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# Autophagy of cancer stem cells is involved with chemoresistance of colon cancer cells

Shaobin Wu [a], Xianwei Wang [a], Jinxiang Chen [a], Yuxiang Chen [b,*]

[a] Department of General Surgery, Xiangya Hospital, Central South University, Changsha 410008, PR China
[b] Hepatobiliary and Enteric Surgery Research Center, Xiangya Hospital, Central South University, Changsha 410008, PR China

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ABSTRACTCT

*Background:* Chemoresistance is a major cause of treatment failure in colon cancer, and cancer stem cells have been found to be involved in the chemoresistance of colon cancer. However, the mechanisms driving the chemoresistance of colon cancer stem cells have not been addressed.
*Methods:* In this study, we investigated the cytotoxicity of paclitaxel in $CD44^+CD24^+$ SW1222 colon cancer cells expressing Cdx1 ($CD44^+CD24^+Cdx1^+$ stem cells) and $CD44^+CD24^+$ HCT116 colon cancer cells expressing wild-type p53 ($CD44^+CD24^+$p53wt stem cells).
*Results:* SW1222 cells were more resistant to paclitaxel-induced cytotoxicity than HCT116 cells. Conversely, HCT-116 cells had higher matrigel colony formation ability than SW1222 cells. The isolated $CD44^+CD24^+Cdx1^+$ cells showed higher resistance to paclitaxel-induced cytotoxicity than $CD44^+CD24^+$-p53wt cells. The resistance of $CD44^+CD24^+Cdx1^+$ cells to paclitaxel is associated with upregulation of Cdx1 and Bcl-2 expression, caspase-3 activity, and the ratio of LC3-II/LC3-I. The sensitivity of $CD44^+$-$CD24^+$p53wt cells to paclitaxel is associated with the downregulation of Bcl-2 expression, upregulation of Bax levels, and upregulation of caspase-3 activity. Silencing of Cdx1 expression and treatment with lysosomal inhibitor bafilomycin A increased paclitaxel-induced cytotoxicity in $CD44^+CD24^+Cdx1^+$ cells. Conversely, overexpression of Cdx1 decreased cell death in $CD44^+CD24^+$p53wt cells. Intratumoral injection of Cdx1 siRNA significantly inhibited tumor growth in a xenograft tumor model inoculated with $CD44^+CD24^+Cdx1^+$ cancer cells.
*Conclusion:* Cdx1 exerts a protective role in colon cancer stem cells, which play a crucial role in chemoresistance to paclitaxel through activation of autophagy. Autophagy is activated though the Cdx1-Bcl-2-LC3 pathway. In contrast, p53 exerts a major role in apoptosis and inhibits autophagy in colon cancer stem cells.



## 1. Introduction

Colorectal cancer is the third most common cancer in the western world [1]. On the other hand, the incidence of CRC in China is much lower but has been on the rise in recent years [2]. Patients with early stage colorectal cancer are usually operable and are primarily treated with surgery. However, most patients with colorectal cancer are diagnosed with advanced stage CRC. Chemotherapy can be used as the primary treatment for advanced disease or as an adjuvant treatment after surgery in cases with lymph node metastasis [3]. However, chemotherapeautic treatment of advanced colorectal cancer showed limited efficacy on the survival of patients because of chemoresistance. Although the molecular and genetic basis of chemoresistance have been intensely studied, the key mechanisms driving chemoresistance have yet to be fully elucidated.

Recently, the role of cancer stem cells (CSC) in chemoresistance has been highlighted. Cancer stem cells are resistant to current chemotherapy and are responsible for relapse and metastasis of tumors [4]. However, the molecular mechanisms involved in chemoresistance of CSC have not been elucidated. More and more studies have suggested that the unusual ability of CSCs to escape antitumor therapy may be related to the role of some key molecules, which characterize their stemness [5]. A recent study demonstrated that the balance between autophagy and apoptosis is involved in the survival of stem cells [6]. For example, the activation of autophagy and inhibition of apoptosis are essential for the maintenance of the haematopoietic stem cell compartment [7].

