5/BP7 cells were seeded in our improved collagen gels as described. To verify whether collagen was able to promote chemoresistance, MMC and DOX were added into culture medium of dish/collagen cultured bladder cancer cells (T24, BP5 and BP7), and cell apoptosis was assessed by Annexin V/PI assay after 48 hours. As expected, analysis of flow cytometry showed that collagen cultured bladder cancer cells (T24, BP5 and BP7) was significantly resistant to DOX (Fig. 1B), when compared to dish cultured group. Notably, enhanced resistance to
MMC was observed in collagen cultured cells (Fig. 1B), suggesting that *in vitro* 3D collagen culture reduced cytotoxicity of chemotherapy in bladder cancer cells. Seeking to assess more clinical relevant between collagen I and chemoresistance development, we acquired information and tumor specimens of 20 bladder cancer patients, and then divided these patients into two groups, including non-recurrent tumor (NR) and recurrent tumor groups (R), according to follow-up visit after standard surgery combing adjuvant chemotherapy. Here, the expression of collagen I associated genes (*COLIA1* and *COLIA2*) in two groups was analyzed by quantified PCR. Of note, we observed that recurrent group displayed elevated expression of *COLIA1* and *COLIA2*, when compared to non-recurrent group (Fig. 1C). Consistently, the protein expression of collagen I in recurrent group was higher than non-recurrent tumors (Fig. 1D). These data suggested that elevated collagen I expression might the causative reason for therapeutic resistance and tumor relapse. Additionally, we assessed the overall survival of bladder cancer patients with high/low *COLIA1/COLIA2* expression by analyzing the Cancer Genome Atlas Program (TCGA) databases. Notably, patients with low levels of *COLIA1/COLIA2* expression had a remarkably longer survival time than those with high levels (Fig. 1E and F). Together, those results suggested that collagen reduced chemotherapy-induced cell apoptosis, correlated with tumor recurrence and poor prognosis in bladder patients.

**Chemotherapy-induced cellular senescence to escape apoptosis in 3D collagen cultured TICs**

Motivated by prior results implicating that collagen reduced cell cytotoxicity-induced...
by chemotherapeutic agents, we next sought to elucidate the mechanism underlying collagen induced chemoresistance. Development of chemoresistance is bound up with diverse biological process, including upregulation of multidrug resistant protein P-glycoprotein and arrest of cell cycle (cell dormancy and senescence).\textsuperscript{13} We firstly examined the expression of multidrug resistant gene \textit{ABCB1} in dish and collagen/MMC cultured T24 cells by quantified PCR, respectively. However, no significant alterations of gene expression were detected for \textit{ABCB1} in bladder cancer cells (Fig. 2A). Similarly, we did not found upregulation of multidrug resistant protein P-glycoprotein at protein level in collagen/MMC cultured cancer cells, when compared to the dish group (Fig. 2B). Those results implicated that collagen reduced chemotherapy inducing cell apoptosis in a multidrug resistant proteins independent manner. Subsequently, we wondered whether collagen played a role in regulating cell cycle to help tumor cells escape from apoptosis. Thus, we assessed cell cycle of dish and collagen cultured bladder cancer cells using AV-PI staining, respectively. Intriguingly, no significant difference was observed in cell cycle between dish and collagen cultured T24 cells, while MMC treated T24 cells (collagen culture) were arrested in the G0/G1 phase (Fig. 2C), suggesting that chemotherapy combining collagen culture induced cell cycle arrest, thus affecting cell apoptosis. As previously reported, a senescence or dormancy-like state could enable tumor cells to be arrested in G0/G1 status, which help tumor cells to escape apoptosis status during chemotherapy. Thus, to assess whether collagen cultured bladder cancer cells entered a dormancy-like state, immunofluorescence assay was performed to examine
dormancy-associated functional protein COUPTF1 and cell proliferation marker Ki-67 expression in collagen/MMC treated T24 cells. Unfortunately, no significant difference was detected in COUPTF1 expression among groups (dish, collagen, MMC or collagen combining MMC, Fig. 2D). This implicated us that T24 cells might progress to premature senescence-like state, instead of dormancy to escape apoptosis. To testify our hypothesis, SA-β-Gal (a senescence marker) assay was conducted to evaluate cell senescence in T24 cells. Remarkably, increased SA-β-Gal positive cells were observed in MMC treated T24 cells (cultured in collagen), whereas the expression of SA-β-Gal was unchanged in mono MMC or collagen cultured group (Fig. 2E). Similarly, elevated expression level of SA-β-Gal was found in DOX treated T24 (collagen culture, Fig. 2F) and BP5/BP7 cells (Fig. 2G). Based on these findings, it was suggested that collagen culture promoted tumor cells to enter a senescent status in the presence of chemotherapeutic agents. Collectively, those results suggested that collagen culture enabled bladder cancer cells progress to senescent state to escape apoptosis induced by chemotherapeutic MMC and DOX.

