RESEARCH ARTICLE

An Epigenetic Mechanism Underlying Doxorubicin Induced EMT in the Human BGC-823 Gastric Cancer Cell

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Abstract

The epithelial to mesenchymal transition (EMT) is a key step during embryonic morphogenesis and plays an important role in drug resistance and metastasis in diverse solid tumors. We previously reported that 48 h treatment of anti-cancer drug doxorubicin could induce EMT in human gastric cancer BGC-823 cells. However, the long term effects of this transient drug treatment were unknown. In this study we found that after 48 h treatment with 0.1 μg/ml doxorubicin, most cells died during next week, while a minor population of cells survived and formed colonies. We propagated the surviving cells in drug free medium and found that these long term cultured drug survival cells (abbreviated as ltDSCs) retained a mesenchymal-like cell morphology, and expressed high levels of EMT-related molecules such as vimentin, twist and β-catenin. The expression of chromatin reprogramming factors, Oct4 and c-myc, were also higher in ltDSCs than parental cells. We further demonstrated that the protein level of p300 was upregulated in ltDSCs, and inhibition of p300 by siRNA suppressed the expression of vimentin. Moreover, the ltDSCs had higher colony forming ability and were more drug resistant when compared to parental cells. Our results suggested that an epigenetic mechanism is involved in the EMT of ltDSCs.

Keywords: Epithelial-mesenchymal transition - gastric cancer cells - drug resistance - metastasis - epigenetic mechanism.

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Introduction

Drug resistance is one of the major obstacles to successful treatment of cancer (Zahreddine and Borden, 2013). Substantial efforts of elucidating the molecular bases of drug resistance have revealed a variety of mechanisms including drug efflux, acquisition of drug binding-deficient mutants of the target, and engagement of alternative survival pathways (Garraway and Janne, 2012). However, recent findings have also revealed non-mutational mechanisms of drug resistance, implicating that acquired drug resistance does not necessarily require heritable genetic alterations (Holohan et al., 2013).

The epithelial to mesenchymal transition (EMT) phenotype is characterized by the loss of cell-to-cell adhesion with the disintegration of tight, adherens, and gap junctions and a phenotypic change from an ''epithelial'' morphology to a motile, fibroblast-like morphology (Zhu et al., 2013). Originally defined as a fundamental process governing morphogenesis in multicellular organisms, EMT seems to play an important role in tumor progression, metastasis and drug resistance in diverse solid tumors (Yang and Weinberg, 2008; Kim et al., 2013). Recent reports showed that mesenchymal subpopulation of cells could spontaneously arising from immortalized human mammary epithelial cell, and the epithelial cells that passed through an EMT acquired the self-renewing trait associated with normal tissue (SCs) and cancer stem cells (CSCs) (Chaffer et al., 2011; Scheel et al., 2011). These data demonstrated the epigenetic traits of EMT in certain cells.

Besides their therapeutic effects, chemotherapeutic agents were also shown to enhance the malignancy of treated cancer cells (Sharma et al., 2010). We previously reported that transient (48 h) treatment of anti-cancer drug doxorubicin (Dox) could induce EMT in human gastric cancer BGC-823 cells (Han et al., 2013). However, the long term effects of this transient drug treatment were unknown. Here we showed that an epigenetic mechanism was involved in doxorubicin induced EMT in BGC-823 cells.

Materials and Methods

Cells culture and reagents

Human gastric cancer cell line BGC-823 was obtained

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from the Institute of Biochemistry and Cell Biology, Shanghai Institute for Biological Science, Chinese Academy of Sciences. Cells were maintained in RPMI 1640 containing penicillin (100 U/ml), streptomycin (100 mg/ml), and 10 % fetal bovine serum (FBS) at 37°C in a humidified incubator supplemented with 5% CO_2 in air. Dox was purchased from Meilun Pharmaceutical Company (Dalian, China) and dissolved in double-distilled water (1.0 g/ml) for storage and diluted with phosphate buffered saline (PBS) before use.

Western blotting

Cells were washed with cold PBS once and harvested by scraping them in RIPA lysis buffer. Protein concentration was determined by the Bradford assay (Beyotime Institute of Biotechnology, Haimen, China). The extracted proteins were separated by SDS polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were first blocked with 5 % (w/v) nonfat milk in TBST and then probed with the indicated primary antibodies at 4°C overnight. After washing four times, the membranes were incubated with the HRP-conjugated secondary antibodies for 1 h. The signals were detected using an enhanced chemiluminescence detection kit (Thermo Scientific, Illinois, USA). Primary antibodies used in this study included mouse monoclonal antibody to β-catenin (Santa Cruz, California, USA), rabbit polyclonal antibody to Vimentin (Biosynthesis Biotechnology, Beijing, China), rabbit polyclonal antibody to p300 (Biosynthesis Biotechnology, Beijing, China), mouse monoclonal antibody to β-actin (Abmart, Shanghai, China).