Autophagy is initiated by the formation of an autophagosome, which then fuses with the lysosomal membrane to deliver its

* Corresponding author. Address: Hepatobiliary and Enteric Surgery Research Center, Xiangya Hospital, Central South University, 87 Xiangya Road, Changsha 410008, PR China. Fax: +86 731 84327987.
*E-mail address:* chenyx008@yahoo.cn (Y. Chen).

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contents into the autolysosome for degradation [8]. During autophagosome formation, microtubule-associated protein light chain 3 I (LC3-I) is conjugated to phosphatidylamine to form LC3-phosphatidylamine (LC3-II). The production of LC3-II is an essential process during the formation of the autophagosome [8,9]. Therefore, the ratio of LC3-II levels to LC3-I levels can be used as a marker to reflect the activation of autophagy. Colon cancer cells expressing wild-type p53 are more sensitive to chemotherapy than cells without functional p53 [10]. The Cdx1 homeobox gene has been demonstrated to stimulate proliferation and induce tumorigenesis in intestinal epithelial cells by being involved in the complex network of signaling comprised of p53 and Bcl-2 [11]. However, involvement of the Cdx1 gene in the chemoresistance of colon cancer has not been reported.

We hypothesize that p53 induced apoptosis increases the sensitization of colon cancer cells to chemotherapy, whereas Cdx1 may play an important role in chemoresistance through activating autophagy signaling. In this study, the roles of Cdx1 and p53 in the chemoresistance of colon cancer cells were investigated in SW1222 cells expressing Cdx1 and HCT116 cells expressing p53. Our study highlights the role of Cdx1 in the resistance of colon cancer stem cells to chemotherapy.

## 2. Materials and methods

### 2.1. Cell culture

SW1222 is a human colorectal adenocarcinoma cell line that does not express p53 but expresses Cdx1. HCT-116 is a human colon carcinoma cell line, which contains a wild-type p53 gene but does not express Cdx1. These two cell lines were acquired originally from ATCC and cultured in DMEM medium (Invitrogen Inc., Carlsbad, CA) with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μg/mL of streptomycin at 37 °C, 5% $CO_2$.

### 2.2. MTT Assay for cell proliferation

The MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay measures cell viability and cell count. Cells in 96-well plate were treated with 50 nM of paclitaxel (Bedford Laboratories, Bedford, OH, USA) for 24 h [10]. The cells were then subjected to MTT assay [12]. The OD values were normalized to the cells without paclitaxel treatment.

### 2.3. Matrigel colony assay

Single cell suspensions were resuspended in a 1:1 mixture of Matrigel (BD Sciences) to medium and plated on 6-cm dishes in a limiting dilution. After 4 weeks of incubation, cells were incubated with 2% Giemsa solution and colonies were counted. Five replicate dishes were plated for each dilution.

### 2.4. Isolation of $CD44^+CD24^+$ cell subsets

The trypsanized cells were suspended at a concentration of $1 \times 10^7$/mL in 37 °C DMEM with 2% FCS. The mouse anti-human CD44-PE and anti-human CD24-FITC monoclonal antibodies (Invitrogen, America) were added to a reaction tube containing 10 ml of cells. The reaction was incubated with antibodies in the dark at room temperature for 30 min. Afterwards, the cells were spun at 1500 rpm/min for 5 min, and cell pellets were washed with PBS twice and re-suspended in PBS solution. The $CD44^+CD24^+$ positive cell population was sorted using a MoFlo cell sorter (Beckman Coulter, USA).