**Integrin-AKT axis induced premature senescence via p21CIP/p53 Pathway**

Given the crucial role of collagen/chemotherapy in regulating cell cycle, we next sought to elucidate the molecular pathway driving cell senescence. As reported in our previous study, biomaterial 3D collagen gels reverted differentiated tumor cells back into CSCs through integrin α2β1/AKT cascade. Importantly, compelling document provided evidence that integrin-Src-AKT axis could mediate cellular senescence by counteracting apoptosis in irradiated tumor cells. Thus, we put forward the
hypothesis that collagen induces upregulation of integrin β1, which leads to the activations of AKT signaling pathway. Subsequently, activated AKT blocks caspase activation and p21 cleavage induced by chemotherapy, eventually resulting in p53/p21 signaling activation and cell senescence, instead of apoptosis in bladder cancer cells (Fig. 3A). To confirm this, western blotting was performed to examine integrin β1/AKT axis in T24 cells with four process modes (dish, collagen, MMC, MMC and collagen combination). As excepted, western blotting analysis revealed both integrin β1 and AKT were notably upregulated in collagen cultured cells (Fig. 3B), indicating that collagen mediated integrin-AKT axis activation. P53 is a critical tumor suppressor gene, and activating p53 and its downstream targets to induce apoptosis is a traditional apoptosis pathway in tumor cells during chemotherapy. P21 is an inhibitor of cyclin-dependent kinase, which has been reported to serve as downstream molecule of p53 to promote cell senescence. Thus, we further examined p53/p21 expression in T24 cells (dish, collagen, MMC, MMC and collagen combination). As shown by western blotting, MMC treatment upregulated p53 expression, and collagen culture mediated p21 activation in MMC treated T24 cells (Fig. 3C). Those results implicated that MMC mediated p53 apoptosis pathway activation, and collagen culture might counteract cell apoptosis and mediate p21 associated cell senescence. Subsequently, in attempt to clarify whether the activation of p53/p21 signaling was affected by integrin-AKT axis, T24 cells were seeded into collagen gels and treated with MMC, following with integrin α2β1 ligand peptide TFA and AKT inhibitor Mir treatment. We found that blockade of integrin or AKT
induced strongly repressed expression of p21, while the protein level of p53 was unchanged (Fig. 3D). Consistently, SA-β-Gal and cell apoptosis analysis suggested that TFA and Mir treatment suppressed cell senescence (Fig. 3E) and increased cell apoptosis (Fig. 3F) in T24 cells (collagen and MMC treatment). Those results suggested that collagen promoted integrin-AKT axis activation to induce premature senescence via p53/p21 pathway to escape MMC-induced cell apoptosis. To further verify our results, expression of integrin β1/AKT and p53/p21 signaling was evaluated in DOX treated BP5 and BP7 cells. The similar results were observed in DOX treated BP5/BP7 cells (Fig. 3G and H). Together, those results suggested that collagen regulated integrin-AKT axis to induce premature senescence via p53/p21 pathway.

**Blockade of integrin/ATK signaling suppressed chemotherapy-induced senescence in vivo**

Stemming from our *in vitro* results, we next became interested in assessing the influence of senescence on chemotherapy *in vivo*. To do this, we firstly checked the expression of collagen-integrin β1-AKT expression in T24-bearing mice *in vivo*. In fact, collagen I, the dominant component in the ECM, has been detected in a wide range of tumor tissues. Consistent to previous reports, a higher expression of collagen I in subcutaneous T24 tumor tissues was found, when compared to dish culture (Fig. 4A). Meanwhile, activation of integrin β1-AKT signaling was observed in T24 tumor tissues (Fig. 4B), which was in line with the elevated expression of collagen I *in vivo*. Next, we treated T24-bearing mice with DOX and MMC by tail vein injection. After 3
days, mice were sacrificed for tumor tissues collection and p53/p21 expression analysis. Accordingly, the protein level of p53 and p21 were both much higher in MMC or DOX treated tumor tissues, when compared to PBS group (Fig. 4C). Those results were in line with hypothesis that activation of integrin enhanced cell senescence through p53/p21 signaling in the presence of chemotherapeutic agents.