RNA isolation and PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's specifications and quantified by spectrophotometer measurement. RNA (2 μg) was reverse transcribed to cDNA with TIANScript M-MLV (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. Primer pairs for human Twist and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were as follows: Twist 5'-GGG AGT CCG CAG TCT TAC-3' (sense) and 5'-CCT GTC TCG CTT TCT CTT T-3' (antisense); GAPDH 5'-ACC ACA GTC CAT GCC ATC TC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). The products were separated in 2 % Agarose gel. Real-time quantitative PCR (qPCR) analyses were performed using SYBR Green-based detection (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol on a Rotor-Gene 3000 instrument (Corbett Life Science). The following primers were used for qPCR: β-catenin 5'- TGG TGC CCA GGG AGA ACC CC -3' (sense) and 5'- TGT CAC CTG GAG GCA GCC CA -3' (antisense); Twist 5'- TCA GCA GGG CCG GAG ACC TA -3' (sense) and 5'- TCC ACG GGC CTG TCT CGC TT -3' (antisense); Vimentin 5'- TGG CCG ACG CCA TCA ACA CC -3' (sense) and 5'- CAC CTC GAC GCG GGC TTT GT -3' (antisense); Oct4 5'- CAG TGC CCG AAA CCC ACA C -3' (sense) and 5'- GGA GAC CCA GCA GCC TCA AA -3' (antisense); c-myc 5'- CGA CCC GGA CGA CGA GAC CT -3' (sense) and 5'- GTT CGG GCT GCC GCT GTC TT -3' (antisense); and β-actin 5'-AGC CTC GCC TTT GCC GAT CC-3' (sense) and 5'-ACA TGC CGG AGC CGT TGT CG-3' (antisense). Relative expression of gene of interest was normalized to β-actin and calculated with $2^{-\Delta\Delta Ct}$ method.

Inhibition of p300 expression by RNAi

The p300 siRNA oligonucleotides (Shi et al., 2009) (sense 5'- CAG AGC AGU CCU GGA UUA Gtt -3' and antisense 5'- CUA AUC CAG GAC UGC UCU Gtt -3') were synthesized by Ribo- Bio Company (Guangzhou, China). A negative control siRNA (sense 5'-UGA AGG AGU UCC UGA UCU Utt-3' and antisense 5'-AAG AUC AGG AAC UCC UUC Att-3') was used as scramble siRNA. siRNA transfection was performed in 6-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Colony forming assay

Cells were suspended in RPMI 1640 medium containing 0.3% noble agar and 10% fetal calf serum at a density of 1000 cells/ml. A layer of this suspension was plated above a solidified medium layer containing RPMI 1640, 0.6% agar and 10% serum in 6-well plates. Cells were incubated for 14 days at 37°C in 5% CO_2. Numbers of colonies with diameters exceeding 100μm were counted under microscope.

Methylthiazol tetrazolium assay

Cells were plated into 96-well plates at 2, 000 cells per well and allowed to adhere overnight. The culture was then replaced with fresh medium containing Dox with indicated concentrations. After 48 h, methylthiazol tetrazolium (MTT) was added into each well. The number of viable cells was determined by the OD value at 570 nm.

Statistical analysis

All experiments were repeated at least in triplicate. Data was reported as mean±SEM. Statistical analyses were performed using Student's t test (*p<0.05; **p<0.01).

Results

Generation of long term cultured drug survival cells

To investigate the long term effect of Dox treatment, BGC-823 cells were seeded into T25 flask at 5×10^4/ml and allowed to adhere for overnight, then the cells were treated with fresh medium containing 0.1μg/ml Dox for 48h. The cells were collected and reseeded in drug-free medium (Figure 1a). 24 h after drug retreatment, we observed the typical morphologic changes related to EMT: the cells displayed a spindle-like shape with obvious filopodia and cell arrangement became irregular (Figure 1a). While most cells died in the next week, a small population survived and formed colonies. These cells were carefully collected and seeded into 6-well plates and passaged before confluence. About 3 weeks after drug retreatment, we could acquire enough cells for subsequent experiments (Figure 1b). We named these cells as long term cultured drug survival cells (abbreviated as ltDSCs).