### 2.5. Western blot

The antibodies for p53, Bax, caspase-3, Bcl-2 and the peroxidase-labeled secondary antibody were purchased from Cell Signaling Technology (Beverley, MA, USA). The anti-Cdx1, anti-LC3-I and LC3-II antibodies were purchased from Sigma–Aldrich (St. Louis, MO, USA). Cells were homogenized and Western blot was performed as previously described [12]. Briefly, 20 μg of total protein was loaded onto a 8% SDS–PAGE gel and transferred to PVDF membranes. After blocking with 5% non-fat milk for 1 h, membranes were then incubated with primary antibody overnight at 4 °C. After washing with PBS, membranes were incubated with HRP-labeled secondary antibody (1:2000 dilution) for 2 h at room temperature the next day. Immunoreactive proteins were detected using a chemiluminescence reagent (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. To control for loading efficiency, the blots were stripped and re-probed with α-tubulin antibody (1:2000 dilution, Sigma–Aldrich, St. Louis, MO, USA).

### 2.6. siRNA silencing of Cdx1 gene expression

Two siRNA sequences that target the human Cdx1 gene were designed as previously described: siRNA1 targeted nucleotides 1054–1072 and siRNA2 targeted nucleotides 1675–1693 of the *CDX1* mRNA sequence (NM_001804.2) [13]. $CD44^+CD24^+$ SW1222 colon cancer cells were passaged 24 h before transfection. Cells in 6-well plates were transfected with a scrambled sequence that did not specifically target any gene using Lipfectamine 2000 by following the user manual (Invitrogen, Grand Island, NY). Forty-eight hours later, the cells were treated with 50 nM of paclitaxel and continually cultured for 24 h. The cells were then subjected to MTT assay or homogenized for Western blot assay as described above.

### 2.7. Construction of Cdx1 expression vector

The Cdx1 expression vector was constructed according to a previously published protocol [14]. Briefly, the human Cdx1 gene was amplified using forward primer: 5′-GGGAATTCAGGTGAGCA GT CG CTGGTCGTC-3′ and reverse primer: 5′-GG GAATT CACA GGAG CCA-CA CTCCCAGCACC-3′, which yielded a 942-base pair product. An EcoRI restriction enzyme site was designed in the primers. The PCR fragment was cloned into pcDNA3.0 vector at the EcoRI site, producing pCdx1 vector expressing Cdx1. The blank pcDNA3.0 vector was used as a negative control.

### 2.8. Tumor growth study

Single $CD44^+CD24^+$ SW1222 cells were resuspended in a 1:1 mixture of Matrigel and serum free medium. A 100-μl suspension containing 1000 cells was injected s.c. into the right hind limbs of Balb/C nude mice (BALB/c, *nu/nu*) weighing 20–22 g (Shanghai Biological Science Institution, Shanghai). After the tumors had grown to about 8 mm in diameter, mice were randomly divided into 3 groups; saline, paclitaxel, and paclitaxel + siRNA injection group. Each group contained 10 animals. In the paclitaxel and paclitaxel + siRNA groups, paclitaxel was i.p. injected at 8 mg/kg every other day for 4 weeks. siRNA1 and siRNA2 mixtures (1:1 mix, a total of 15 μg of siRNA in 50 μl saline) were intratumorally injected once a week for 4 weeks. Tumors were measured in two dimensions every 5 days and tumor volume ($V$) was calculated using the following formula: $V = (1/2)\, S^2 \times L$ ($S$, the shortest dimension; $L$, the longest dimension) [15]. Animals were euthanized when the subcutaneous tumor reached a size that required euthanasia (40 days after first injection). All animal experiments were conducted under an approved protocol from the Central South Univer-

sity and performed in accordance with the animal care guidelines of the Chinese Council.