Subsequently, we established subcutaneous T24-bearing mice model, following with MMC and integrin α2β1 ligand peptide TFA treatment (day 20), for cell senescence and apoptosis assay. 48 hours later, reduced SA-β-Gal positive cells (Fig. 4D), and enhanced cell apoptosis (Fig. 4E) were found in mice treated with MMC/TFA combination, when compared to MMC group. Additionally, TFA and MMC combination efficiently halted tumor growth (Fig. 4F), and extended survival time of tumor bearing mice (Fig. 4G). Those results supported the notion that blockade of integrin/ATK signaling suppressed chemotherapy-induced senescence in vivo, resulting in an improved tumor suppressive effect.

**Discussion**

Development of chemoresistance is intimately linked with the microenvironment which comprises both neoplastic cells and stromal components. The bidirectional communications between tumor cells and the stroma are thought to initiate events that contribute to drug resistance. A large body of information exists regarding the role of collagen, the main constituents of the ECM, in modulating the response of tumors to diverse treatments. SooHyeon et al. reported that ovarian cancer cells within...
collagen-based hydrogel were less vulnerable to the induction of apoptosis by antitumor agents, resulting in a higher level of drug resistance. It was also demonstrated that collagen mediated tumor initiating cells-like characteristics and decreased the sensitivity to 5-fluorine and paclitaxel in gastric cancer. In a study of lung carcinoma, chemo-resistant patients exhibited enhanced expression of COL1A1, which might serve as an independent prognostic factor. As for bladder cancer, we established an improved 3D culture system and found that collagen gels had an inhibitory effect on cell apoptosis induced by MMC and DOX. Clinical data further revealed that elevated collagen I expression in patients with recurrent bladder cancer. What’s more, analysis of TCGA database correlated a survival benefit with low levels of COL1A1/COL1A2 in bladder cancer patients. The above results highlighted that collagen promoted drug resistance in bladder cancer and was closely associated with tumor relapse.

Based on the crucial role of collagen, we sought to elucidate the specific mechanism whereby collagen suppressed chemotherapy-mediated cell apoptosis. Evidence is accumulating that apoptosis is not the only way in which tumor cells respond to drug stimuli. Tumor cells failing to undergo apoptosis may enter into a terminally arrested state termed as premature senescence. A panel of markers participate in defining a cell as being senescent, including SA-β-gal reactivity, altered expressions of mediators of cell cycle checkpoints, as well as lack of the cell-cycle-associated Ki67 protein. Despite losing the ability to proliferate and migrate, senescent cells remain viable over an extended period of time and secrete a plethora of proteins known as the
senescence-associated secretory phenotype (SASP), which influences the TME beneficial to tumor progression. Indeed, studies have identified premature senescence as a key player in modulating the responses to chemotherapy.\textsuperscript{22, 23} Research on breast cancer demonstrated that drug treatment triggered the generation of senescent cells from which multidrug-resistant colonies with aggressive stem-like phenotype emerged.\textsuperscript{24} In vivo experiments also confirmed that after doxorubicin administration, breast cancer developed characteristics of senescence with the secretion of cytokines that led to the phosphorylation of signal transducer and activator of transcription 3 (STAT3), a substantial contributor to chemoresistance.\textsuperscript{25} In addition, colorectal mouse models suggested that late disease recurrence was associated with the senescent state in response to drug stimuli, and interruption of this state augmented the apoptotic response of colorectal cancer cells to SN38, hinting at the reversibility of chemotherapy-induced senescence.\textsuperscript{26} In the current study, we first ruled out the influence of multidrug resistant protein and found that collagen played a role in regulating cell cycle to help bladder cancer cells evade apoptosis. We further demonstrated that cellular senescence, rather than dormancy, was responsible for the cell cycle arrest in the presence of collagen, finally resulting in low sensitivity to chemotherapy. Meanwhile, MMC or DOX treatment caused an elevation in SA-β-Gal levels in bladder cancer cells under this 3D culture condition, which provided proof of the involvement of senescent state in chemoresistance development. Compared to previous work, we initially addressed the relationship between chemotherapy and cellular senescence in 3D culture model, underscoring that drug administration in
conjunction with collagen culture enabled bladder cancer cells to enter premature senescence-like state, thus impairing the curative effect of cytotoxic agents. Also, our study offered new explanations for collagen-mediated chemoresistance in bladder cancer and added further insights into the role of collagen in tumor development.