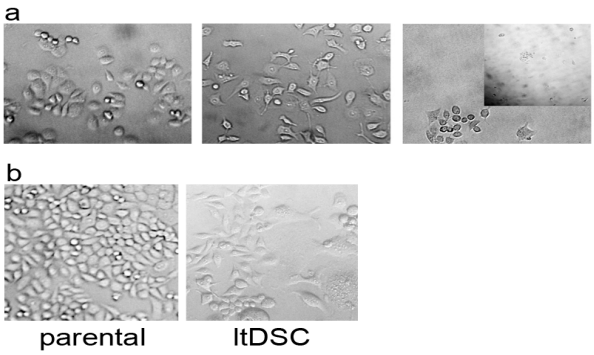

Figure 1. Generation of ltDSCs. After 48 h Dox treatment **(a, left)**, the cells were collected and plated into drug free medium **(a, middle)**. About 1 week later most of the cells died and a few survived cells formed small colonies **(a, right)**. These colonies were propagated in drug free medium for 2 to 3 weeks **(b, right)** until enough cells were harvested for subsequent experiments

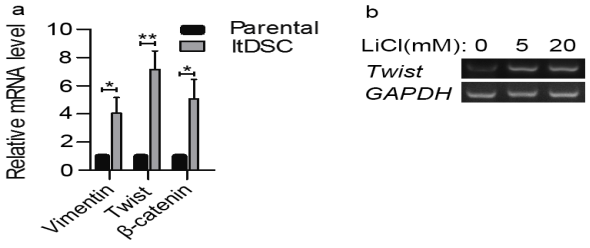

Figure 2. EMT Status were Maintained in ltDSCs. A) mesenchymal cell markers were expressed at high levels in ltDSCs in comparison to parental cells. **B)** 48 h treatment of LiCl induced Twist transcription in BGC-823 cells

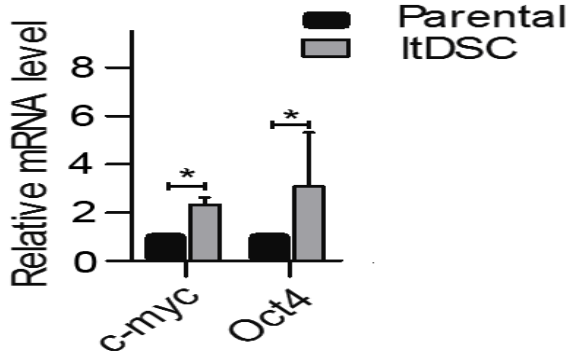

Figure 3. ltDSCs Expressed High Levels of Chromatin Reprogramming Factors

EMT status were maintained in ltDSCs

As shown in Figure 1b, the ltDSCs remained the morphologic characteristics of EMT. RT-qPCR assays demonstrated that ltDSCs expressed higher levels of mesenchymal cell markers such as Vimentin and Twist when compared to parental cells (Figure 2a). These data indicated that the EMT phenotype was inherited in ltDSCs. We previous reported that β-catenin signaling was activated after 48 h Dox treatment (Han et al., 2013). Here we also found that the mRNA level of β-catenin was higher in ltDSCs than parental cells (Figure 2a), suggesting the sustained activation of β-catenin signaling in ltDSCs. GSK3β phosphorylates β-catenin and leads to its degradation. The mRNA level of Twist was increased after 48 h treatment of LiCl, a GSK3β inhibitor, which further indicated the important role of β-catenin in EMT (Figure 2b).

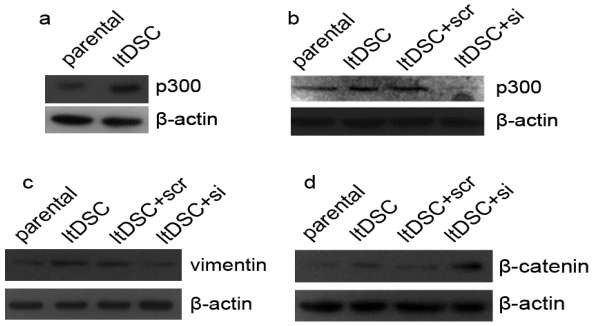

Figure 4. Maintenance of EMT in ltDSCs was dependent on p300. A) ltDSCs expressed higher level of p300 than parental cells. **B)** p300 expression in ltDSCs was suppressed by siRNA. **C)** p300 siRNA inhibited the expression of Vimentin in ltDSCs. **D)** the expression of β-catenin was further increased by p300 siRNA in ltDSCs. scr: scramble siRNA, si: p300 siRNA