### 2.9. Statistical Analysis

Data were analyzed using the Statistical Package for the Social Science, version 14.0 (Chicago, IL). The tumor growth data was analyzed by one-way analysis of variance followed by Bonferroni paired *t*-test to assess statistical significance between different treatment groups. The two tailed student *t-test* was used for statistical analysis of MTT data. All data are presented as mean ± standard error of the mean. A $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Cell viability assay

A previous study indicated that p53 plays a crucial role in the sensitization of colon cancer cells to chemotherapy [10]. SW1222 is a colon cancer cell line that does not express p53 but expresses Cdx1. In contrast, HCT116 cells express wild-type p53 but does not express Cdx1. We treated these two types of cells with 50 nM of paclitaxel for 24 h. MTT assay showed that paclitaxel induced 61.4% cell death in HCT116, but only 24.1% cell death in SW1222 (Fig. 1A), suggesting that SW1222 cells are more resistant to paclitaxel compared to HCT116 cells ($p < 0.001$). To further investigate whether the resistance of SW1222 cells to paclitaxel is associated with higher presence of cancer stem cells, we performed a cancer stem cell assay. The matrigel colony assay (Fig. 1B) showed that SW1222 had lower colony-forming ability (78 colonies per 1000 cells) than HCT116 (187 colonies per 1000 cells) ($p < 0.001$). We further analyzed the sensitization of $CD44^+CD24^+$ cells isolated from SW1222 and HCT116 cells to paclitaxel. 50 nM of paclitaxel induced significantly more cell death in $CD44^+CD24^+$ HCT116 cells compared to $CD44^+CD24^+$ SW1222 cells (Fig. 1C, $p < 0.001$). These findings suggest that the cancer stem cells isolated from SW1222 are more resistant to paclitaxel.

### 3.2. Signaling transduction responsible for paclitaxel resistance

The $CD44^+CD24^+$ SW1222 and HCT116 cells were treated with or without 50 nM of paclitaxel for 2 h. Cell lysates were subjected to Western blot assay. Paclitaxel significantly increased Cdx1, caspase-3, Bcl-2, and LC3-II protein expression, but significantly decreased LC3-I expression in $CD44^+CD24^+$ SW1222 cells. No p53 protein expression was detected in $CD44^+CD24^+$ SW1222 cells (Fig. 2). In $CD44^+CD24^+$ HCT116 cells, no Cdx1 protein expression was detected. Paclitaxel significantly increased p53, Bax, and caspase-3 expression, and significantly decreased Bcl-2 expression but had no effect on LC3-I and LC3-II expression (Fig. 2).

### 3.3. Cdx1 plays a key role in resistance to paclitaxel-induced cytotoxicity in SW1222 CSC cells

To investigate whether the resistance to paclitaxel of $CD44^+CD24^+$ SW1222 cells is associated with Cdx1 expression, siRNAs were used to silence Cdx1 expression. $CD44^+CD24^+$ SW1222 cells were transfected with or without siRNA for 24 h and then treated with 50 nM of paclitaxel for 24 h. Both siRNA1 and siRNA2 significantly increased the amount of cell death in $CD44^+CD24^+$ SW1222 cells compared to paclitaxel alone ($p < 0.001$). To further explore whether Cdx1 exerted its role through regulating autophagy, we measured LC3-I and LC3-II expression in $CD44^+CD24^+$ SW1222 cells transfected with or without siRNA. Transfection of siRNA significantly increased LC3-I expression and decreased LC3-II expression (Fig. 3B). To further verify the role of autophagy in the resistance of CSC to paclitaxel, $CD44^+CD24^+$ SW1222 cells were treated with or without the lysosomal inhibitor bafilomycin A (BafA) for 24 h and then subjected to MTT assay. BafA significantly sensitized the effect of paclitaxel (Fig. 3C, $p < 0.001$). These findings suggested that Cdx1 plays a key role in autophagy, which is responsible for the resistance of $CD44^+CD24^+$ SW1222 cells to paclitaxel.