The above findings prompted us to unveil the specific molecular pathways driving cellular senescence. Senescence activation is executed by intracellular and extracellular signals and highly depends on the engagement of the ECM. Integrins, transmembrane receptors interacting with the matrix elements, are involved in control of complicated cellular behaviors, including adhesion, proliferation, migration, survival, and cell fate transitions. Upon ligand binding, integrins recruit intracellular proteins and activate downstream signals, such as Ras-ERK, PI3K/AKT, and Yes1 associated transcriptional regulator (YAP)/tafazzin (TAZ) pathways. Importantly, compelling findings have established the role of integrins in cellular senescence. β1-integrin activation was proved to be essential for Tenascin-C-induced senescence of fibroblasts, which secreted soluble factors driving the malignant transformation of epithelial cells. What’s more, when exposed to ionizing radiation, lung carcinoma cells might undergo a shift from cell apoptosis to premature senescence via the integrin α6β4-Src-AKT signaling pathway. Consistently, our in vitro and in vivo experiments confirmed that collagen enhanced the activation of integrin-AKT axis to mediate a state of premature senescence in bladder cancer, leading to low reactivity to cytotoxic agents. We further identified the p53/p21 pathway, a core regulator of cell cycle, as the downstream molecules of integrin signals. Notably, inhibitors and
monoclonal antibodies of integrins have been demonstrated to dampen tumor progression and disrupt angiogenesis in mice models. More importantly, integrin-targeted drugs (e.g. cilengitide, etaracizumab, CNTO 95) are being extensively investigated in cancer clinical trials with promising results. Both clinical and experimental data has suggested the tumor suppressive effects of cilengitide (an RGD pentapeptide αvβ3 and αvβ5 integrin inhibitor) in clinical glioblastoma, and E7820 (an integrin α2β1 inhibitor) in combination with erlotinib for non-small cell lung cancer treatment. Here, our study determined the efficacy of integrin α2β1 ligand peptide TFA in combination with MMC, which hindered bladder cancer growth and prolonged survival time of tumor bearing mice. On the basis of these results, our study illustrated that (1) the expression of collagen was correlated with tumor recurrence and could be potentially translated into a prognostic marker for bladder cancer. (2) Bladder cancer cells cultured in collagen gels were skewed toward premature senescence rather than cellular apoptosis in response to chemotherapeutic agents. (3) The induction of senescent state was dependent on integrin-AKT axis. (4) The suppression of tumor growth could be achieved by MMC (or other chemotherapeutic agents) in cooperation with an integrin α2β1 ligand peptide, which opened up possibilities for targeting integrins in oncological treatment.

In brief, our study indicated that chemotherapy triggered premature senescence to evade apoptosis in bladder cancer cells within collagen gels. We provided evidence for the integrin-AKT axis being a regulator of senescence via p52/p21 pathway. Targeting integrin signals may provide a feasible strategy to improve the clinical
treatment for bladder cancer.

Author contributions

Data curation: Linghui Deng, Kun Jin, Xianghong Zhou, Zilong Zhang, Liming Ge, Xingyu Xiong, Xingyang Su, and Di Jin; Formal analysis: Linghui Deng, Kun Jin, Xianghong Zhou, Qiming Yuan, Chichen Zhang, Yifan Li, Haochen Zhao, and Qiang Wei; Writing-original draft: Linghui Deng, Kun Jin, and Xianghong Zhou; Conceptualization, Methodology, Writing-review & editing: Shi Qiu and Lu Yang.

Conflict of interests

The authors declare that they have no conflict of interest.