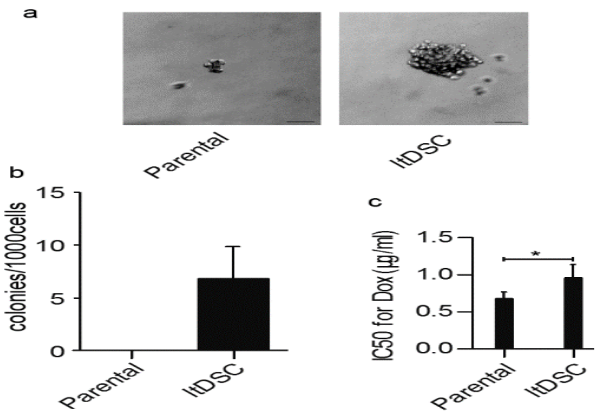

Figure 5. ltDSCs were More Malignant than Parental Cells. ltDSC showed enhanced colony forming ability (a, b) and drug resistance (c) in comparison to parental cells. Bars represent 100 μm

ltDSC express high levels of chromatin reprogramming factors

Since ltDSCs were expanded from a small group of drug survival cells and maintained EMT after drug retreatment, we speculated that an epigenetic mechanism was involved in the maintenance of EMT in ltDSCs. Indeed, we found that the expression of chromatin reprogramming factors, c-myc and Oct4, were significantly higher in ltDSCs than parental cells (Figure 3). However, we couldn't detect the expression of Sox2 and Klf4 in ltDSCs. These were not due to the bad primer qualities for Sox2 and Klf4, since they could effectively amplify these two genes from the total mRNA of human acute lymphoid leukemia MOLT4 cells (data not shown).

Maintenance of EMT in ltDSCs was dependent on p300

Acetyltransferase p300 catalyses the acetylation of histones, thereby facilitates gene transcription (Iyer et al., 2004). We found that ltDSCs expressed higher levels of p300 than their parental cells (Figure 4a). To further investigate the role of p300 in the maintenance of EMT, we used p300 specific small interference RNA to knockdown p300 expression in ltDSCs. Figure 4b showed the siRNA significantly suppressed the expression of p300 in ltDSCs. In response to p300 knockdown, the expression of Vimentin in ltDSCs was downregulated (Figure 4c),

suggesting the maintenance of EMT phonotype in ltDSCs was dependent on high level of p300 expression. To our surprise, the protein level of β-catenin was further upregulated after p300 siRNA treatment (Figure 4d).

ltDSCs were more malignant compared to parental cells

Previous studies reported that cancer cells could acquire new traits such as enhanced proliferation and drug resistance through EMT (Sarkar et al., 2009; Singh and Settleman, 2010; Haslehurst et al., 2012). We also found that ltDSCs had higher colony forming ability than parental cells. In soft agar assay, ltDSCs formed 6.8±3.1 colonies/1000 cells, whereas parental cells couldn't form colony with diameter exceeding 100 μm (Figure 5a and b). Our MTT assay also showed that ltDSCs were more resistant to Dox when compared to parental cells (Figure 5c). These evidences suggested that ltDSCs were more malignant than parental cells.

Discussion

In this study, we explored the long term effect of Dox treatment on BGC-823 cells. We showed that while majority of the cells eventually died after 48 h Dox treatment, a small group of cells survived and expanded in drug free medium. These long term cultured DSC still exhibited a typical mesenchymal morphology and expressed high levels of mesenchymal cell markers and chromatin reprogramming factors. Our previous study showed that 48 h Dox treatment could induced EMT in BGC-823 cells (Han et al., 2013), which precluded the possibility that the EMT phenotype of ltDSCs was caused by DNA mutation in such short time of drug exposure. Together, our data suggested that an epigenetic mechanism was involved in the maintenance of EMT in ltDSCs.

Histone acetylation is one of the epigenetic mechanisms in regulating gene expression (Suva et al., 2013). We showed that the high level of Vimentin in ltDSCs was sensitive to p300 knockdown, indicating that histone acetylation played a role in the maintenance of EMT in ltDSCs, although the details need further investigation. We previously identified the critical role of β-catenin signaling in Dox induced EMT in BGC-823 cells (Han et al., 2013). Here we found that β-catenin signaling was continuously activated in ltDSCs. However, the expression of β-catenin was further elevated after treatment of p300 siRNA. Besides histones, p300 catalyses many non-histone proteins, including β-catenin (Wolf et al., 2002). Indeed, Li et al reported that acetylation at Lysine 49 was required for β-catenin phosphorylation (Li et al., 2008), whereas downregulation of histone deacetylation resulted in β-catenin degradation in colon cancer cells (Yang et al., 2008; Mak et al., 2012).

After 48 h Dox treatment, the survived cells had increased colony forming ability and drug resistance even expanded in drug free medium, suggesting that ltDSCs were more malignantly when compared to parental cells. Considering the frequent phenomenon of drug resistance and minimal residual disease in cancer chemotherapy, our study emphasized the need of new tactics targeting epigenetic reprogramming in ltDSCs.

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