### 3.4. Overexpression of Cdx1 increased resistance to paclitaxel-induced cytotoxicity in HCT116 CSC cells

To further validate the role of Cdx1 in resistance, Cdx1 was artificially expressed in $CD44^+CD24^+$ HCT116 cells. $CD44^+CD24^+$ HCT116 cells were transfected with Cdx1 expression vector (pCDX1) or blank pcDNA3.0 vector (pBlank). 24 h later, the transfected cells were treated with 50 nM of paclitaxel for 24 h. MTT assay showed that transfection of pCdx1 vector significantly decreased the amount of cell death in $CD44^+CD24^+$ HCT116 cells (28.6%) compared to cells transfected with blank vector (47.5%) (Fig. 4A). Western blot showed that transfection of Cdx1 expression vector caused overexpression of Cdx1 protein, which was accompanied by a decrease in LC3-I expression and increase in LC3-II level (Fig. 4B). These results suggest that artificial Cdx1 expression in $CD44^+CD24^+$ HCT116 CSC cells increased resistance to paclitaxel.

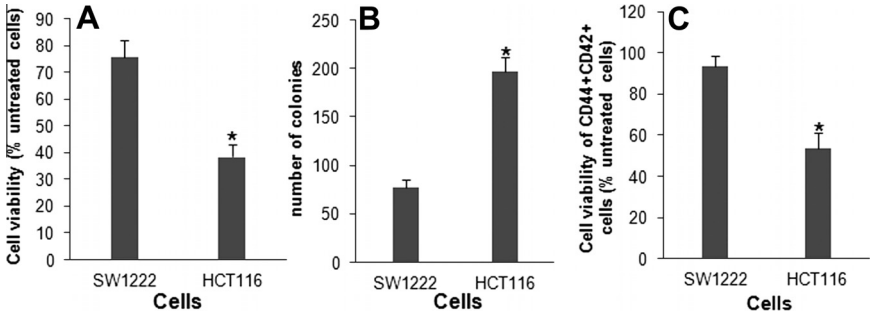


**Fig. 1.** MTT assay of cell viability and Matrigel colony assay. (A) MTT assay of SW122 and HCT116 cells treated with 50 mM of paclitaxel. Results represented percentage of viability to control cells without paclitaxel treatment. $^*p < 0.001$ *vs.* SW1222 cells. $N = 5$. (B) Matrigel colony assay. Singled cell suspension was resuspended with 1:1 Matrigel to medium and then incubated for 4 weeks. Results represented colonies per 1000 cells. $^*p < 0.001$ *vs.* SW1222 CSC cells. $N = 5$. (C) MTT assay of CSCs. The $CD44^+CD24^+$ cells were isolated by MoFlo cell sorter and re-seeded for 24 h, followed by treatment with 50 nM paclitaxel for 24 h. Paclitaxel induced significantly more cell death in HCT116 CSC cells. $p < 0.001$, $n = 10$.