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Fig. 1 Collagen reduced chemotherapy-induced cell apoptosis and correlated with tumor recurrence in patients. A, schematic diagram of primary tumor cells culture (15 tumor tissues, BP1~15). B, cell apoptosis of T24, BP5 and BP7 (dish or collagen culture) treated with MMC (0.5 μg/ml, 48 hours) or DOX (1 μg/ml, 48 hours). C, relative expression of COL1A1 and COL1A2 at mRNA level in bladder tumor tissues from 20 patients, which was divided into recurrent (R, n=10) and non-recurrent (NR, n=10) groups. D, immunohistochemical staining of collagen I in bladder tumor tissues from 20 patients, which was divided into recurrent (R, n=10) and non-recurrent (NR, n=10) groups. The scale bar was 50 μm. E, overall survival analysis of bladder cancer patients with high/low level COL1A1 expression (n=406) using TCGA database. F, overall survival analysis of bladder cancer patients with high/low level COL1A2 expression (n=406) using TCGA database.
Fig. 2 Chemotherapy induced cellular senescence to escape apoptosis in 3D collagen cultured TICs. A, Relative \textit{ABCB1} expression at mRNA level in T24 cells cultured in dish or collagen gels. B, western blotting of P-glycoprotein (P-gp) in T24 cells cultured in dish or collagen gels. C, cell cycle analysis of T24 cells (cultured in dish or collagen gels) treated with PBS or MMC (0.5 μg/ml, 48 hours), using PI staining. D, immunofluorescence of Ki67 and COUP TF1 in T24 cells (cultured in dish or collagen gels).
dish or collagen gels) treated with PBS or MMC (0.5 μg/ml, 48 hours), the scale bar was 30 μm. Fluorescence intensity was determined by Image J 6.0 software. E, SA-β-Gal staining of T24 cells (cultured in dish or collagen gels) treated with PBS or MMC (0.5 μg/ml, 48 hours), the scale bar was 30 μm. F, SA-β-Gal positive cells analysis of T24 cells (cultured in dish or collagen gels) treated with PBS or DOX (1 μg/ml, 48 hours). G, SA-β-Gal positive cells analysis of BP5 and BP7 cells (cultured in dish or collagen gels) treated with PBS or MMC (0.5 μg/ml, 48 hours).
Fig. 3 Integrin-AKT axis induced premature senescence via p21/p53 pathway. A, schematic diagram of integrin reducing chemotherapy-induced cell apoptosis and promoting premature senescence through p53/p21 signaling. B, western blotting of ITGB1, phosphorylated AKT and total AKT in T24 cells (cultured in dish or collagen gels) treated with PBS or MMC (0.5 μg/ml, 48 hours). C, western blotting of p53 and p21 in T24 cells (cultured in dish or collagen gels) treated with PBS or MMC (0.5 μg/ml, 48 hours). D, western blotting of p53 and p21 in T24 cells (collagen culture, 0.5 μg/ml MMC treatment) pre-treated with PBS, Mir (10 nM) or TFA (1 μM). E, SA-β-Gal positive cells analysis of T24 cells (collagen culture, 0.5 μg/ml MMC treatment) pre-treated with PBS, Mir (10 nM) or TFA (1 μM). F, cell apoptosis of T24 cells (collagen culture, 0.5 μg/ml MMC treatment) pre-treated with PBS, Mir (10 nM) or TFA (1 μM). G, western blotting of ITGB1, phosphorylated AKT, total AKT, p53
and p21 in BP5 cells (cultured in dish or collagen gels) treated with PBS or DOX (1 μg/ml, 48 hours). H, western blotting of ITGB1, phosphorylated AKT, total AKT, p53 and p21 in BP7 cells (cultured in dish or collagen gels) treated with PBS or DOX (1 μg/ml, 48 hours).
Fig. 4 Blockade of integrin/ATK signaling suppressed chemotherapy-induced senescence in vivo. A, immunofluorescence of collagen in dish-cultured T24 or tumor tissues from T24 bearing mice. The scale bar was 50 μm. B, western blotting of ITGB1, phosphorylated AKT and total AKT in dish-cultured T24 or tumor tissues from T24 bearing mice. C, western blotting of p53 and p21 in tumor tissues from T24 bearing mice treated with PBS, MMC (5 mg/kg) or DOX (5 mg/kg) by tail vein injection. D, SA-β-Gal positive cells analysis in tumor tissues from T24 bearing mice treated with PBS, MMC (5 mg/kg), TFA (2.5 mg/kg) or combination. E, cell apoptosis in tumor tissues from T24 bearing mice treated with PBS, MMC (5 mg/kg), TFA (2.5 mg/kg) or combination. F, tumor volume of T24 bearing mice treated with PBS, MMC (5 mg/kg), TFA (2.5 mg/kg) or combination. G, overall survival of T24 bearing mice treated with PBS, MMC (5 mg/kg), TFA (2.5 mg/kg) or combination. n.s, no significant difference, *, p<0.05, **, p<0.01.