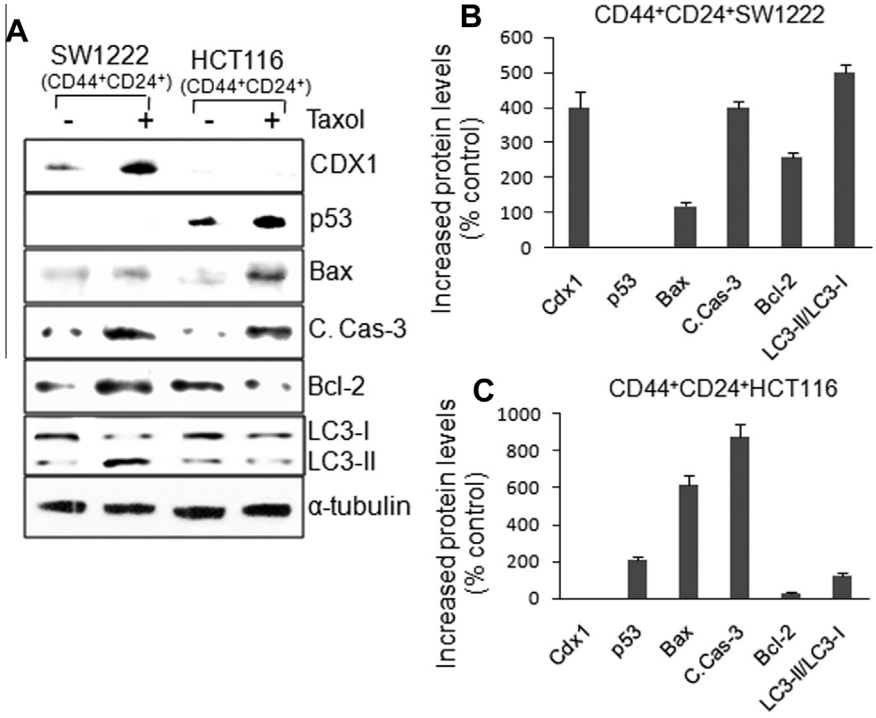


**Fig. 2.** Apoptosis and autophagy associated molecule levels. $CD44^+CD24^+$ Cells were treated with or without 50 nM of paclitaxel. (A) Representative of Western blots of protein levels. (B) Relative protein levels in $CD44^+CD24^+$ SW1222 cells. (C) Relative protein levels in $CD44^+CD24^+$ HCT116 cells. C. Cas-3: cleaved caspase-3. $N = 5$.

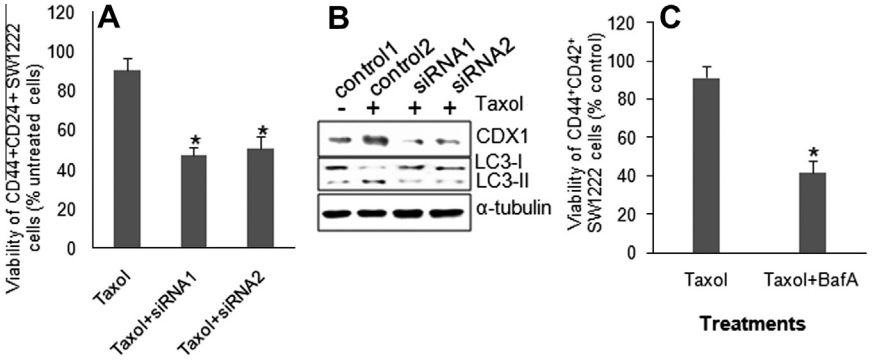


**Fig. 3.** Inhibition of Cdx1 expression in $CD44^+CD24^+$ SW1222 cells. (A) MTT assay in $CD44^+CD24^+$SW1222 cells transfected with siRNA of Cdx1. Cells were transfected with siRNA1 or siRNA2 for 24 h and then treated with 50 nM of paclitaxel for 24 h. Results presented as percentage of viability to untreated cells. $p < 0.001$, $n = 5$. (B) Western blot. Transfection of siRNA significantly reduced Cdx1 expression and reversed the effect of paclitaxel on LC3-I and LC3-II expression. (C) MTT assay of $CD44^+CD24^+$ SW1222 cells treated with or without the lysosomal inhibitor bafilomycin A (BafA). $p < 0.001$, $n = 5$.

### *3.5. Silencing of Cdx1 expression sensitized the therapeutic effect of paclitaxel in xenograft $CD44^+CD24^+$ SW1222 tumor models*

To investigate the role of Cdx1 in chemoresistance in vivo, we tested the role of Cdx1 siRNAs on tumor growth inhibition in a xenograph CSC animal model. Injection of paclitaxel alone significantly inhibited tumor growth 35 days after first injection. Injection of a mixture of siRNA1 and siRNA2 (1:1) significantly inhibited tumor growth 20 days after first injection (Fig. 4C). However, the inhibitory effect was statistically significant starting from day 25 compared to paclitaxel injection alone. These results indicated that Cdx1 plays a crucial role in the chemoresistance of cancer stem cells.

## 4. Discussion

Resistance to chemotherapy is a major obstacle hindering effective therapy of advanced colon cancer; however, the underlying mechanisms have not yet to be elucidated. Cancer stem cells are currently acknowledged to play a key role in the resistance of cancer cells to chemotherapy and radiotherapy. However, the mechanisms for the involvement of cancer stem cells in chemoresistance have not been well documented. Paclitaxel has been widely used to treat a variety of cancers, including colon cancer, but it often induces resistance. In this study, we demonstrated that colon cancer CSCs expressing Cdx1 are more resistant to paclitaxel than CSCs expressing p53. The observed chemoresistance is associated with the paclitaxel-induced upregulation of Cdx1 expression and activation of autophagy-associated signaling in $CD44^+CD24^+Cdx1^+$ stem cells. In contrast, colon cancer CSCs expressing p53 are more sensitive to paclitaxel through the activation of apoptosis-associated signaling. Our study highlighted the role of Cdx1 in the activation of autophagy and the protective role of autophagy in colon cancer stem cells.

In the clinic, p53 mutations have been found in 34–45% of colon and rectal tumors [16]. Patients with colorectal cancer expressing

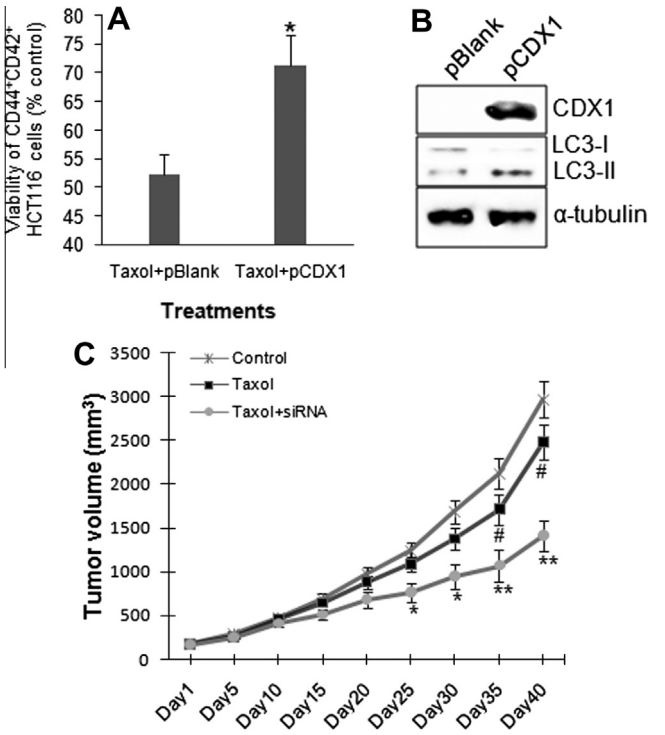


**Fig. 4.** Artificial expression of Cdx1 in $CD44^+CD24^+$ HCT116 cells and inhibition of tumor growth of Cdx1 siRNAs in vivo. (A) MTT assay of $CD44^+CD24^+$HCT116 cells transfected with or without Cdx1 expression vector. Transfection of Cdx1 expression vector significantly reduced cell death induced by paclitaxel. $p < 0.001$, $n = 5$. (B) Representative of Western blot assay of Cdx1, LC3-I and LC3-II protein levels. (C) SW1222 CSC growth in vivo. 1000 $CD44^+CD24^+$ SW1222 cells were injected s.c. into the right hind limbs of mice. After the tumors had grown to about 8 mm in diameter, mice were injected with saline (control), paclitaxel (Taxol) and paclitaxel + siRNA (Taxol + siRNA). $N = 10$.

mutated p53 exhibited significantly poorer prognosis when treated with chemotherapy compared to patients with wild-type p53 [16]. Also, p53-mutated colon cancer cells have been revealed to be relatively more resistant to paclitaxel compared to colon cancer cells expressing wild-type p53 [17]. Importantly, we found that SW1222 cells were more resistant to paclitaxel than HCT116 cells. The higher resistance of SW1222 cells to paclitaxel was not due to the presence of a large amount of $CD44^+CD24^+Cdx1^+$ stem cells, but due to the high resistance ability of stem cells. The higher resistance of $CD44^+CD24^+Cdx1^+$ stem cells was also associated with the upregulation of Cdx1 expression induced by paclitaxel. Conversely, $CD44^+CD24^+$p53wt stem cells were relatively more sensitive to paclitaxel and were associated with high expression of p53 induced by paclitaxel. The upregulation of p53 expression inhibited Bcl-2 expression, upregulated Bax expression, and upregulated caspase-3 activity but had no effect on the ratio of LC3-II/LC3-I. Therefore, the p53-Bcl-2-Bax-Cas-3 pathway leads to cell apoptosis in $CD44^+CD24^+$p53wt stem cells. In $CD44^+CD24^+Cdx1^+$ stem cells, upregulation of Cdx1 expression had limited effect on Bax level, but increased caspase-3 activity and Bcl-2 expression, as well as increased the ratio of LC3-II/LC3-I. Therefore, the Cdx1-Bcl-2/Cas-3 pathway activated autophagy in $CD44^+CD24^+Cdx1^+$ stem cells. This suggested that activation of autophagy in cancer stem cells plays a key role in stem cell survival. The role of Cdx1 in the activation of autophagy in colon cancer stem cells and subsequent survival and chemoresistance was further validated by the following: (1) silencing of Cdx1 expression sensitized $CD44^+CD24^+Cdx1^+$ stem cells to paclitaxel-induced cell death through reversal of paclitaxel-induced upregulation of LC3-II and downregulation of LC3-I levels; (2) the lysosomal inhibitor bafilomycin A increased paclitaxel-induced death of $CD44^+CD24^+Cdx1^+$ stem cells; (3) artificial expression of Cdx1 gene increased the resistance to paclitaxel of $CD44^+CD24^+$p53wt stem cells; and (4) silencing of Cdx1 expression in vivo sensitized the inhibitory effect of paclitaxel on the growth of $CD44^+CD24^+Cdx1^+$ tumor cells.

Autophagy is a physiological mechanism that eliminates toxic wastes or damaged cellular components in response to stress. A number of studies have reported that autophagy is activated in cancer cells in response to various anticancer therapies, and autophagy has been considered an alternative way to kill apoptosis-resistant tumor cells [18]. However, a recent study revealed that activation of autophagy results in inhibition of apoptosis [7]. In this study, artificial expression of Cdx1 in $CD44^+CD24^+$p53wt stem cells inhibited cell death from paclitaxel treatment, suggesting that activation of autophagy inhibited apoptosis. Autophagy signaling can be activated by multiple signaling pathways and usually shares the same pathway with apoptosis. In this study, paclitaxel activated caspase-3 in both the $CD44^+CD24^+Cdx1^+$ and $CD44^+CD24^+$p53wt stem cells although it exhibited divergent effects on Bax and Bcl-2 expression in the two types of cells. The LC3-I and LC3-II levels can be used as markers to reflect the activation of autophagy. We demonstrated that paclitaxel increased LC3-II, but decreased LC3-I expression in $CD44^+CD24^+Cdx1^+$ stem cells. A previous study demonstrated that Cdx1 can activate Bcl-2, and Bcl-2 can stimulate LC3-II production. We therefore propose that CDX1-Bcl-2-LC3-II signaling is an important pathway involved in the activation of colon cancer stem cells.

In conclusion, Cdx1 exerts a protective role in colon cancer stem cells through activation of autophagy, which is involved in chemoresistance of colon cancer to anticancer agents. The activation of autophagy inhibits apoptosis though the Cdx1-Bcl-2-LC3 pathway. In contrast, p53 exerts a major role in anticancer agents-induced apoptosis, which inhibits autophagy in colon cancer stem cells. Our study highlighted the role of autophagy in the chemoresistance of colon cancer.

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